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Biosynthesis of Folates by *Streptococcus thermophilus* and *Lactobacillus delbruekii* ssp. *bulgaricus*

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ABSTRACT

The folate synthesizing ability of yogurt bacteria, including *Streptococcus thermophilus* MC and ATCC 19258 and *Lactobacillus delbrueckii* ssp. *bulgaricus* 448 and 449, were evaluated. The major effort of this study focused on increasing the folate contents of yogurt bacteria fermented milk by adding lactose or calcium chloride. The addition of 2% lactose in reconstituted non-fat dry milk increased folate synthesis. Although folates increased only about 3 to 7% for yogurt cultures incubated for 6 hr, folates increased 12 to 198% for yogurt bacteria grown for 18 hr. The folate content decreased for reconstituted milk fermented with *S. thermophilus* MC or *L. bulgaricus* 448 or 449 when 0.02% calcium chloride was added. The folate level increased only for reconstituted milk fermented with *S. thermophilus* ATCC 19258 when calcium chloride was added. The addition of 2% lactose increased the cell counts for yogurt bacteria grown for 6 and 18 hr. On the other hand, the results of the cell counts from the addition of 0.02% calcium chloride were not consistent. However, the cell counts increased for both *L. bulgaricus* strains. The folate levels and cell counts during refrigerated storage at 4˚C were determined for reconstituted non-fat dry milk fermented with these bacteria. Folate levels decreased about 9 to 28% at the end of the second week. The viable yogurt bacterial counts remained stable for the 2-week shelf life for all four strains tested in this study. To determine the effect of folate on protecting cells from the cytotoxicity of oxidant H_2O_2, Intestine 407 was used in this study for evaluation. The cell viability of Intestine 407 was increased due to the inhibition of oxidant H_2O_2 cytotoxicity by folate and Intestine 407 cells demonstrated higher viability when treated with higher concentration of folate.

Key words: *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, folates, cell viability

INTRODUCTION

Folates are important in human nutrition. Research findings suggest that folates play a significant role in the reduction of neural tube defects during pregnancy[5,16]. It has also been reported that an increased consumption of folic acid lowers the levels of plasma homocysteine, which is an emerging risk factor for coronary heart disease. Folates, therefore, may play a protective role in the reduction of coronary heart disease[2,7,10]. Recent studies also find that folates may lower the risk of cancer, where they function as cofactors in reactions involving 1-carbon transfer during the biosynthesis of nucleic acids[1,14].

An exogenous supply of folic acid is necessary to prevent nutritional deficiency due to the incapability of synthesizing folates by mammalian cells[14]. Dairy products represent one of the important dietary sources of folates. Yogurt, which is fermented with lactic acid bacteria *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (*L. bulgaricus*), is one of the most popular dairy products. It is a nutritious food reported to be beneficial for human health. The nutritional and health benefits of cultured yogurt include: anti-allergic effect to milk protein, enhancement of bio-availability of calcium and other nutrients, improvement of lactose intolerance, presence/colonization of active culture in the digestive tract, control of gastrointestinal infections, stimulation of immunological system, anticarcinogenic actions, growth stimulation, reduction of serum cholesterol, and longevity[4]. Folate synthesizing lactic cultures can be used in yogurt to increase folate contents and provide consumers with an even healthier product.

High-performance liquid chromatography (HPLC) has been used to measure folates in several foods[8,9,17]. The HPLC method was used in our previous study[18] to determine folates synthesized by lactic acid bacteria in fermented milk. In this study, the folate levels of yogurt were also determined using the HPLC method.

The objective of this research was to increase the folate levels in yogurt by adding lactose or calcium chloride in reconstituted non-fat dry milk. The influence of lactose or calcium chloride on cell counts of yogurt bacteria was studied at the same time. As mentioned previously, folates play significant roles as cofactors in reactions involving 1-carbon transfer during the biosynthesis of nucleic acids[1,14]. This functionality is expected to help living cells in DNA replication and repair. The effect of folates on protecting Intestine 407 cells from the cytotoxicity of hydrogen peroxide, which can cause oxidative damage to cell DNA, was also investigated.

MATERIALS AND METHODS

I. *Yogurt Bacterial Strains*

Yogurt bacteria including *Lactobacillus delbrueckii* ssp. *bulgaricus* 448 and 449, and *Streptococcus thermophilus* MC and ATCC 19258 were obtained from our frozen stock cul-

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ture collection. *L. bulgaricus* strains were propagated in MRS broth (Difco Laboratories, Detroit, MI, USA) and *S. thermophilus* strains were grown in M17 broth (Difco Laboratories, Detroit, MI, USA) at 37˚C. All strains were serially transferred at least three times prior to use in these studies. For viable cell counts, *L. bulgaricus* strains were enumerated on MRS agar plates and *S. thermophilus* strains were enumerated on M17 agar plates following anaerobic incubation (BBL GasPak anaerobic system; Becton Dickinson and Company, Cockeysville, MI, USA) at 37˚C for 2 to 3 d.

II. *Effect of Lactose or Calcium Chloride on Folate Synthesis*

Reconstituted non-fat dry milk (10%) with lactose (0, 2%) or calcium chloride (0, 0.02%) were inoculated with yogurt cultures at 2% and incubated at 37˚C. Aliquots were taken at 0, 6, and 18 hr and the folate levels were determined using the HPLC method described below. The cell counts were also determined.

III. *Changes of Folate Contents and Cell Counts in Fermented Milk during the Storage*

Reconstituted non-fat dry milk was inoculated with yogurt bacteria at 2% and incubated at 37˚C for 6 hr[18] and then stored at 4˚C. Aliquots were removed initially and at 1 and 2 weeks and the folate contents were analyzed using the HPLC method described below. The viable cell counts were also determined.

IV. *Folate Analysis by HPLC Method*

Six mL of the reconstituted non-fat dry milk fermented with yogurt bacteria was added to 10 mL of extraction buffer (0.1 M phosphate buffer containing 0.5% sodium ascorbate). The mixture was placed in a boiling water bath for 15 min and then centrifuged at 4000 xg for 10 min. To 3 mL supernatant, 0.4 mL human plasma (Sigma, St. Louis, MO) was added and the mixture was incubated at 37˚C for 1 hr under continuous rotation. The reaction was stopped by placing the samples in boiling water for 5 min. The extract was centrifuged at 27000 xg for 10 min, filtered through a 0.45-μm filter, and used directly or stored at -20˚C until use. The chromatographic analysis was performed using a Hitachi Model L-6200 HPLC system (Tokyo, Japan). Distilled and deionized water was used for mobile phase. The mobile phase was HPLC grade methanol (15%) in 0.05 M KH_2PO_4. All solvents were filtered through 0.45-μm filters prior to use. The C_{18} Hypersil™ (25 cm × 4.6 mm I.D., 5 μm spherical packing; Runcorn, UK) was used as the analytical column. A Hamilton cartridge system (Hamilton Co., Reno, Nevada), equipped with a 25 mm × 2.3 mm I.D. guard column packed with a 10-μm C_{18} silica packing material, was installed ahead of the analytical column. Flow rate through the analytical column was 0.4 mL/min. The column was flushed with ddH_2O, followed by methanol, after each working day. Fluorescence detector (Hitachi Model F-1050, Tokyo, Japan; Ex = 295 nm, Em = 365 nm) was used for detection. The entire HPLC system was operated at an ambient temperature. Folates reported were the total of tetrahydrofolate, 5-methyltetrahydrofolate, and 5-formyltetrahydrofolate[18]. All the observed folate values in tables were calculated by subtracting folate values at 0 hr.

V. *Intestine 407 Cell Culture*

Intestine 407 (CCRC 60022) cells were purchased from Culture Collection and Research Center (CCRC; Hsinchu, Taiwan). Cells were grown in basal medium eagle (BME) supplemented with Earle's BSS and fetal bovine serum (Gibco BRL, Life Technologies, Grand Island, NY) and incubated in a humidified atmosphere of air/CO_2 (95:5 v/v) at 37˚C. Confluent cells were washed twice with PBS. One mL of trypsin-EDTA (0.05% trypsin and 0.53 mM EDTA) was added and left for 5 min. Two mL of BME was added and cells were harvested by centrifugation at 1000 xg for 5 min.

VI. I*nhibition of Oxidant H_2O_2 Cytotoxicity to Intestine 407 Cells by Folate*

The inhibition of oxidant H_2O_2 cytotoxicity to Intestine 407 cells by folate was determined by the MTT colorimetric assay[3,6,11,15]. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenol tetrazolium bromide (MTT; Sigma, St. Louis, MO) was used as a tetrazolium salt. One mL of BME was inoculated with 5 × 10^4 Intestine 407 viable cells and incubated under the conditions described previously for 16 to 18 hr. One mL of H_2O_2 (0.006 or 0.6%) was added. Folate at 0, 100, 200, 400, and 800 μg/mL was then added. After 24 hr of reaction at 37˚C, the solution was mixed with 1 mL of MTT (2 mg/mL) and incubated at 37˚C for 2.5 hr. One mL of dimethyl sulfoxide was added to dissolve the blue crystals and absorbance was read at 492 nm. The percentage increase of Intestine 407 cell viability due to the inhibition of oxidant H_2O_2 cytotoxicity by folate was defined as follows: {[A_{492}(Int 407 treated with H_2O_2 and folate)- A_{492}(Int 407 treated with H_2O_2)]/A_{492}(Int 407 treated with H_2O_2)} X 100%.

RESULTS AND DISCUSSION

As illustrated in Table 1, the addition of 2% lactose in reconstituted non-fat dry milk increased folate synthesis. Although folate increase only ranged from approximately 3 to 7% for yogurt cultures incubated for 6 hr, folates increased 12 to 198% for yogurt bacteria grown for 18 hr. As shown in Table 1, folate synthesis decreased dramatically for yogurt bacteria propagated for an extended incubation time of 18 hr in reconstituted milk, without the extra addition of lactose. This is because lactic acid bacteria do not only synthesize folates, but also utilize folates[12], especially when a nutrient such as lactose is exhausted. The addition of 2% lactose did not only increase the folate levels for yogurt cultures incubated for 6 hr, but also elevated the folate synthesis (almost

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Table 1. Effect of lactose and calcium chloride on folate synthesis by yogurt bacteria[a]

Folate (ng/mL) / Strain	No lactose or calcium chloride added		2% Lactose added[b]		0.02% Calcium chloride added[b]	
	6hr	18hr	6hr	18hr	6hr	18hr
S. thermophilus ATCC 19258	46.7	19.4	50.1 (+7.3)	57.9 (+198)	48.6 (+4.1)	27.1 (+39.7)
S. thermophilus MC	59.6	45.2	62.2 (+4.4)	50.4 (+11.5)	52.1 (-12.6)	29.6 (-34.5)
L. bulgaricus 449	62.8	22.8	64.5 (+2.7)	55.0 (+141)	56.1 (-10.7)	14.9 (-34.6)
L. bulgaricus 448	68.5	22.8	70.7 (+3.2)	58.8 (+131)	53.8 (-21.5)	21.2 (-16.5)

[a] Data reported was obtained from experiments repeated three times.
[b] Values in parenthesis are percentage (%) changes.

Table 2. Effect of lactose and calcium chloride on viable cell counts of yogurt bacteria[a]

Cell count (cfu/mL) / Strain	No lactose or calcium chloride added		2% Lactose added[b]		0.02% Calcium chloride added[b]	
	6hr	18hr	6hr	18hr	6hr	18hr
S. thermophilus ATCC 19258	5.7×10^8	2.0×10^9	1.1×10^9 (+93)	5.5×10^9 (+175)	3.9×10^8 (-32)	1.6×10^9 (-20)
S. thermophilus MC	8.0×10^7	2.3×10^9	1.2×10^8 (+50)	6.0×10^9 (+161)	4.7×10^8 (+488)	1.9×10^9 (-17)
L. bulgaricus 449	1.6×10^8	9.5×10^8	2.7×10^8 (+69)	1.1×10^8 (+16)	3.5×10^8 (+119)	2.2×10^9 (+132)
L. bulgaricus 448	3.1×10^8	9.4×10^8	1.2×10^9 (+287)	1.5×10^9 (+60)	4.7×10^8 (+0.52)	1.9×10^9 (+102)

[a] Data reported was obtained from experiments repeated three times.
[b] Values in parenthesis are percentage (%) changes.

Table 3. Changes of folate contents in yogurt bacteria fermented milk during storage at 4˚C[a]

Folate (ng/mL) / Strain	Fresh	1 week[b]	2 weeks[b]
S. thermophilus ATCC 19258	38.7	36.8 (-4.9)	35.4 (-8.5)
S. thermophilus MC	36.2	34.4 (-5.0)	33.0 (-8.8)
L. bulgaricus 449	51.8	44.2 (-14.7)	37.2 (-28.2)
L. bulgaricus 448	33.7	33.0 (-2.1)	27.2 (-19.3)

[a] Data reported was obtained from experiments repeated three times.
[b] Values in parenthesis are percentage (%) changes.

triple) for bacteria grown for 18 hr. Therefore, in addition to playing a significant role in fermentation, lactose is also important for folate synthesis by yogurt bacteria.

To further increase folate contents, 0.02% of calcium chloride was added to reconstituted skim milk. According to the study by Reif *et al.*[13], the addition of calcium chloride would increase the folate levels in cheese, which were also fermented with lactic acid bacteria. However, in the study by Rief *et al*. different species of lactic acid bacteria and incubation conditions were used in cheese fermentation. The folate contents decreased for reconstituted milk fermented with *S. thermophilus* MC or *L. bulgaricus* 448 or 449. The folate level increased only for reconstituted milk fermented with *S. thermophilus* ATCC 19258 in this study (Table 1). Therefore, the addition of calcium chloride is not recommended for the purpose of increasing folate synthesis by yogurt bacteria. Generally speaking, the use of different strains, media, incubation temperature, and propagation time will affect the synthesis levels of folates and other metabolites.

The addition of 2% lactose increased the cell counts for yogurt bacteria grown for 6 and 18 hr (Table 2). However, the results of the cell counts from the addition of 0.02% calcium chloride were not consistent, while the cell counts increased for both *L. bulgaricus* strains (Table 2).

The addition of 2% lactose increased both folates and viable cells in milk fermented with yogurt bacteria for 6 hr and 18 hr as mentioned previously (Tables 1 and 2). However, the productivity of folates per cell increased only for *S. thermophilus* ATCC 19258 and *L. bulgaricus* 448 and 449 grown for 18 hr. *L. bulgaricus* 449 propagated for 18 hr had the highest productivity increase among the strains tested. Despite low productivity, the increase of cell counts is a choice to increase total folates available.

The changes of folate contents and viable cell counts in yogurt bacteria fermented milk during storage are shown in Table 3. The folate levels and cell counts during refrigerated storage at 4˚C were determined initially and every week for 2 weeks for reconstituted non-fat dry milk fermented with yogurt bacteria. The typical shelf life of cultured yogurt is usually about 2 weeks. Folate levels decreased about 9 to 28% at the end of second week. The folate content of milk fermented with *L. bulgaricus* 449 was reduced more than that in milk fermented with the other three yogurt strains. The viable yogurt bacterial counts remained quite stable for the 2-week shelf life for all strains tested in this study.

To determine the effect of folates on protecting cells from the cytotoxicity of oxidant H_2O_2, Intestine 407 was used in this study for evaluation. Intestine 407 cells were treated with 0 to 800 μg/mL of folate. The cell viability of Intestine 407 was increased due to the inhibition of oxidant H_2O_2 cytotoxicity by folates as shown in Table 5. Intestine 407 cells demonstrated higher viability when treated with higher concentration of folates.

In our previous study, lactic acid bacteria including

Table 4. Changes of viable cell counts in yogurt bacteria fermented milk during storage at 4˚C[a]

Cell count (cfu/mL) / Strain	Fresh	1 week[b]	2 weeks[b]
S. thermophilus ATCC 19258	6.2×10^8	5.9×10^8 (-4.8)	5.2×10^8 (-11.9)
S. thermophilus MC	6.7×10^7	6.1×10^7 (-9.0)	5.1×10^7 (-16.4)
L. bulgaricus 449	5.6×10^8	4.9×10^8 (-12.5)	3.8×10^8 (-22.4)
L. bulgaricus 448	4.7×10^8	2.7×10^8 (-31.9)	1.9×10^8 (-40.6)

[a] Data reported was obtained from experiments repeated three times.

[b] Values in parenthesis are percentage (%) changes.

Table 5. Inhibition of oxidant H_2O_2 cytotoxicity to Intestine 407 cells by folate[a]

Cells / Folate (μg/mL)	A_{492} for cells treated with 0.006% H_2O_2 and folate	Viability change (%)[b]	A_{492} for cells treated with 0.6% H_2O_2 and folate	Viability change (%)[b]
0	0.112[a]		0.083[a]	
100	0.119[a]	+0.8	0.085[a]	+0.4
200	0.136[a]	+3.0	0.089[a]	+1.1
400	0.154[b]	+5.2	0.090[a]	+1.4
800	0.168[b]	+6.9	0.099[a]	+3.1

[a] Data reported were means of experiments repeated three times. Values in the same column with different letter superscripts are significantly different ($P < 0.05$).

[b] The percentage increase of Intestine 407 cell viability was defined as follows: $\{[A_{492}(\text{Int 407 treated with } H_2O_2 \text{ and folate}) - A_{492}(\text{Int 407 treated with } H_2O_2)] / A_{492}(\text{Int 407 treated with } H_2O_2)\} \times 100\%$.

yogurt bacteria were found to be able to synthesize folates in fermented milk. These bacteria synthesized the maximum levels of folates when they were propagated at 37˚C for 6 hr. The effort of this study was focusing on increasing the folate contents of fermented milk by adding lactose or calcium chloride. The results of this study indicate that lactose helps increase the cell counts and folate levels for milk fermented with yogurt bacteria. The results also show that folate helps protect cells from the cytotoxicity of oxidant H_2O_2, which can cause oxidative damage to cells, including their nucleic acids. Folates function as cofactors in reactions involving 1-carbon transfer during the biosynthesis of nucleic acids[1,14]. Folates are, therefore, important in DNA replication and repair. This explains what was observed in this study. Although further *in vivo* study is needed to prove the effect of folates in humans, the results of this study demonstrate the potential of folates to help protect cells from oxidation. The improvement of cell viability can help improve health.

Yogurt and other fermented dairy products have been consumed for centuries in Western countries, and are also becoming popular with consumers in Taiwan. These probiotic bacteria have been reported to have health-promoting characteristics that make these microorganisms desirable for use in the production of dairy and other food products. These microorganisms are potential candidates for production of natural folate supplements or of functional foods that help provide healthy living and increase longevity.

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嗜熱鏈球菌及保加利亞乳桿菌合成葉酸之探討

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摘 要

本研究中探討嗜熱鏈球菌菌株MC與ATCC 19258及保加利亞乳桿菌菌株448與449合成葉酸之能力。在此研究中於發酵所用牛乳中添加乳糖與氯化鈣以期提高這些菌株合成葉酸的量。由實驗結果發現，乳糖之添加的確可提高菌株所合成的葉酸，雖然對培養6小時的菌株只提昇葉酸合成3至7%，但對培養18小時的菌株所合成之葉酸可提高12至198%。而氯化鈣的添加卻導致葉酸合成之降低，只有嗜熱鏈球菌菌株MC之葉酸合成提高。乳糖的添加同時使培養6小時與18小時之菌株其菌數增加，而氯化鈣之添加則對各菌株菌數的增減有不同之效果，但兩株保加利亞桿菌菌數皆增加。以這些菌株發酵之發酵乳探討其葉酸的量與菌數於4°C下儲存之變化，結果發現，葉酸在第二週時降低9至28%，菌數在此兩週保存期限內則維持穩定。本研究中同時以細胞株Intestine 407探討葉酸於氧化劑過氧化氫之細胞毒性下保護細胞之效果，實驗結果發現，葉酸可抑制氧化劑過氧化氫之細胞毒性，且當葉酸濃度提高時細胞株Intestine 407展現較高之存活率。

關鍵詞：嗜熱鏈球菌，保加利亞乳桿菌，葉酸，細胞存活率