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Effect of multispecies probiotics on irritable bowel syndrome: A randomized, double-blind, placebo-controlled trial

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irritable bowel syndrome, microbiota, probiotics.

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Abstract

Background and Aim: The efficacy of treatment with multispecies probiotics on irritable bowel syndrome (IBS) symptoms and the alterations of gut microbiota in patients who have taken probiotics were investigated.

Methods: This randomized, double-blind, placebo-controlled trial involved 49 IBS patients (probiotics: 25, placebo: 24) diagnosed according to the Rome III criteria. Patients were randomly assigned to two groups: either to receive multispecies probiotics (a mixture of *Bifidobacterium longum*, *B. bifidum*, *B. lactis*, *Lactobacillus acidophilus*, *L. rhamnosus*, and *Streptococcus thermophilus*) twice a day for 4 weeks or to receive a placebo twice a day for 4 weeks. The primary efficacy end-point was the proportion of participants whose IBS symptoms were substantially relieved at week 4. Secondary end-points were the intensity of abdominal pain/discomfort, bloating, stool frequency/consistency, alterations in fecal microflora over the 4 weeks. Fecal microflora were analyzed in 34 patients (probiotics: 17, placebo: 17) by quantitative real-time polymerase chain reaction assays.

Results: The proportion of patients whose IBS symptoms were substantially relieved at week 4 was significantly higher in the probiotics group than in the placebo group: 68.0% (17/25) *versus* 37.5% (9/24) ($P < 0.05$). Secondary end-points such as improvement in abdominal pain/discomfort and bloating occurred in the probiotics group but not in the placebo group. Fecal analysis revealed that *B. lactis*, *L. rhamnosus*, and *S. thermophilus* had increased significantly in the probiotics group after 4 weeks and that *B. lactis* had increased in the placebo group.

Conclusions: Multispecies probiotics are effective in IBS patients and induce the alterations in the composition of intestinal microbiota.

Introduction

Irritable bowel syndrome (IBS) is a functional gastrointestinal disease that presents as abdominal pain or discomfort with abnormalities of stool consistency and frequency. IBS is a common chronic gastrointestinal disorder and results in reduced health-related quality of life.[1]

The pathophysiology of IBS is not completely understood but probably involves a variety of factors. These include gut motor dysfunction, visceral hypersensitivity, dysregulation of the brain-gut axis, post-infectious bowel changes, altered intestinal microbiota, and psychological factors.[2] Attempts to treat patients with IBS have been based on different approaches, depending on the different factors involved.[3]

There is a growing interest in the relationship between gut microbiota and human health and disease.[4] Alterations in intestinal microbiota (employing probiotics, prebiotics, synbiotics and antibiotics) are used in attempts to treat gastrointestinal disorders including IBS.[5] Probiotics are effective in the treatment of IBS symptoms, but the most effective species are unclear.[6,7]

The composition of gut microbiota in patients with IBS is different to that in healthy people,[8] and this fact underpins the use of probiotics in IBS treatment. However, although treatment with multispecies probiotics rather than a single organism relieve some IBS symptoms, it is not clear which organisms induce the change in intestinal microbiota.[6]

The aim of this randomized, double-blind, placebo-controlled trial was to investigate the efficacy of multispecies probiotics in treating IBS. We assessed the effects of multispecies probiotics on IBS symptoms in comparison with placebo and evaluated alterations in gut microbiota after probiotics therapy by analyzing fecal microflora.

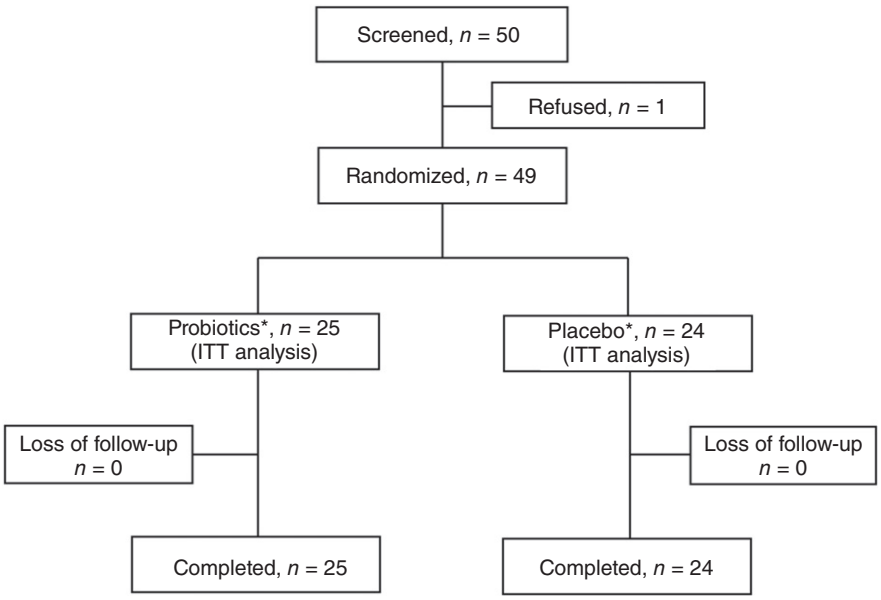

Figure 1 Consolidated Standards of Reporting Trials flowsheet. Probiotics: a probiotic mixture (total 5×10^9 colony-forming unit) containing *Bifidobacterium bifidum, B. lactis, B. longum, Lactobacillus acidophilus, L. rhamnosus,* and *Streptococcus thermophiles*. Participants were randomly assigned to receive either one capsule of probiotics or one capsule of placebo twice daily for 4 weeks. ITT, intention to treat.

Methods

Study patients. Patients who were eligible for this study were aged 19–75 years and were diagnosed with IBS according to the Rome III diagnostic criteria. A colonoscopy or barium enema study had been performed in all patients within the previous 5 years. Exclusion criteria included a history of organic bowel disease (e.g. colon cancer, intestinal tuberculosis and inflammatory bowel disease), acute or chronic liver/kidney disease, significant allergic disorders (e.g. asthma), previous major abdominal surgery other than appendectomy, uncontrolled thyroid disease, and acute illness within the previous 2 weeks. No alcoholics, pregnant or nursing women were included. Patients who were using probiotics, prebiotics, synbiotics, antibiotics, corticosteroids, antidepressants, antihistamines, non-steroidal anti-inflammatory drugs, and other drugs that affect intestinal motility (e.g. laxatives, antidiarrheals, prokinetics, and antispasmotics) were excluded.

All enrolled participants received comprehensive information about this study, and informed consent was obtained before any study-related processes began.

Study design and procedures. The study was conducted at Hanyang University Hospital in Korea between March 2011 and August 2011, and was approved by the Clinical Research Ethics Committee of the Hanyang University Hospital of Korea (2010-04-009). After a 2-week run-in period, enrolled patients were randomly assigned to receive either one capsule (500 mg) of LacClean Gold-S (Cell Biotech, Co. Ltd, Gimpo, Korea; a multispecies probiotics) or one capsule (500 mg) of a placebo twice daily (total dosage 1000 mg/day) for 4 weeks (Fig. 1). The patients were instructed to take the study product between meals because the increased gastric pH is more favorable for the ingested bacteria. LacClean Gold-S is a capsule-form probiotics containing six species of live bacteria. The six strains of probiotics were *Bifidobacterium bifidum* (KCTC 12 199BP), *Bifidobacterium lactis* (KCTC 11 904BP), *Bifidobacterium longum* (KCTC 12 200BP), *Lactobacillus acidophilus* (KCTC 11 906BP), *Lactobacillus rhamnosus* (KCTC 12 202BP), and *Streptococcus thermophilus* (KCTC 11 870BP). A total of 5×10^9 viable cells in a lyophilized powder form were included in each capsule and constituted 13.1% (w/w) of the total weight (500 mg/capsule). The amount of probiotics equally consisted in each of the six strains. The dose was determined based on previous studies where the daily doses were between 5×10^7 and 3.6×10^{11} colony forming units (CFUs)/day, and $\geq 5 \times 10^9$ CFUs/day has been suggested.[9–11] The placebo powder contained the same "other ingredients" as the active medication and maltodextrin instead of bacteria. OY Lee and KN Lee enrolled the patients for this study. Patients were allocated to the probiotics or placebo group using a computer-generated randomization schedule with a 1 : 1 allocation ratio. Dr. Jun generated the random allocation sequence, and no one but him knew the allocation sequence. The practice nurse gave a questionnaire and explained the protocol to the patients. The nurse did not know the allocation sequence and met the patients in regular sequence. The patient received the medication from the clinical pharmacist. No one could differentiate the two drugs without the sequence information. Stool samples for fecal microflora analysis were obtained immediately before the start of treatment and at the end of the 4 weeks of treatment. Fecal microbiota was analyzed only from patients who agreed to the stool sample collection.

IBS symptoms were assessed by examiners and patients at baseline and week 4 using a questionnaire. Global relief of IBS symptoms, drug compliance, and adverse events were evaluated by a questionnaire after the 4 weeks of treatment.

Measurements

Efficacy measurements. The primary efficacy end-point was the proportion of patients who experienced global relief of IBS symptoms after the 4-week treatment. Efficacy was estimated by

Table 1 Oligonucleotides used for quantitative real-time polymerase chain reaction assays

Target groups or species	Primer names	Primer sequences (5'-3')	Reference
Total bacteria	341F	CCTACGGGAGGCAGCAG	Nakamura *et al.*[35]
	543R	ATTACCGCGGTGCTGG	
Bifidobacterium group	F	TCGCGTC(C/T)GGTGTGAAAG	Rinttila *et al.*[36]
	R	CCACATCCAGC(A/G)TCCAC	
Bifidobacterium longum	BlonF	CAGTTGATCGCATGGTCTT	Ramirez-Farias *et al.*[37]
	BlonR	TACCCGTCGAAGCCAC	
Bifidobacterium bifidum	BiBIF-1	CCACATGATCGCATGTGATTG	Matsuki *et al.*[38]
	BiBIF-2	CCGAAGGCTTGCTCCCAAA	
Bifidobacterium lactis	BlactF	CCCTTTCCACGGGTCCC	Malinen *et al.*[39]
	BlactR	AAGGGAAACCGTGTCTCCAC	
Lactobacillus group	F	AGCAGTAGGGAATCTTCCA	Rinttila *et al.*[36]
	R	CACCGCTACACATGGAG	
Lactobacillus rhamnosus	LU-5	CTAGCGGGTGCGACTTTGTT	Song *et al.*[40]
	RhaII	GCGATGCGAATTTCTATTAT	
Lactobacillus acidophilus	F-acid-IS	GAAAGAGCCCAAACCAAGTGATT	Haarman and Knol[41]
	R-acid-IS	CTTCCCAGATAATTCAACTATCGCTTA	
Streptococcus thermophilus	ST-F	ACGGAATGTACTTGAGTTTC	Tilsala-Timisjarvi and Alatossava[42]
	ST-R	TTTGGCCTTTCGACCTAAC	
Escherichia coli subgroup	F	GTTAATACCTTTGCTCATTGA	Malinen *et al.*[13]
	R	ACCAGGGTATCTAATCCTGTT	
Bacteriodes group	Bac303F	GAAGGTCCCCCACATTG	Ramirez-Farias *et al.*[37]
	Bfr-Fmrev	CGCGACTTGGCTGGTTCAG	
Clostridium.perfringens	F	ATGCAAGTCGAGCGA(G/T)G	Malinen *et al.*[13]
	R	TATGAGGTATTAATCT(C/T)CTTT	

the response (yes or no) to the question: "Compared with your health before treatment started, have overall IBS symptoms improved during the past seven days?"

Secondary efficacy end-points were: (i) intensity of abdominal pain/discomfort and bloating, for which patients scored their worst symptoms over the previous 24 h on a numeric scale from 0 to 10; (ii) defecation frequency defined as the average number of episodes per week; (iii) stool consistency using the 7-point Bristol Stool Form Scale (BSFS); and (iv) alterations in the composition of fecal microflora as a result of treatment.

Compliance and safety assessments. Drug compliance was defined as the ratio of number of drugs taken to the number of drugs prescribed. All adverse reactions were reported.

Fecal samples. Patients enrolled in the study provided fecal specimens at the beginning and end of the study. These were collected in sterile containers, brought to the laboratory in a frozen condition, and stored at −80°C until analysis.

Preparation of genomic DNA from reference strains and fecal samples. Bacterial genomic DNA from pure cultures or fecal samples was prepared using an AccuPrep Genomic DNA extraction kit (Bioneer, Daejeon, Korea). Genomic DNA was extracted from 1 mL of pure culture according to the manufacturer's instructions.

Real-time quantitative polymerase chain reaction. Real-time quantitative polymerase chain reaction (PCR) was carried out using a LightCycler 480 (Roche, Germany), and the group and species-specific primers for PCR are listed in Table 1. The primers were synthesized commercially by Bioneer, and their specificity was previously verified using DNA from closely or distantly related bacteria. Quantitative PCR was performed in 96-well plates in final volumes of 20 μL consisting of 1 μL of fecal DNA, 0.5 μL of primers (10 pmol each), 10 μL SYBR Green I master (Roche, Mannheim, Germany), and 8 μL of H_2O. PCR amplification involved: pre-incubation at 94°C for 4 min followed by 55 cycles of amplification (denaturation at 94°C for 15 s, primer annealing at 55°C for 15 s, and elongation at 72°C for 20 s). Melting curves were obtained by heating samples from 50 to 90°C at a rate of 5°C/s.

Statistical analysis. The sample size for this study was calculated assuming a 40% difference in the primary end-point between two groups.[12] From this assumption, we calculated that a total of 48 patients would have a statistical power of 80% and a two-sided α risk of 0.05. We planned to enroll 50 patients, as we expected some participants to dropout of the study.

Efficacy and safety were assessed by intention-to-treat (ITT) analysis. The ITT analysis included all participants who had taken any medication, and dropouts were regarded as non-responders. All significance tests were two-sided, and a *P* value of less than 0.05 was regarded as significant. All statistical analyses were performed using SPSS for Windows release 18.0 (SPSS, Inc., Chicago, IL, USA).

Results

Participants. Of enrolled 50 patients, 49 (98%) patients were randomized to either probiotics or a placebo for 4 weeks. One

Table 2 Baseline characteristics of patients

	Probiotics group (n = 25)	Placebo group (n = 24)
Age (years, mean ± SD)	45.9 ± 13.7	43.1 ± 15.1
Gender (male/female)	11/14	6/18
IBS subtype (IBS-D/IBS-C/IBS-M)	14/9/2	12/11/1
Abdominal pain (mean ± SD)	3.2 ± 1.7	3.1 ± 1.7
Abdominal discomfort (mean ± SD)	3.9 ± 1.6	3.5 ± 1.5
Abdominal bloating (mean ± SD)	4.2 ± 1.5	3.8 ± 2.3
Stool frequency/week (mean ± SD)	6.9 ± 4.7	6.3 ± 4.6
Stool form (BSFS) (mean ± SD)	4.6 ± 1.8	4.1 ± 1.8

There were no statistically significant differences between the two groups.
Abdominal pain, discomfort, and bloating were assessed on a numeric rating scale from 0 to 10, with symptoms increasing from 0 to 10.
BSFS, Bristol Stool Form Scale; IBS, irritable bowel syndrome; IBS-C, IBS with constipation; IBS-D, IBS with diarrhea; IBS-M, mixed-type IBS; *n*, number; SD, standard deviation.

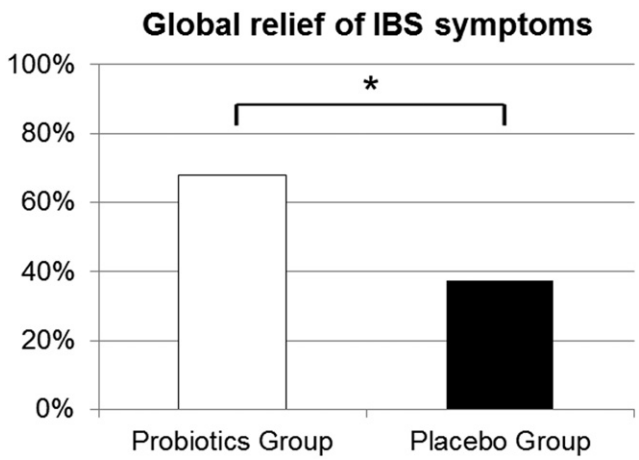

Figure 2 Comparison of global relief of irritable bowel syndrome (IBS) symptoms in the probiotics and placebo groups after 4 weeks of treatment. More patients in the probiotics group experienced global relief of IBS symptoms than in the placebo group: 68.0% (17/25) *versus* 37.5% (9/24) (P = 0.03). *P < 0.05.

patient refused to participate in this study; 49 patients (25 in the probiotics group and 24 in the placebo group) completed the study (Fig. 1).

Table 2 shows the baseline characteristics of the participants. Age, gender, IBS subtype, intensity of abdominal pain/discomfort, bloating, stool frequency, and consistency (according to the BSFS) were not different between the two groups.

Primary efficacy end-points. The proportion of patients who received global relief of IBS symptoms at week 4 is shown in Figure 2. There was a significantly higher response rate in the probiotics group than in the placebo group: 68.0% (17/25) *versus* 37.5% (9/24) (P = 0.03).

Secondary efficacy end-points. Relative to baseline, the intensity of abdominal pain (0–10 rating scale) at week 4 was significantly reduced in the probiotics group (3.2 ± 1.7→2.0 ± 1.9, P < 0.01), but not in the placebo group (3.1 ± 1.7→2.6 ± 1.4, P = 0.13) (Table 3). The intensity of abdominal discomfort and bloating was also reduced in the probiotics group but not in the placebo group. However, there was no significant difference in stool frequency and consistency between baseline and week 4 in either group. The change of abdominal pain relative to baseline was greater in the probiotics group than the placebo group, but it does not satisfy the statistical significance (–37.1 ± 46.3% *vs* –9.2 ± 57.1%, P = 0.07) (Table 4).

Table 4 The change of IBS symptoms between week 0 and week 4

	Change rates (%)		P value*
	Probiotics	Placebo	
Abdominal pain (mean ± SD)	–37.1 ± 46.3	–9.2 ± 57.1	0.07
Abdominal discomfort (mean ± SD)	–28.7 ± 42.6	–17.8 ± 37.8	0.35
Abdominal bloating (mean ± SD)	–22.4 ± 49.0	–20.3 ± 30.5	0.86
Stool Frequency/week (mean ± SD)	–2.4 ± 35.0	32.6 ± 125.1	0.19
Stool Form (BSFS) (mean ± SD)	0.5 ± 30.4	–3.3 ± 33.2	0.68

*P < 0.05 for comparison between probiotics and placebo group.
Change rates (%) = (week 4 score – week 0 score)/week 0 score × 100.
BSFS, Bristol Stool Form Scale; IBS, irritable bowel syndrome; SD, standard deviation.

Table 3 Comparison of IBS symptoms in baseline and week 4 (ITT analysis)

	Probiotics group (n = 25)		P value*	Placebo group (n = 24)		P value*
	Baseline	Week 4		Baseline	Week 4	
Abdominal pain (mean ± SD)	3.2 ± 1.7	2.0 ± 1.9	< 0.01	3.1 ± 1.7	2.6 ± 1.4	0.13
Abdominal discomfort (mean ± SD)	3.9 ± 1.6	2.9 ± 2.2	< 0.01	3.5 ± 1.5	2.9 ± 1.3	0.17
Abdominal bloating (mean ± SD)	4.2 ± 1.5	3.0 ± 1.9	< 0.01	3.8 ± 2.3	3.1 ± 1.5	0.12
Stool Frequency/week (mean ± SD)	6.9 ± 4.7	5.7 ± 2.5	0.06	6.3 ± 4.6	6.1 ± 3.9	0.10
Stool Form (BSFS) (mean ± SD)	4.6 ± 1.8	4.4 ± 1.6	0.17	4.1 ± 1.8	3.8 ± 1.6	0.87

*P < 0.05 for comparison between baseline and week 4.
Abdominal pain, discomfort and bloating were assessed on a numeric rating scale from 0 to 10, with symptoms increasing from 0 to 10.
BSFS, Bristol Stool Form Scale; ITT, intention to treat; *n*, number; SD, standard deviation.

Table 5 Changes in fecal microbiota between baseline and week 4 in the multispecies probiotics and placebo groups

	Probiotics (n = 17)			Placebo (n = 17)		
	Baseline	Week 4	P value*	Baseline	Week 4	P value*
Total bacteria	11.56 ± 0.32	11.64 ± 0.40	0.44	11.15 ± 0.74	11.45 ± 0.37	0.21
Bifidobacterium group	9.34 ± 0.69	9.37 ± 0.66	0.79	8.53 ± 1.25	8.90 ± 1.31	0.29
Bifidobacterium longum	8.81 ± 0.89	8.75 ± 0.73	0.53	8.15 ± 1.31	8.25 ± 1.48	0.72
Bifidobacterium bifidum	5.38 ± 1.11	5.28 ± 1.14	0.33	5.30 ± 1.22	5.36 ± 1.45	0.84
Bifidobacterium lactis	6.09 ± 1.23	7.57 ± 1.22	< 0.01	5.99 ± 0.52	6.54 ± 0.87	0.04
Lactobacillus group	8.38 ± 0.80	8.43 ± 0.82	0.57	7.76 ± 1.37	7.82 ± 0.99	0.88
Lactobacillus rhamnosus	2.80 ± 1.69	5.05 ± 1.43	< 0.01	2.82 ± 1.49	2.97 ± 1.17	0.22
Lactobacillus acidophilus	3.28 ± 0.77	3.65 ± 0.90	0.21	3.29 ± 1.12	2.93 ± 0.13	0.34
Streptococcus thermophilus	4.81 ± 0.87	5.35 ± 1.28	0.04	5.08 ± 1.47	5.20 ± 1.53	0.76
Escherichia coli subgroup	6.72 ± 1.06	7.11 ± 1.31	0.23	6.45 ± 1.10	6.41 ± 0.89	0.87
Bacteroides group	6.61 ± 1.18	7.06 ± 1.22	0.17	6.23 ± 1.06	6.87 ± 1.18	0.07
Clostridium perfringens	10.08 ± 0.18	10.02 ± 0.87	0.20	9.98 ± 1.18	10.02 ± 0.11	0.39

*$P < 0.05$ by paired t-test for the difference between baseline and week 4.
Values are mean ± standard deviation, and the unit of measurement is $\log_{10}$ cells/gram in feces.

Fecal microflora was analyzed by real-time quantitative PCR to identify any alterations in intestinal microbiota after treatment with multispecies probiotics. Fecal microflora counts for each group were evaluated immediately before the start of treatment and at the end of treatment. Fecal microflora were analyzed in the 34 patients (17 each in the probiotics and placebo groups) who agreed to the collection of stool samples. Changes in the composition of fecal bacteria over the 4-week period are summarized in Table 5.

Compared with baseline, counts of *B. lactis*, *L. rhamnosus* and *S. thermophilus* at week 4 had increased in the probiotics group (*B. lactis*: $6.09 \pm 1.23 \rightarrow 7.57 \pm 1.22$ $\log_{10}$ cells/g in feces, $P < 0.01$; *L. rhamnosus*: $2.80 \pm 1.69 \rightarrow 5.05 \pm 1.43$, $P < 0.01$; *S. thermophilus*: $4.81 \pm 0.87 \rightarrow 5.35 \pm 1.28$, $P = 0.04$). Meanwhile placebo group showed the increase of *B. lactis* counts ($5.99 \pm 0.52 \rightarrow 6.54 \pm 0.87$ $\log_{10}$ cells/g in feces, $P = 0.04$). Counts of *B. longum, B. bifidum, L. acidophilus, and Escherichia coli* subgroup, and *Clostridium perfringens* and *Bacteroides* group were unchanged in both groups.

Compliance and adverse events. The mean percentage of drugs taken to drugs prescribed was 96% in the probiotics group and 94% in the placebo group ($P > 0.05$). No adverse events or serious adverse events occurred in either group.

Discussion

A randomized, double-blind, placebo-controlled clinical trial of IBS was done for 4 weeks. Compared with placebo, multispecies probiotics were effective for global relief of IBS symptoms as well as for various secondary end-points (i.e. abdominal pain/discomfort and bloating). In addition, probiotics and placebo had different effects on the composition of fecal microbiota.

Multispecies probiotics was used for the treatment of IBS in our study: three *Bifidobacterium* species, two *Lactobacillus* species, and one *Streptococcus* species. The reason is as follows. The level of *Bifidobacteria* and *Lactobacilli* species was lower in IBS patients compared with healthy persons.[13,14] Also, *S. thermophillus* showed the reduction of tumor necrosis factor-alpha caused by lipopolysaccharide in the intestinal barrier.[15] Then, several studies showed that the supplement of *Lactobacillus*, *Bifidobacterium* species, or mixtures including species of the genera was effective in alleviating symptoms of IBS.[16]

In this study, the multispecies probiotics were more effective than the placebo group in terms of the primary efficacy end-point. Secondary end-points were achieved in the probiotics group but not placebo group. This finding is consistent with previous data from multispecies probiotics treatment of IBS.[17–19] Multispecies probiotics may have a variety of different beneficial effects on IBS symptoms because each species act in a particular way on the gastrointestinal tract, and two or more species acting together may have a synergistic effect. However, the changes in stool frequency and consistency in the probiotics group was similar to those in the placebo group. This may be because the patients had three different subtypes of IBS (IBS with diarrhea, IBS with constipation, and mixed-type IBS) rather than a single subtype. The changes of IBS symptoms relative to baseline were not significantly greater in the probiotics group compared with the placebo group. Although the change of abdominal pain was more improved in the probiotics group, it did not reach the statistical significance (–37% *vs* –9.2%, $P = 0.07$). This result seems to be caused by the relatively low number of subjects in this study (the sample size of each arm was 25 patients) because the calculation of sample size was performed based on the primary end-point not the secondary end-points.

To investigate the alterations in intestinal microbiota, fecal microflora was analyzed in this study. Interestingly, numbers of *B. lactis*, *L. rhamnosus*, and *S. thermophilus* increased after week 4 in the probiotics group, whereas only the number of *B. lactis* increased in the placebo group. In other words, only three of the species in the probiotics mixture remained in the gut after 4 weeks even though there were six species in the mixture. Our findings differ from previous observations. Kajander *et al*. reported that *Bifidobacterium* species decreased after treatment with a probiotic mixture of *L. rhamnosus*, *B. breve*, and

Propinibacterium freudenreichii.[20] Firmesse *et al*. reported no difference in the composition of gut microbiota after treatment with *L. rhamnosus*.[21] We found no significant change in the *E. coli* subgroup, *C. perfringens*, or the *Bacteroides* group after treatment, whereas Lyra *et al*. reported elevated levels of *C. thermosuccinogenes* following multispecies probiotics treatment that included *Bifidobacterium* and *Lactobacillus* species.[22]

Based on the changes in fecal microbiota after probiotics, we thought the relationship between the alterations in gut microflora and the alleviation of IBS symptoms. There is some difficulty in analyzing the direct correlation between IBS symptoms and the alterations of gut microflora because the total number of subjects in fecal analysis ($n = 34$) was smaller than that of subjects with probiotics or placebo treatment ($n = 49$). In spite of this discrepancy, we cautiously assumed that the alleviations of IBS symptoms in probiotics groups after 4 weeks were probably associated with significant increases in *B. lactis*, *L. rhamnosus*, and *S. thermophilus*. Interestingly, compared with baseline, the counts of *B. lactis* at week 4 were increased in the placebo group, although placebo did not show the improvement of IBS symptoms. Maybe, the diet induced the alteration of *B. lactis* in the placebo group. Despite the drugs that affect intestinal microbiota (e.g. probiotics, prebiotics, synbiotics and antibiotics) were equally restricted in both probiotics and placebo groups, it is very difficult to control the dietary habits in all participants during 4 weeks. So, it seemed that the efficacy of probiotics on IBS symptoms may be associated with the synergistic effect of three strains (*B. lactis*, *L. rhamnosus*, and *S. thermophilus*) rather than that of single strain (*B. lactis*). Also, we can consider the metabolic effect of probiotic strains on gastrointestinal tract. Several studies demonstrated that the probiotic supplement including *Lactobacilli* and *Streptococcus* species induced the increase of short-chain fatty acids (SCFAs) in colon lumen and then decreased fecal pH.[23,24] The change of SCFA and fecal pH are considered to contribute the improvement of gut motility. We assumed that *L. rhamnosus* and *S. thermophilus* in the probiotics group could improve IBS symptoms by the change of SCFA and fecal pH.

To identify the alterations of gut microbiota, quantitative reverse transcription–PCR (qRT-PCR) was used in our study. Among the commonly used molecular techniques, for example denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis, terminal-restriction fragment length polymorphism, and dot blot hybridization, qRT-PCR provides a rapid, precise quantification of the genus or species, and has been applied to human feeding studies.[25–27] Recently, new approaches to gut microbiota have been tried other than commonly used method such as 16S rDNA. So called metagenomic sequencing can reveal the combined genomes of the gut microflora, non-cultured ones, and functional dysbiosis beyond compositional dysbiosis. In spite of these merits, however, it is not widely used, and there is no information on microbial expressed functions.[27] The global changes of intestinal mirobiota can provide us with a role of the probiotics in IBS. Determining the global changes of fecal microflora after probiotics supplement was limited because complete sequencing was not done. There is a need to perform in-depth methods including a complete sequencing for intestinal microflora after probiotic supplement in future studies.

Several reports demonstrated that IBS patients had more temporal instability of fecal microbiota than healthy controls.[28,29] In this study, concordance of PCR-DGGE using fecal DNAs from each group was done to evaluate the compositional change in the fecal microbiota between before and after treatment. PCR-DGGE was done in some patients, but not all. The placebo group showed a lower concordance rate of DGGE profiles than the probiotics group between before and after treatment, but it did not reach the significant difference statistically ($P = 0.086$). Although all the fecal samples of each group were not analyzed, the result indicates that the test product contributed to the maintenance of the compositional stability of the intestinal microbiota. The clinical improvements in this study may be associated with the maintenance of the compositional stability of the intestinal microbiota.

Our study has some limitations. First, fecal microflora were analyzed in only 75.6% of the patients (34/49) because we only analyzed stool samples from those who consented. As mentioned earlier, it was not easy to assess a direct relationship between alterations in gut microbiota and improvements in IBS symptoms in patients who have taken probiotics supplements. Even though the present study has this weak point, the probiotics group showed alleviations of IBS symptoms such as abdominal pain and bloating with increases in the counts of *B. lactis*, *L. rhamnosus*, and *S. thermophilus*. Second, we evaluated the intestinal microbiota by fecal microflora analysis. There are two methods for analyzing gut microbiota: fecal microflora analysis reflects the composition of the luminal intestinal microbiota, while culture of intestinal tissue reflects that of the mucosal-associated intestinal microbiota. Parkes *et al*. reported that luminal microbiota were associated with gas production through carbohydrate fermentation, whereas mucosal-associated microbiota might play a role in immune responses to microbes.[30] Despite these theoretical differences, the luminal and mucosal-associated intestinal microbiota in IBS patients were found to be similar.[31] Third, a validated quality of life was not measured in this study, although we checked a global relief of IBS symptoms after treatment. Several reports indicated that probiotics improved quality of life in patients with IBS.[32,33] Our study focused on the improvement of IBS symptoms and gut microbiota alterations after probiotic supplement. Fourth, we did not perform a separate analysis of therapeutic effect on IBS according to gender or IBS subtypes. The reason is as follows. There were a small number of subjects present in some subgroup (e.g. the number of women in placebo group was six), although baseline characteristics of the participants were not statistically different between the probiotic and placebo group (Table 2). In case of the small sample sizes in subgroup analyses, the statistical power is not enough because of increase in type I error.[34]

In conclusion, multispecies probiotics given to IBS patients are effective in the global relief of IBS symptoms as well as in alleviating abdominal pain, discomfort and bloating. Furthermore, the multispecies probiotics induced the alterations of intestinal microbiota. These findings support that probiotics therapy is effective by mechanism of gut microbiota alterations in IBS.

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