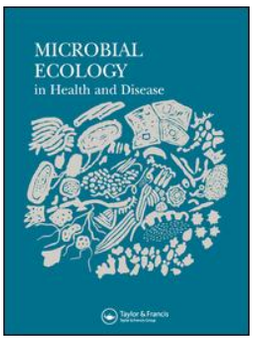

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Effect of *Bifidobacterium longum* BB536 yogurt administration on the intestinal environment of healthy adults.

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A yogurt supplemented with *B. longum* BB536 was administered at 250 ml per day for 2 weeks to six healthy volunteers. The effects on the fecal microflora, fecal putrefactive substances, fecal enzymatic activities and fecal properties were examined and compared with the effects of standard yogurt. A significant increase ($p < 0.05$) in the proportion of *Bifidobacterium* in the fecal microflora was observed following ingestion of yogurt containing *B. longum* BB536. The numbers of *Lactobacillus* also increased significantly ($p < 0.05$). *Clostridium* sp. and total aerobic bacteria in the feces tended to decrease. The level of some putrefactive substances, including ammonia ($p < 0.05$), indole and *p*-cresol decreased, whereas the levels of short chain and volatile fatty acids increased significantly ($p < 0.01$). Urease activity decreased concomitant with the decrease in ammonia levels. These findings suggested that administration of yogurt containing *B. longum* BB536 was effective to improve the intestinal environment. Similar effects were observed with standard yogurt but they were less evident than in the case of yogurt containing *B. longum* BB536. *Key words*: bifidobacteria, *Bifidobacterium longum*, yogurt, fecal flora, intestinal environment.

INTRODUCTION

Bifidobacteria are well known human intestinal bacteria. They represent the predominant member of the microflora in the intestine of infants (1). The number of bifidobacteria decrease with age and *Bacteroides* and other intestinal bacteria replace bifidobacteria to become the dominant members of the intestinal microflora in mature adults. Such change in microflora influences the intestinal environment, because bifidobacteria and lactobacilli are known to create a favorable intestinal environment by suppressing the proliferation of unfavorable bacteria (2). The intestinal environment affects human health (2). Bifidobacteria have been used as a probiotic supplement in pharmaceutical products, health foods and dairy products. Many *Bifidobacterium*-supplemented dairy products have been marketed since the 1980s especially in Japan (3) and European countries (4). These now account for a considerable share of the dairy products market. Among such products, Bifidus yogurt is the major product sold.

Bifidobacteria comprise more than 30 species, and *B. longum* is the most common species found in the intestines of humans of all ages, from infant to adult. *B. longum* BB536 was initially isolated from the feces of a healthy infant (5) and has been used in many dairy products. Ingestion of *B. longum* BB536 has been reported to reduce cancer risk, (6, 7) enhance immunity (8) and increase bone density in animal experiments (9). In this report, the authors studied the effects of a yogurt supplemented with *B. longum* BB536 on the intestinal environment of human volunteers by analyzing the intestinal microflora, levels of putrefactive substances, enzyme activities and fecal characters. These effects were compared with those of standard yogurt that was not supplemented with *B. longum* BB536.

MATERIALS AND METHODS

Yogurt

The yogurt supplemented with *B. longum* BB536 (BB536 yogurt) was prepared by Morinaga Milk Industry Co. Ltd., and is a plain yogurt containing 3% milk fat and 9.5% non-fat milk solid contents. It was prepared using cultures of *B. longum* BB536, *Streptococcus thermophilus* STH450 and *Lactobacillus delbrueckii* subsp. *bulgaricus* LBU108. The standard yogurt used as the control diet was prepared using the same bacterial cultures but without *B.*

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longum BB536. The BB536 yogurt contained more than 2×10^7/ml of viable *B. longum* BB536, as enumerated by the selective MGLP-medium (10) incubated under anaerobic condition.

Subjects and experimental schedule

Six healthy volunteers (two males, four females; aged 21–42 years) were informed of the content of the study (purpose and procedures) and gave consent to participate the test. The experimental duration was eight weeks which was divided into four consecutive periods: [1] Yogurt free period A (Control period A: weeks 1 and 2), [2] BB536 yogurt administration period (BB536 yogurt period: weeks 3 and 4), [3] Yogurt free period B (Control period B: weeks 5 and 6), [4] Standard yogurt administration period (Standard yogurt period: weeks 7 and 8). During the BB536 yogurt period and the standard yogurt period each subject ingested 250 ml of the assigned yogurt per day. The number of viable *B. longum* BB536 in 250 ml of BB536 yogurt was more than 5×10^9. Through the entire experimental period the volunteers did not consume any liquid milk products, bifidogenic oligosaccharides, or fermented dairy products prepared using lactic acid bacteria.

Collection of feces

Fecal analysis was performed twice during the each of the four consecutive periods (usually at the end of each week). Whole portions of freshly voided feces were collected and transported anaerobically. Fecal samples were kept at 5°C until analysis. Microflora and biochemical analyses were performed within 12 hours after excretion. Specimens for the analysis of putrefactive products were frozen at -20°C immediately after weighing, and specimens for analyses of organic acids and ammonia were frozen in 2% perchloric acid at -20°C after weighing. The frozen samples were thawed prior to analysis.

Fecal microflora and enzyme activities analyses

The methods of fecal microflora analysis were basically identical to those reported by Mitsuoka (11, 12). The media used are shown in Table I. After thorough mixing of a fecal specimen, serial dilutions were made in anaerobic diluent. Then 0.05 ml aliquots of the appropriate dilutions were spread onto three non-selective agar plates (modified Eggerth-Gagnon [EG] agar and glucose-blood-liver [BL] agar for anaerobes, and Trypticase soy [TS] agar with 5% blood for aerobes), as well as 11 selective agar plates (media for bifidobacteria [BS], eubacteria [ES], bacteroides [NBGT], lactobacilli [LBS], *C. perfringens* s[NN], veillonella [VS], enterobacteria [DHL], streptococci [TATAC], staphylococci [PEES], yeast [P] and clostridia [CC: EG medium supplemented with 0.001% crystal violet and 0.001% colimycin]). One plate each of EG, BL, BS, ES, NBGT, LBS, NN and CC media were incubated at 37°C for three days in an anaerobic steel-wool jar (13) filled with an atmosphere of oxygen-free CO_2. TATAC, PEES, PDA, DHL and TS media were incubated aerobically at 37°C for 1 or 2 days. After incubation, each plate was examined for bacterial colonies. Identification of 13 bacterial groups, yeast and molds was based on colonial and cellular morphologies, gram-reaction, spore formation and aerobic growth. For each bacterial species identified, the colony count per gram of wet feces was calculated and converted into a logarithmic equivalent. Total viable count was calculated from the sum of the counts of all the bacterial species.

All procedures for determination of enzymatic activities except for nitroreductase were conducted under aerobic

Table I

Media and culture methods

Medium	Main enumerated organisms	Incubation methods	Incubation times (days)
Non-selective media			
EG agar	Anaerobes	Steel wool jar	3
BL agar	Anaerobes	Steel wool jar	3
TS agar	Aerobes	Aerobic condition	1–2
Selective media			
BS agar	*Bifidobacterium*	Steel wool jar	3
ES agar	*Eubacterium*	Steel wool jar	3
NBGT agar	*Bacteroidaceae*	Steel wool jar	3
VS agar	*Veillonela*	Steel wool jar	3
CC agar	*Clostridium*	Steel wool jar	3
NN agar	*Clostridium perfringens*	Steel wool jar	3
LBS agar	*Lactobacillus*	Steel wool jar	3
DHL agar	*Enterobacteriaceae*	Aerobic condition	1–2
TATAC agar	*Streptococcus*	Aerobic condition	1–2
PEES agar	*Staphylococcus*	Aerobic condition	1–2
P agar	Yeast and Molds	Aerobic condition	1–2

Table II

Changes of bacterial numbers in the fecal microflora

	Control A	BB536 yogurt	Control B	Control yogurt
Enterobacteriaceae	7.9 ± 0.9[a] (100)[b]	7.6 ± 0.7 (100)	7.9 ± 0.6 (100)	7.7 ± 0.5 (100)
Streptococcus	7.3 ± 0.8 (100)	$6.8 \pm 0.8^{\dagger}$ (100)	7.2 ± 0.5 (100)	8.0 ± 0.5 (100)
Staphylococcus	3.8 ± 0.7 (75)	3.2 ± 0.7 (75)**	3.5 ± 0.9 (83)	3.2 ± 0.7 (75)
Yeast	3.2 ± 0.6 (33)	2.8 ± 0.7 (67)	2.6 ± 0.4 (25)	3.5 ± 1.1 (58)
Lactobacillus	5.7 ± 0.7 (75)	7.1 ± 1.0* (58)	6.4 ± 1.1 (75)	5.1 ± 1.5 (67)
Bifidobacterium	9.8 ± 0.4 (92)	10.0 ± 0.1 (100)	9.8 ± 0.3 (100)	9.9 ± 0.4 (100)
Eubacterium	9.7 ± 0.6 (100)	9.7 ± 0.6 (100)	9.7 ± 0.2 (100)	9.9 ± 0.2 (100)
Bacteroidaceae	10.4 ± 0.2 (100)	10.3 ± 0.1 (100)	10.3 ± 0.3 (100)	10.2 ± 0.2 (100)
Peptococcaceae	8.6 ± 2.4 (67)	8.9 ± 0.8 (83)	9.3 ± 0.4 (100)	9.1 ± 0.6 (92)
Clostridium perfringens	5.0 ± 0.3 (58)	4.8 ± 1.9 (25)	5.6 ± 1.5 (50)	4.9 ± 1.3 (25)
Clostridium others	7.1 ± 1.4 (75)	6.4 ± 1.5 (75)	7.5 ± 0.4 (83)	7.4 ± 0.5 (50)
Veillonella	5.1 ± 1.8 (83)	5.8 ± 1.2 (67)	5.8 ± 1.5 (75)	5.6 ± 0.7 (75)
Others	7.6	5.9 ± 1.7 (25)		5.0 ± 3.1 (17)
Total aerobic bacteria	8.4 ± 0.6	7.9 ± 0.7	8.1 ± 0.4	8.5 ± 0.4
Total counts	10.6 ± 0.1	10.6 ± 0.1	10.5 ± 0.2	10.6 ± 0.2

[a] Bacterial counts expressed as mean $\pm$SD of $\log_{10}$ CFU/gram of wet feces.
[b] Frequency of occurrence (%)
*,† Significantly different ($p < 0.05$) compared with control period A(*) and control yogurt period (†).
** Significantly different ($p < 0.05$) compared with Control period B (Chi-square test).

conditions. Fecal samples were suspended in 0.1 M phosphate buffer (pH 7.0) and then centrifuged at $3000 \times$ g for 2 min. The supernatant was used for assays of urease and β-glucuronidase activities. The supernatant was sonicated for 10 min prior to determination of tryptophanase activity. Urease activity was determined by measuring the ammonia concentration after incubation at 37 for 20 min with 10 mM urea as substrate, using an ammonia test kit (Wako Pure Chemical Industries Osaka Japan) (14). Beta-glucuronidase activity was determined by measuring the amount of nitrophenol released after incubation at 37°C for 20 min with 3 mM *p*-nitrophenyl-D-glucuronide as substrate (15). Tryptophanase activity was determined by measuring the indole concentration after incubation at 37° for 60 min with 8 mM tryptophan (pH 7.5) as substrate (16). Nitroreductase activity was measured by using anaerobic (nitrogen replaced) phosphate buffer (0.1 M, pH 7.0) (15).

Determination of fecal putrefactive products and fatty acids

Fecal putrefactive products (phenol, *p*-cresol, 4-ethylphenol, indole and skatole) were measured by a slight modification of the methods of Yoshihara (17, 18). Fecal samples were homogenized in 30 mM potassium phosphate buffer (pH 8.5) and iso-propylphenol was added at a final concentration of 50 mM as internal standard. The homogenate was steam-distilled and analyzed by gas chromatography. The concentrations of short chain fatty acids and ammonia in the feces were measured as follows. One gram of fecal sample was homogenized in 9 ml of 2% perchloric acid and then centrifuged at $20000 \times$ g for 20 min. The supernatant was used for determination of short chain fatty acid by high pressure liquid chromatography, and ammonia using an ammonia test kit (Wako Pure Chemical Industries).

Statistical analysis and other

Results are presented as mean $\pm$SD. The statistical significance of differences was examined by Student's *t*-test and $p < 0.05$ were regarded as significant. For the analysis of bacterial counts, positive values are taken into account. Chi-square test or Fisher's exact probability test was used to analyze frequency of occurrence. This study was performed in accordance with the Helsinki Declaration. The ethical committee of Morinaga Milk Industry Co. Ltd. reviewed the protocol and approved the study.

RESULTS

The two analytical data obtained from the first and the second fecal samples in each test period showed some minor random fluctuations. Therefore they were averaged and compared with the values of the other test periods.

Fecal microflora

Table II shows the changes of bacterial counts and Table III shows the proportion (percentage) of the main bacterial groups in the fecal microflora during the four experimental periods. The average numbers of *Bifidobacterium* and *Lactobacillus* were high during the period in which BB536 yogurt was ingested. The increase in numbers of *Lactobacillus* was significant compared to the

control period A. The proportion of *Bifidobacterium* increased significantly ($p < 0.05$) during the period in which BB536 yogurt was ingested compared to the control period A (Table III), although the change in number of these bacteria was modest. The average proportion of *Lactobacillus* in the BB536 yogurt period was 0.15% and higher than in the other periods (Control A: 0.002%, Control B: 0.08%, Standard yogurt: 0.04%) but the increase was not significant. The numbers of *Enterobacteriaceae* were lower (not significantly) during both yogurt ingestion periods. *Streptococcus* decreased significantly ($p < 0.05$) during the BB536 yogurt period compared to the standard yogurt period. The frequency of occurrence of *C. perfringens* was lower (not significantly) following the ingestion of either BB536 yogurt or standard yogurt. Other *Clostridium* was lower (not significantly) during BB536 yogurt consumption.

Fecal putrefactive substances, enzyme activities and other characteristics

Table IV shows the changes in major fecal putrefactive substances (ammonia, indole and *p*-cresol), enzymatic activities relating to the production of putrefactive substances, and other fecal characteristics. Ammonia concentration decreased during the BB536 yogurt period, and increased significantly when consumption of BB536 yogurt was stopped. The level again decreased when standard yogurt was consumed but it was higher compared to the BB536 yogurt period. Indole and *p*-cresol concentrations changed in a similar manner as for ammonia; the concentrations during the BB536 period were lower than in other periods. Urease activity was lowest in the BB536 yogurt period, compatible with the results of ammonia concentration. The levels of β-glucosidase and nitroreductase also were lowest in the BB536 yogurt period but the changes were small. Fecal moisture tended to increase following yogurt ingestion in both instances. Fecal pH did not change markedly throughout the entire experimental period.

Fecal short chain fatty acids

The changes in levels of fecal short chain and volatile fatty acids are shown in Table V. Total levels of short chain fatty acids increased significantly ($p < 0.01$) following ingestion of BB536 yogurt and declined slowly when ingestion of this yogurt was stopped. A slight increase was observed following the consumption of standard yogurt. The levels of volatile fatty acids changed in the same manner as for total short chain fatty acids; they increased ($p < 0.01$) when BB536 yogurt was consumed and decreased when consumption was stopped.

DISCUSSION

The intestinal environment is a complex ecosystem reflecting the total physiological condition of the host intestine. This environment can be monitored by examining the fecal microflora, residues generated by the host digestive processes, and metabolites of intestinal bacteria. Intestinal bacteria metabolize food residues and physiological excreta of the host and produce many metabolites such as organic acids, bile acid metabolites, putrefactive substances and gases. These substances influence the intestinal conditions. In this report, the effect of BB536 yogurt consumption on the intestinal environment was studied, by examining fecal microflora, putrefactive substances and enzymes, and other fecal components and characters.

Ingestion of BB536 yogurt at 250 ml per day influenced the intestinal environment of healthy volunteers. The changes in microflora observed following the ingestion of BB536 yogurt included an increase in the proportion of *Bifidobacterium*, an increase in number of *Lactobacillus*. It is notable that *Lactobacillus* increased together with *Bifidobacterium* by ingestion of BB536. Also some minor changes were observed in other bacterial species. These changes in the intestinal microflora were not marked but seemed sufficient to modify the intestinal environment. Ballongue et al., (19) observed a marked increase in number of fecal *Bifidobacterium* and a decrease in numbers of antagonistic bacteria following the ingestion of *B. longum* BB536. However, Benno et al., (20) observed only a slight

Table III

Changes of the proportions of bacterial populations in fecal microflora

	Control A	BB536 yogurt	Control B	Standard yogurt
Bifidobacterium	20.2 ± 13.4[a]	27.6 ± 11.0*†	21.0 ± 11.1	22.4 ± 11.2
Eubacterium	18.0 ± 11.3	18.1 ± 8.9	17.1 ± 7.3	22.1 ± 8.2
Bacteroidaceae	54.2 ± 12.1	48.5 ± 14.8	53.0 ± 11.5	44.9 ± 13.1
Peptococcaceae	10.5 ± 12.1	5.9 ± 3.6	7.3 ± 5.3	9.7 ± 8.2
Total aerobic bacteria[b]	0.7 ± 1.1	0.5 ± 0.8	0.9 ± 0.5	1.9 ± 1.3

[a] Population expressed as mean ± SD of percentage of bacteria count of group to total bacterial count.
[b] *Enterobacteriaceae*, *Streptococcus* and *Staphylococcus*.
*,† Significantly different ($p < 0.05$) compared with control period A (*) and control period B (†).

Table IV

Influence of yogurt administration on fecal enzyme activities, putrefactive substances and other characteristics

	Control A	BB536 yogurt	Control B	Standard yogurt
Enzyme activity in feces				
Urease (μmol NH_3/h/g)	414 ± 231[a]	368 ± 179	539 ± 326	438 ± 221
Tryptophanase (μmol indole/h/g)	2.59 ± 0.83	2.90 ± 1.14	3.19 ± 1.23	2.56 ± 0.89
β-glucuronidase (μmol *p*-NP/h/g)	29.9 ± 22.9	24.6 ± 12.9	27.6 ± 21.7	21.2 ± 13.7
β-glucosidase (μmol *p*-NP/h/g)	57.3 ± 32.3	36.4 ± 23.9	53.1 ± 23.6	41.7 ± 15.6
Nitroreductase (μmol *p*-aminophenol/h/g)	1.30 ± 0.51	1.25 ± 0.59	1.64 ± 0.32	1.55 ± 0.72
Metabolites in feces				
Ammonia (μmol/g)	33.3 ± 16.1	24.5 ± 9.2[†]	38.1 ± 14.8	31.2 ± 21.6
Indole (μg/g)	42.0 ± 18.5	34.5 ± 16.8	56.1 ± 17.1	39.9 ± 21.3
p-Cresol (μg/g)	95.6 ± 51.9	73.2 ± 24.7	106.8 ± 33.8	90.7 ± 53.1
Feacal characteristic				
moisture (%)	74.8 ± 6.4	78.8 ± 4.6	74.9 ± 2.8	77.9 ± 1.5
pH	6.73 ± 0.30	6.66 ± 0.23	6.62 ± 0.31	6.69 ± 0.23

[a] Data expressed as mean $\pm$ SD

[†] Significantly different ($p < 0.05$) compared with control period B by Student's *t*-test.

increase in number of *Bifidobacterium* following the ingestion of freeze dried *B. longum*. Although the difference of administered products in Ballongue's and Benno's studies may be one reason of the difference in microflora changes, another reason of this difference may reflect a difference in the pre-study bifidobacterial counts in the volunteers. While the counts in the study by Benno et al were around 10.0 ($\log_{10}$ cfu) per gram of feces, those in the study by Ballongue et al were around 6.2 ($\log_{10}$ cfu) per gram. In our study, the bifidobacterial counts of the volunteers during the control periods were around 9.8 ($\log_{10}$ cfu) per gram. This high initial number of *Bifidobacterium* may be the reason why the effect of BB536 yogurt on the *Bifidobacterium* number was not marked. However significant increase in the proportion of *Bifidobacterium* was detected. The small microbial changes observed in this study may reflect a stable microflora in our healthy subjects.

Ammonia, indole and *p*-cresol concentrations in the feces were lowest during the period in which BB536 yogurt was consumed. Urease activity changed concomitant with changes in ammonia levels. Araya-Kojima et al., (21) reported inhibitions of growth and ammonia production by intestinal putrefactive bacteria when these bacteria were co-cultured with *B. longum* BB536. Their report also indicated that compared to the putrefactive bacteria, the enzymes in *B. longum* BB536 involved in ammonia production (urease and amino acid deaminase) were lower in level and weaker in activity, whereas the enzymes involved in ammonia assimilation (glutamine synthetase,

Table V

Influence of yogurt administration on the fecal short chain fatty acid concentrations

	Control A	BB536 yogurt	Control B	Standard yogurt
Total SCFA	77.2 ± 17.2[a]	119.0 ± 23.0**	107.8 ± 21.0	110.5 ± 24.5*
Total VFA[b]	71.6 ± 14.2	113.3 ± 21.1**	100.9 ± 18.8	104.9 ± 23.7*
Acetic	40.5 ± 6.9	65.7 ± 11.6**	56.7 ± 18.8	66.0 ± 15.3**
Propionic	15.7 ± 4.3	22.5 ± 7.4	22.5 ± 4.9	19.5 ± 5.7
Butyric	13.6 ± 5.2	22.9 ± 5.5*	18.0 ± 7.8	17.5 ± 5.9
iso-Butyric	1.9 ± 0.8	2.2 ± 1.1	3.7 ± 2.7	1.9 ± 0.9
Valeric	2.8 ± 1.8	3.2 ± 1.1	2.9 ± 2.0	2.6 ± 1.6
iso-Valeric	2.7 ± 1.0	2.2 ± 1.2	3.2 ± 1.1	2.8 ± 1.3
Caproic	1.0 ± 0.6	1.1 ± 0.8	1.1 ± 0.7	0.6 ± 0.4

SFCA; short chain fatty acids, VFA; volatile fatty acids

[a] Data expressed as mean $\pm$ SD of μmol fatty acid/gram of wet feces.

[b] Acetic, propionic, butyric and iso-butyric acid

*, ** Significantly different (*, $p < 0.05$; **, $p < 0.01$) compared with control period A.

glutamate synthetase and glutamate dehydrogenase) had much higher activities. Therefore, it is likely that the change of fecal ammonia level in the BB536 yogurt period reflects the changes of the fecal flora. The changes of indole and *p*-cresol levels may also be caused by changes of the fecal flora.

The levels of short chain (and volatile) fatty acids in the feces increased markedly following the ingesting of BB536 yogurt. However, the pH was stable during the whole experimental periods. It is difficult to explain why the increase in organic acid levels (and also the decreased in ammonia level) did not influence the fecal pH. Diversity of diet may be the reason but further study is required concerning this point. The fecal moisture content tended to increase when either BB536 yogurt or standard yogurt was ingested. Intestinal organic acids are known to promote peristalsis and secretion of moisture (22, 23). The increase in moisture is considered to reflect the increase in organic acid levels and it may facilitate excretion. Consumption of BB536 yogurt may be expected to alleviate constipation.

Summarizing the entire effects of consuming BB536 yogurt, an improvement in the intestinal environment was achieved. Although the fecal microflora did not change markedly, changes in proportion or number of bifidobacteria, *Lactobacillus*, *Clostridium* and *Streptococcus* were observed. The levels of putrefactive substances in the feces decreased and the levels of organic acids increased significantly. Some improvement in the intestinal environment was observed with the standard yogurt, but the effect was less evident compared to the BB536 yogurt.

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