The Effects of a Synbiotic Fermented Milk Beverage Containing *Lactobacillus casei* Strain Shirota and Transgalactosylated Oligosaccharides on Defecation Frequency, Intestinal Microflora, Organic Acid Concentrations, and Putrefactive Metabolites of Sub-Optimal Health State Volunteers: A Randomized Placebo-Controlled Cross-Over Study

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We evaluated the effects of ingestion of a synbiotic fermented milk beverage containing *Lactobacillus casei* strain Shirota (LcS) at $3 \times 10^{10}$ and transgalactosylated oligosaccharides (GOS) at 2.5 g per 80 ml (once a day, 2 weeks) on the defecation frequency in 35 female university students with constipation as well as the defecation frequency, intestinal microflora, and the levels of putrefactive metabolites in elderly persons in whom the intestinal microflora and the levels of putrefactive metabolites were abnormal in a placebo-controlled double-blind study. In the female students, the defecation frequency after 1 week of synbiotic fermented milk beverage ingestion was significantly higher than that after 1 week of placebo ingestion or before ingestion. In the elderly persons, the fecal *Bifidobacterium* and *Lactobacillus* bacterial counts after 1 and 2 weeks of synbiotic fermented milk beverage ingestion were significantly higher than those after placebo ingestion (p<0.05 and p<0.01, respectively). The fecal lecithinase-positive *Clostridium* bacterial count after 1 week of synbiotic fermented milk beverage ingestion and the fecal Enterobacteriaceae bacterial counts after 1 and 2 weeks of synbiotic fermented milk beverage ingestion were significantly lower than those after placebo ingestion (p<0.05). The acetic acid levels after 1 and 2 weeks of synbiotic fermented milk beverage ingestion were significantly higher than those after placebo ingestion (p<0.01). The stool pH values after 1 and 2 weeks of synbiotic fermented milk beverage ingestion and the ammonia and phenol levels after 2 weeks of synbiotic fermented milk beverage ingestion were significantly lower than those after placebo ingestion (p<0.05). These results suggest that ingestion of the synbiotic fermented milk beverage containing LcS and GOS improves the stool quality, intestinal microflora, and intestinal environment.

Key words: synbiotics; fermented milk beverage; bowel movements; fecal microflora; organic acid, putrefactive metabolite

INTRODUCTION

In Japan, increases in the consumption of European/American food and stress have recently elevated the incidences of intestinal disorders (25). In particular, the incidence of colorectal cancer has increased more than 2-fold during the past 10 years. It is indicated that colorectal cancer will soon comprise the highest mortality risk among various cancers, as demonstrated in Europe and the United States, exceeding the incidence of gastric cancer (29). Furthermore, infectious intestinal diseases represented by infection with *Escherichia coli* O-157 are an important issue (20). Along with a recent increase in people’s interests in intestine and enteric bacteria, some types of beneficial bacteria contained in yogurts, fermented milk, or other fermented foods have been recognized as the medical entity of probiotics (24). Recently, a collaborative FAO/WHO working group for preparing guidelines for evaluating probiotics defined
probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (24). Prebiotics represented by oligosaccharides are a non-digestive food component that contributes to the host’s health status by promoting the proliferation of probiotics and beneficial enteric bacteria or increasing their activity (11). In addition, when the two components are combined to achieve synergistic effects they are called synbiotics (10). Several studies have reported that foods containing Lactobacillus casei strain Shirota (LcS), known as a probiotic, or transgalactosylated oligosaccharides (GOS), a prebiotic, improve the intestinal microflora by increasing beneficial enteric bacteria and decreasing harmful bacteria, regulate the intestinal environment by decreasing the contents of putrefactive metabolites, and condition bowel movements in humans by increasing the defecation frequency (14, 16–18, 27). However, no study has reported the bowel movement-conditioning effects of a synbiotic food containing both LcS/other types of Lactobacillus and a prebiotic.

In this study, we conducted a placebo-controlled double-blind study using a synbiotic fermented milk beverage containing LcS at 3 \times 10^{10} and GOS at 2.5 g per 80 ml bottle and a placebo (also 80 ml). The synbiotic fermented milk beverage was made from liquid GOS, sugar, skim milk powder, liquid glucose/fructose, soybean polysaccharides, a flavoring agent, vitamin C, and vitamin E. The energy, protein, lipid, carbohydrate, sodium, vitamin C, and vitamin E contents per bottle (80 ml) were 57 kcal, 1.0 g, 0.1 g, 13.6 g, 18 mg, 30 mg, and 3 mg, respectively. The LcS bacterial count at ingestion was 3 \times 10^{10} or more. Placebo was prepared basically with the same ingredients and nutritional contents and with the same taste, color, pH and energy (by adjusting glucose and sugar compositions) as for the synbiotic fermented milk beverage except that it contained neither L. casei strain Shirota nor GOS; Acidic taste was adjusted by addition of lactic acid.

**MATERIALS AND METHODS**

**Subjects**

The student study: We conducted a screening test for 2 weeks on 90 female university students aged 19 to 22 years, and selected 35 who reported a defecation frequency of 9 times or less per 2 weeks. We excluded students with food allergy, those frequently skipping meals, and those with serious diseases. In addition, we excluded those periodically taking intestinal drugs or agents that may influence bowel movement-conditioning effects.

The elderly person study: The subjects were 20 healthy elderly persons in whom the intestinal microflora and environment were abnormal and we investigated the bowel movement-conditioning effects of the symbiotic fermented milk.

**Test diet**

We employed two test diets: a synbiotic fermented milk beverage containing LeS at 3 \times 10^{10} and GOS at 2.5 g per 80 ml bottle and a placebo (also 80 ml). The synbiotic fermented milk beverage was made from liquid GOS, sugar, skim milk powder, liquid glucose/fructose, soybean polysaccharides, a flavoring agent, vitamin C, and vitamin E. The energy, protein, lipid, carbohydrate, sodium, vitamin C, and vitamin E contents per bottle (80 ml) were 57 kcal, 1.0 g, 0.1 g, 13.6 g, 18 mg, 30 mg, and 3 mg, respectively. The LcS bacterial count at ingestion was 3 \times 10^{10} or more. Placebo was prepared basically with the same ingredients and nutritional contents and with the same taste, color, pH and energy (by adjusting glucose and sugar compositions) as for the synbiotic fermented milk beverage except that it contained neither L. casei strain Shirota nor GOS; Acidic taste was adjusted by addition of lactic acid.

**Study schedule and ingestion of the test diets**

We conducted a placebo-controlled double-blind cross-over study (Fig. 1). The study period was 9 weeks: Non-ingestion Period 1 (2 weeks), Ingestion Period 1 (2 weeks), Non-ingestion Period 2 (3 weeks), and Ingestion Period 2 (2 weeks). During the ingestion period, the test diet at 1 bottle per day was given to the subjects. The subjects were instructed to ingest the test diet at a specific time every day, and not to change their normal daily activities such as dietary and exercise habits.

**Examination methods**

Survey by diary: During the study period, the subjects recorded a diary regarding the defecation frequency, defecation hour, stool quantity, stool quality, health status, presence or absence of test diet ingestion, ingestion hour, and contents of meals every day by the 24 hr remembering method. Concerning the stool quantity, a sample for determining the stool quantity (a column measuring 1.5 cm in diameter and 5 cm in length) was delivered to the subjects, and they recorded the stool quantity as the number of sample bottles used (including the first decimal place). Furthermore, the subjects were instructed to avoid ingestion of milk products, foods containing oligosaccharides, and fermented soybeans as well as excessive ingestion of other milk products during the study period. However, there was no dietary, alcohol, or drug restriction. They recorded alcohol or drugs in a questionnaire. We excluded subjects who did not have the test diet on 3 or more of 28 days (period of test diet ingestion)(rate of test diet ingestion: less than 90%), and analyzed the study results.
Stool test

1. Sample collection and transport

In the elderly person study, stools were collected on the final day of Non-ingestion Period 1, at the ends of Weeks 1 and 2 of Ingestion Period 1, on the final day of Non-ingestion Period 2, and at the ends of Weeks 1 and 2 of Ingestion Period 2 (total: 6 times) for a stool test (Fig. 1). Immediately after collection, stools were stored in an anaerobic state using an Anaero Pack Kenki (MITSUBISHI GAS CHEMICAL COMPANY, INC., Tokyo), and the fecal microflora were investigated within 24 hr after collection.

2. Microflora test

Stools were weighed in an anaerobic glove box (CO₂: 5%, H₂: 4%, N₂: 91%), and homogenized. A 0.5 g sample was mixed with 4.5 ml of anaerobic transport medium (Lab lemco powder (Becton, Dickinson and Company, USA), 1% w/v; Bact Agar (Becton, Dickinson and Company), 0.05% w/v; sodium thioglycollate, 0.075% w/v; glycerin, 10% w/v; KH₂PO₄, 0.0225% w/v; K₂HPO₄, 0.0225% w/v; NaCl, 0.045% w/v; (NH₄)₂SO₄, 0.0225% w/v; CaCl₂, 0.00225% w/v; MgSO₄, 0.00225% w/v; Na₂CO₃, 0.3% w/v; L-cysteine 1 hydrate (Wako Pure Chemical Industries, Ltd.), 0.05% w/v; and resazurin, 0.0001% w/v; pH 7.4 to 7.6) to prepare a stool solution (dilution ratio: 10). This solution was serially diluted with an anaerobic dilution buffer (BACT Agar (Becton, Dickinson and Company), 0.05% w/v; Tween 80, 0.05% w/v; KH₂PO₄, 0.0225% w/v; K₂HPO₄, 0.0225% w/v; NaCl, 0.045% w/v; (NH₄)₂SO₄, 0.0225% w/v; CaCl₂, 0.00225% w/v; MgSO₄, 0.00225% w/v; Na₂CO₃, 0.3% w/v; L-cysteine HCl (Wako Pure Chemical Industries, Ltd.), 0.05% w/v; and resazurin, 0.0001% w/v) in multiples of 10. Various concentrations of samples at 0.05 ml were inoculated to various types of medium. A total of 10 items, i.e., total obligate anaerobe count, Bacteroidaceae, Bifidobacterium, lecithinase-positive Clostridium, Lactobacillus, Enterobacteriaceae, Enterococcus, Staphylococcus, Candida, and LeS, were examined using the following agar plate media, respectively: M10 (3), NBGT (1), TOS propionic acid agar (pH 5.7) (Eiken Chemical Co. Ltd., Tokyo), CW (Nikken BioMedical Laboratory, Inc., Kyoto), modified LBS (1), DHL (Nissui Pharmaceutical Co., Ltd., Tokyo), COBA (23), MSE (Nikken BioMedical Laboratory, Inc.), Sabouraud dextrose (Nikken BioMedical Laboratory, Inc.), and LLV agar medium (30). Anaerobic culture was performed using M10, NBGT, TOS propionic acid agar (pH 5.7), CW, and modified LBS agar medium. Using the other media, aerobic culture was performed. After culture at 37°C for a specific period (1 to 5 days), the number of colonies was measured. The colonies appearing in various media were morphologically classified, and Gram’s staining of the representative colonies was performed to examine the bacterial morphology. In the colonies appearing in COBA and MSE media, a catalase test was conducted, and Enterococcus and Staphylococcus were confirmed, respectively. LeS was identified by enzyme-linked immunosorbent assay (ELISA) with a strain-specific monoclonal antibody (30). The bacterial count was expressed as the logarithmic mean per gram of stool ± standard deviation. The lower detection limit was 2.3, and the detection rate was calculated as the number of positive samples/the number of tested samples.

3. Measurement of the fecal concentrations of organic acids

A portion of the homogenized stool was isolated, weighed, mixed with 0.15 M perchloric acid at a 4-fold volume, and reacted at 4°C overnight. Then, the mixture was centrifuged at 4°C at 15,000 rpm for 10 min, and the supernatant was filtrated with a 0.45-μm membrane filter.
(Millipore Japan, Tokyo), and sterilized. The concentrations of organic acids in this sample were measured using a Waters high-performance liquid chromatography (HPLC) system (Waters 432 Conductive Detector, Waters, USA) and a Shodex Rspack KC-811 column (Showa Denko, Tokyo) [13]. We prepared a standard mixed solution consisting of 1 to 20 mM succinic acid, lactic acid, formic acid, acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid, and calculated the concentrations of organic acids based on the standard curve.

<4> Stool pH and water content

The stool pH was measured by directly inserting the glass electrode of a D-51 pH meter (Horiba Seisakusho Co. Ltd., Tokyo) into the homogenized stool. The stool water content was calculated as weight differences between before and after freeze-drying of a portion of the stool.

<5> Analysis of fecal putrefactive metabolites

We measured the fecal levels of indole, ammonia, phenol, and p-Cresol. A stool sample weighing approximately 2.5 g was mixed with 0.1 M phosphate buffer (PB) (pH 5.5) at 9 times the stool weight, homogenized with glass beads, and filtrated with gauze. A 10-fold serial dilution of this sample was used for measurement. To measure the levels of phenol and p-Cresol, the sample was mixed with PB at 9 times the stool weight, and homogenized using a stomacher (Organo Corporation, Tokyo). The stool suspension was stored at –30°C until measurement. Various putrefactive metabolites were measured as described below. 1) Indole: A coloring reaction test was performed immediately after the dilutions were prepared. To 1.5 ml of a coloring solution prepared by dissolving 14.7 g of p-dimethyl aminobenzaldehyde in a sulfuric acid/alcohol mixture (52 ml of concentrated sulfuric acid and 948 ml of 95% ethanol) or a control coloring solution (the sulfuric acid/alcohol mixture), 0.3 ml of a 70-fold dilution of a stool sample was added. The mixture was immediately stirred, reacted at room temperature for 20 min, and centrifuged at 1,390 × G for 10 min. The supernatant was placed on a microplate at 0.2 ml/well, and the absorbance at 570 nm was determined using a fluorescence detector (excitation wavelength: 260 nm, measuring wavelength: 305 nm) and a UV detector (270 nm).

Statistical analysis

Non-ingestion Periods 1 and 2 were established before Ingestion Periods 1 and 2, respectively. The values obtained at Week 2 of Non-ingestion Period 1 and at Week 3 of Non-ingestion Period 2 were analyzed as pre-ingestion values. The survey parameters in the diary were analyzed using the weekly collected data. The results were compared between the synbiotic fermented milk beverage group and the placebo group before ingestion and after 1 and 2 weeks of ingestion. In addition, the pre-ingestion values were compared with the values after 1 and 2 weeks of ingestion.

The defecation frequency, number of days with bowel movements, stool quantity, and fecal microflora were analyzed using non-parametric paired Wilcoxon’s rank sum test. The bacterial detection rate was analyzed using Fisher’s direct probability test. The stool pH and water content were analyzed using the paired t-test. We employed SPSS Ver. 11 software (SPSS Japan Inc., Tokyo). p<0.05 was regarded as significant.

RESULTS

Student study

Influence on the defecation frequency, number of days with bowel movements, and stool quantity: The mean age, height, body weight, and body mass index (BMI) of the 35 female students enrolled in the study were 19.4 ± 0.8 years, 158.3 ± 4.2 cm, 51.2 ± 6.6 kg, and 20.6 ± 2.2,
respectively.

Before ingestion, the mean defecation frequency per week was 4.0 ± 1.5 times and 4.4 ± 2.2 times in the synbiotic fermented milk beverage and placebo groups, respectively (Table 1). There were no significant differences in the defecation frequency, number of days with bowel movements, or stool quantity between the two groups. The defecation frequency and number of days with bowel movements after 1 week of synbiotic fermented milk beverage ingestion were significantly higher than the values after 1 week of placebo ingestion (p<0.05, respectively). After 2 weeks of ingestion, there were no significant differences in any parameters between the two groups. There were no significant differences in stool quantity after 1 or 2 weeks of test diet ingestion between the two groups. The defecation frequency and number of days with bowel movements after 1 week of synbiotic fermented milk beverage ingestion were significantly higher than the values before ingestion (p<0.05, respectively). However, there were no significant changes in the placebo group. After 2 weeks of ingestion, there were no marked changes in these parameters in comparison to the pre-ingestion values in either group. However, the means of during the 2-week ingestion period in the synbiotic milk beverage group were significantly higher than the means before ingestion and after 1 and 2 weeks of placebo ingestion (data not shown).

**Elderly person study**

Influence on the defecation frequency, number of days with bowel movements, and stool quantity: The mean age, height, body weight, and BMI of the 20 subjects (5 males, 15 females) were 74.4 ± 6.6 years, 156.0 ± 8.2 cm, 58.1 ± 10.8 kg, and 23.8 ± 3.8, respectively.

Before ingestion, the mean defecation frequency per week was 8 times or more in the synbiotic fermented milk beverage and placebo groups (Table 2). There were no significant differences in the defecation frequency, number of days with bowel movements, or stool quantity after ingestion of the test diet between the two groups. After ingestion, there were no marked changes in comparison to the pre-ingestion values.

Influence on fecal microflora: There was no significant difference in the fecal microflora before ingestion between the synbiotic fermented milk beverage and placebo groups (Table 3). However, the Bifidobacterium and Lactobacillus bacterial counts after 1 week of ingestion in the synbiotic fermented milk beverage group were significantly higher than those in the placebo group (p<0.05 and p<0.01, respectively). These values were also significantly higher than the pre-ingestion values (p<0.05 and p<0.01, respectively). In addition, these counts after 2 weeks of synbiotic fermented milk beverage ingestion significantly differed from those in the placebo group or the pre-ingestion values. In the synbiotic fermented milk beverage group, the Bifidobacterium bacterial count further increased after 2 weeks of ingestion compared to that after 1 week of ingestion. After 1 and 2 weeks of ingestion, ingested LcS was collected at bacterial counts of 7.2 ± 0.8 and 7.4 ± 1.9 (Log) per gram of stool, respectively. After 1 and 2 weeks of synbiotic fermented milk beverage ingestion, the Lactobacillus bacterial counts were 7.8 ± 0.7, significantly higher than the collected LcS levels (p<0.05, respectively). In the synbiotic fermented milk beverage group, the lecithinase-positive Clostridium and Enterobacteriaceae bacterial counts after 1 week of ingestion were significantly lower than those in the placebo group (p<0.05, respectively). After 2 weeks of ingestion, the Enterobacteriaceae bacterial count in this group remained lower than that in the placebo group (p<0.05). There were no significant differences in the total obligate anaerobe count or Bacteroidaceae, Enterococcus, Staphylococcus, or Candida counts between the two groups.

Influence on fecal levels of organic acids: Before ingestion of the test diet, there were no significant differences in the fecal levels of organic acids between the synbiotic fermented milk beverage and placebo groups. After 1 week of ingestion, the total organic acid, acetic acid, and butyric acid levels in the synbiotic fermented milk beverage group were significantly higher than those in the placebo group (p<0.05, p<0.01, and p<0.05, respectively)(Table 4). After 2 weeks of ingestion, the fecal level of acetic acid in the synbiotic fermented milk beverage group remained higher than that in the placebo group (p<0.01). Simultaneously, the fecal level of succinic acid was lower than that in the placebo group (p=0.06).

Influence on the stool pH and water content: The stool pH values after 1 and 2 weeks of ingestion in the synbiotic fermented milk beverage group were significantly lower than those in the placebo group (p<0.05, respectively)(Table 5). There were no significant differences in the water content between the two groups (Table 5).

**Influence on fecal putrefactive metabolites:** There were no significant differences in the fecal levels of putrefactive metabolites before ingestion or after 1 week of ingestion between the synbiotic fermented milk beverage and placebo groups (Table 5). However, after 2
weeks of ingestion, the fecal levels of ammonia and phenol in the synbiotic fermented milk beverage group were significantly lower than the values in the placebo group (p<0.05, respectively). There were no significant differences in the fecal levels of indole or p-Cresol.

DISCUSSION

Deguchi et al. conducted a study with a GOS-containing beverage in females (age: 17 to 29 years) and males (age: 19 to 45 years) with mild constipation, and reported that the defecation frequency and number of days with bowel movements in the group taking 5 g of GOS were significantly higher than those in the placebo group, but there were no significant changes in the group taking 2.5 g of GOS (6). In this study, we gave a synbiotic fermented milk product containing LcS and GOS to female university students with mild constipation (age: 19 to 22 years), and the defecation frequency and number of days with bowel movements after 1 week of ingestion in the synbiotic fermented milk beverage group were higher than those in the placebo group, suggesting that this product markedly improved the defecation frequency via synbiotic coordination between LcS and GOS, because the GOS content of this product was 2.5 g. However, after 2 weeks of ingestion, there was no improvement in the defecation frequency, possibly because continuous ingestion of oligosaccharides promoted proliferation of Bifidobacterium in the large intestine, which rapidly metabolizes oligosaccharides, restricting large intestine osmotic pressure. Essentially, a low molecular weight GOS-related increase in large intestine osmotic pressure and the promotion of peristalsis related to organic acids produced via the fermentation of Lactobacillus and intrinsic Bifidobacterium may be involved in the action mechanism by which synbiotic fermented milk improves defecation problems (21). Recently, Matsumoto et al. investigated the intestine-conditioning effects of a
Table 3. Effect of synbiotic fermented milk beverage containing L. casei strain Shirota and transgalactosylated oligosaccharides on fecal microflora in the elderly persons

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Before ingestion</th>
<th>Week 1 of ingestion</th>
<th>Week 2 of ingestion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Symbiotic milk</td>
<td>Placebo</td>
<td>Symbiotic milk</td>
</tr>
<tr>
<td>Total anaerobes</td>
<td>10.2 ± 0.3 (17/17)</td>
<td>10.2 ± 0.4 (17/17)</td>
<td>10.3 ± 0.2 (17/17)</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>8.3 ± 1.2 (17/17)</td>
<td>8.3 ± 1.5 (17/17)</td>
<td>8.9 ± 1.1b, c (17/17)</td>
</tr>
<tr>
<td>Clostridum (L+)a</td>
<td>4.1 ± 1.6 (11/17)</td>
<td>4.2 ± 1.4 (10/17)</td>
<td>3.5 ± 1.3b (9/17)</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>7.0 ± 0.4 (17/17)</td>
<td>6.7 ± 1.6 (17/17)</td>
<td>7.8 ± 0.7dd, cc (17/17)</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>6.9 ± 1.5 (17/17)</td>
<td>7.2 ± 1.2 (17/17)</td>
<td>6.6 ± 1.1b (17/17)</td>
</tr>
<tr>
<td>Enterococcus</td>
<td>7.2 ± 1.1 (17/17)</td>
<td>6.9 ± 1.5 (17/17)</td>
<td>6.9 ± 1.2 (17/17)</td>
</tr>
<tr>
<td>Staphylococcus</td>
<td>3.6 ± 0.9 (10/17)</td>
<td>3.4 ± 0.9 (9/17)</td>
<td>3.2 ± 0.8 (11/17)</td>
</tr>
<tr>
<td>Candida</td>
<td>4.3 ± 1.2 (12/17)</td>
<td>4.2 ± 1.2 (12/17)</td>
<td>4.1 ± 1.1 (12/17)</td>
</tr>
<tr>
<td>L. casei strain Shirota ND</td>
<td>ND (0/17)</td>
<td>ND (0/17)</td>
<td>7.2 ± 0.8 (17/17)</td>
</tr>
</tbody>
</table>

Organisms: Synbiotic fermented milk beverage containing L. casei strain Shirota and transgalactosylated oligosaccharides on their concentration in the elderly persons.

Table 4. Effect of synbiotic fermented milk beverage containing L. casei strain Shirota and transgalactosylated oligosaccharides on the concentration of organic acids in the elderly persons

<table>
<thead>
<tr>
<th>Item</th>
<th>Before ingestion</th>
<th>Week 1 of ingestion</th>
<th>Week 2 of ingestion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Symbiotic milk</td>
<td>Placebo</td>
<td>Symbiotic milk</td>
</tr>
<tr>
<td>Total organic acids</td>
<td>125 ± 37 (17/17)</td>
<td>110 ± 38 (17/17)</td>
<td>129 ± 37 (17/17)</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>5.9 ± 10.7 (13/17)</td>
<td>6.5 ± 10.7 (12/17)</td>
<td>1.7 ± 1.4 (14/17)</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>0 (0/17)</td>
<td>2.7 (2/17)</td>
<td>3.0 ± 1.1 (4/17)</td>
</tr>
<tr>
<td>Formic acid</td>
<td>1.1 ± 0.3 (5/17)</td>
<td>1.8 ± 2.6 (9/17)</td>
<td>1.1 ± 0.4 (5/17)</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>67 ± 24 (17/17)</td>
<td>60.6 ± 22.6 (17/17)</td>
<td>72 ± 18a (17/17)</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>22 ± 7 (17/17)</td>
<td>15.7 ± 8.1 (17/17)</td>
<td>24 ± 10 (17/17)</td>
</tr>
<tr>
<td>iso-Butyric acid</td>
<td>2.4 ± 0.9 (15/17)</td>
<td>1.8 ± 1.1 (14/17)</td>
<td>2.1 ± 1.0 (15/17)</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>23 ± 12 (17/17)</td>
<td>15.7 ± 11.4 (17/17)</td>
<td>24 ± 16b (17/17)</td>
</tr>
<tr>
<td>iso-Valeric acid</td>
<td>4.1 ± 2.3 (15/17)</td>
<td>3.7 ± 2.6 (11/17)</td>
<td>3.3 ± 2.6 (15/17)</td>
</tr>
<tr>
<td>Valeric acid</td>
<td>2.5 ± 0.8 (13/17)</td>
<td>2.4 ± 1.6 (12/17)</td>
<td>2.4 ± 1.2 (12/17)</td>
</tr>
</tbody>
</table>

The results are expressed as the mean μmol and S.D. per gram of feces. The results in parentheses are the numbers of samples in which the bacteria were detected/the number of samples examined. ND: Not detected (<2.3 Log10 c.f.u. and S.D. per g). aLecithinase positive Clostridium. bp<0.05, bpb<0.01: Significant difference between the synbiotic fermented milk beverage and the placebo (Wilcoxon signed-rank test).

The results are expressed as the mean Log10 c.f.u. and S.D. per gram of feces. The results in parentheses are the number of samples in which the organic acids were detected/the number of samples examined.  

fermented milk beverage containing L. casei strain Shirota at 4 × 10¹⁰ bacteria, and reported that the defecation frequency after 1 and 2 weeks of ingestion was significantly higher than that before ingestion. However, in their study, ingestion of the beverage decreased the fecal levels of organic acids (17). Ingestion of fermented milk may promote the absorption of organic acids in the intestinal tract. In the future, whether changes in the intestinal levels of organic acids influence constipation alleviation should be further investigated.

Mitsuoka et al. investigated fecal microflora in 72 healthy elderly persons aged 65 to 86 years, and compared it with that in 29 healthy adults aged 20 to 64 years. In the elderly persons, the Bifidobacterium bacterial count and detection rate were significantly lower, and the lecithinase-positive Clostridium bacterial count and detection rate were significantly higher (19). In that study, a pre-ingestion test confirmed abnormalities in the intestinal flora and environment in the elderly persons leading a normal healthy daily life with a mean age of 74 years; the Bifidobacterium bacterial count was lower, the detection rates for lecithinase-positive Clostridium and Candida were higher, and the ammonia and p-Cresol levels were higher than the reference values for healthy adults. In the
Table 5. Effect of synbiotic fermented milk beverage containing L. casei strain Shirota and transgalactosylated oligosaccharides on fecal pH, water content, and putrefactive metabolites in the elderly persons

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Before ingestion</th>
<th>Week 1 of ingestion</th>
<th>Week 2 of ingestion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Synbiotic milk</td>
<td>Placebo</td>
<td>Synbiotic milk</td>
</tr>
<tr>
<td></td>
<td>beverage</td>
<td></td>
<td>beverage</td>
</tr>
<tr>
<td>pH</td>
<td>6.7 ± 0.3 (17/17)</td>
<td>6.8 ± 0.4 (17/17)</td>
<td>6.7 ± 0.3 (17/17)</td>
</tr>
<tr>
<td>Water content (％)</td>
<td>73.0 ± 6.9 (17/17)</td>
<td>75.5 ± 10.6 (17/17)</td>
<td>71.3 ± 12.4 (17/17)</td>
</tr>
<tr>
<td>Indole</td>
<td>1.7 ± 1.0 (17/17)</td>
<td>1.8 ± 2.4 (17/17)</td>
<td>1.3 ± 0.7 (17/17)</td>
</tr>
<tr>
<td>Ammonia</td>
<td>675 ± 352 (17/17)</td>
<td>597 ± 442 (17/17)</td>
<td>525 ± 302 (17/17)</td>
</tr>
<tr>
<td>Phenol</td>
<td>4.5 ± 7.0 (14/17)</td>
<td>3.6 ± 5.5 (13/17)</td>
<td>3.9 ± 7.2 (16/17)</td>
</tr>
<tr>
<td>p-Cresol</td>
<td>78 ± 55 (15/17)</td>
<td>72 ± 68 (15/17)</td>
<td>66 ± 67 (17/17)</td>
</tr>
</tbody>
</table>

The results are expressed as the mean and S.D. in μmol/g feces for indole, in μg/g feces for ammonia, Phenol and p-Cresol. The results in the parentheses are the number of samples in which the putrefactive metabolites were detected/the number of samples examined.

\*p<0.05: Significant difference between the synbiotic fermented milk beverage and the placebo (Student’s paired t-test).

\*p<0.05: Significant difference between the synbiotic fermented milk beverage and the placebo (Wilcoxon signed-rank test).

In the elderly person study, no subject showed constipation prior to ingestion of the test diet. However, in future studies, the correlation between disordered intestinal flora/environment and constipation should be examined in a larger number of elderly persons.

In the elderly person study, the stool Bifidobacterium bacteria count after 1 week of ingestion in the synbiotic fermented milk beverage group was significantly higher than that in the placebo group, and further increased after 2 weeks of ingestion. In the synbiotic fermented milk beverage group, the counts of harmful bacteria such as lecinthinase-positive Clostridium and Enterobacteriaceae were significantly lower than the values in the placebo group. In addition, the stool pH value was significantly lower, and the level of acetic acid was significantly higher. The GOS contained in this synbiotic fermented milk beverage product is a Bifidobacterium-selective growth factor (12), and the main metabolite of Bifidobacterium is acetic acid. Acetic acid has potent bactericidal activity against pathogenic Enterobacteriaceae (4, 8, 9, 22), and several studies have suggested that the bactericidal actions of acetic acid are achieved in a non-dissociation state (4, 7). Based on these findings, Bifidobacterium proliferating in the large intestine, increasing the ammonia and phenol levels after ingestion of the test diet were similar to the reference values for healthy adults. However, the ammonia and p-Cresol levels were markedly higher (synbiotic fermented milk beverage group: 675 ± 352, 78 ± 55, placebo group: 597 ± 442, 72 ± 68, respectively). It is known that intestinal ammonia and p-Cresol are produced via the enteric bacteria-related decomposition of amino acids (2, 15). The intestinal ammonia and p-Cresol levels increase with a high-protein diet-related increase in the substrates utilized by enteric bacteria (5). Therefore, in the elderly subjects, an age-related reduction of digestive tract function such as digestion/absorption and peristalsis may have allowed a massive influx of the substrates into the large intestine, increasing the ammonia and p-Cresol levels. In the synbiotic fermented milk beverage group, the fecal ammonia and phenol levels were significantly lower than the values in the placebo group. The production of ammonia and phenol is inhibited at a low pH (26, 28). Therefore, concerning the mechanism by which ingestion of synbiotic fermented milk beverage inhibits the fecal production of putrefactive metabolites, an increase in the intestinal organic acid (acetic acid) level may reduce ammonia/phenol-producing bacteria isolated in the human intestine, including L. casei, metabolized GOS (12). Therefore, synbiotic fermented milk beverage GOS may have increased intrinsic Lactobacillus; however, the mechanism should be investigated in a future study.

Matsumoto et al. measured the fecal levels of putrefactive metabolites in 22 healthy adults (21 males, 1 female) with a mean age of 39 ± 10 years, and reported that the indole, ammonia, phenol, and p-Cresol levels were 1.8 ± 0.7 μmol/g, 297 ± 203 μg/g, 3.2 ± 3.2 μg/g, and 29 ± 41 μg/g, respectively (18). In the elderly person study, the fecal indole and phenol levels before ingestion of the test diet were similar to the reference values for healthy adults. However, the ammonia and p-Cresol levels were markedly higher (synbiotic fermented milk beverage group: 675 ± 352, 78 ± 55, placebo group: 597 ± 442, 72 ± 68, respectively). It is known that intestinal ammonia and p-Cresol are produced via the enteric bacteria-related decomposition of amino acids (2, 15). The intestinal ammonia and p-Cresol levels increase with a high-protein diet-related increase in the substrates utilized by enteric bacteria (5). Therefore, in the elderly subjects, an age-related reduction of digestive tract function such as digestion/absorption and peristalsis may have allowed a massive influx of the substrates into the large intestine, increasing the ammonia and p-Cresol levels. In the synbiotic fermented milk beverage group, the fecal ammonia and phenol levels were significantly lower than the values in the placebo group. The production of ammonia and phenol is inhibited at a low pH (26, 28). Therefore, concerning the mechanism by which ingestion of synbiotic fermented milk beverage inhibits the fecal production of putrefactive metabolites, an increase in the intestinal organic acid (acetic acid) level may reduce ammonia/phenol-producing bacteria.
such as Enterobacteriaceae, Clostridium, and Staphylococcus, and restrict an elevation of pH, as observed in the placebo group, inhibiting the production of putrefactive metabolites.

In this study, ingestion of the synbiotic fermented milk product containing LcS and GOS improved defecation problems in the students with mild constipation (increases in the defecation frequency and number of days with bowel movements) and the intestinal flora and environment in the elderly persons (an increase in beneficial bacteria, a decrease in harmful bacteria, and decreases in the levels of putrefactive metabolites). Our study is the first report in which the intestine-conditioning actions of symbiotic beverage, such as improvement in defecation frequency, an increase in beneficial bacteria, a decrease in harmful bacteria, and improvement in the intestinal environment, were demonstrated when compared with the placebo beverage in the same ingestion test. In future studies, the roles of LcS and GOS in synbiotic fermented milk-related improvement in the stool quality and intestinal flora/environment should be analyzed.

REFERENCES


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