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Full Paper

Combination of poly-γ-glutamic acid and galactooligosaccharide improves intestinal microbiota, defecation status, and relaxed mood in humans: a randomized, double-blind, parallel-group comparison trial

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The genus *Bifidobacterium* comprises beneficial intestinal bacteria that play a crucial role in the regulation of human health. Traditional prebiotics are known to increase intestinal bifidobacteria by supplying a carbon source necessary for their growth. However, intestinal bifidobacteria need not only a carbon source but also a nitrogen source for growth. Moreover, the growth of bifidobacteria is known to be inhibited in a culture medium that does not contain glutamic acid. Based on these reports, we hypothesized that the combined intake of traditional prebiotics and glutamic acid would be beneficial for growth of bifidobacteria in the gut. In this study, we investigated the effects of the combination of galactooligosaccharide (GOS; traditional prebiotic material) and poly-γ-glutamic acid (γ-PGA; source of glutamic acid) and only GOS on the intestinal microbiota and health conditions (including intestinal regulation, mood status, gastrointestinal condition, skin condition, and sleep quality) in a randomized, double-blind, parallel-group comparison trial in healthy subjects. The combined intake of GOS and γ-PGA significantly increased the prevalence of *B. longum* compared to the intake of GOS alone. A minimum effective dose of 2.0 g GOS and 0.3 g γ-PGA improved defecation and mood status. We revealed the combined effects of GOS and γ-PGA on intestinal microbiota as well as physical condition and concluded that the delivery of glutamic acid to the large intestine with traditional prebiotics is useful as an advanced prebiotic.

Key words: intestinal regulation, intestinal environment, bifidobacteria, galactooligosaccharide, poly-γ-glutamic acid

INTRODUCTION

It is estimated that 40 trillion bacteria live in the human intestine, encompassing nearly 1,000 types [1]. Recently, extensive studies have revealed the close relationships between gut microbiota and the physiological functions of the host, such as maintenance of the intestinal barrier function, nutrient absorption, and regulation of the immune system [2, 3]. With the recent development of new technologies for gut microbiome research, such as next-generation sequencing (NGS), investigating the effects of the gut microbiota on human health and host gene expression has become a booming area of research and presents a new paradigm of opportunities for medical and food applications, personalized diet, and lifestyle recommendations [4–7].

A disturbance in the intestinal environment results in constipation, which is defined as unsatisfactory defecation resulting from infrequent stools, difficult stool passage, or both [8]. Constipation remains difficult to treat and has a clinically important deleterious effect on health-related quality of life. Functional gastrointestinal disorders are linked to an increased prevalence of concomitant anxiety and depression [9]. Therefore, studies on the successful treatment of mood disorders caused by colonic motility are drawing attention.

Bifidobacteria are one of the most well-known beneficial bacteria associated with human health. They have an impact on intestinal regulation, body fat reduction, hyperlipidemia improvement, glucose tolerance improvement, anticancer effects, infection prevention, regulation of skin condition, sleep quality,

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and inflammatory bowel disease improvement and remission [10–15]. Hence, many researchers have attempted to identify effective food components that help increase the number of intestinal bifidobacteria.

Bacteria need minimal nutrients for their growth, namely water, carbon and nitrogen sources, and some minerals [16, 17]. Traditional prebiotics, such as galactooligosaccharides (GOS), are effective food ingredients for increasing intestinal bifidobacteria [18, 19]. GOS is an indigestible oligosaccharide produced from lactose by β-galactosidases and is used as a carbon source for bacterial growth. In addition, a report showed that the growth of several species of the genus *Bifidobacterium* was inhibited in culture medium that did not contain glutamic acid (Glu), suggesting that Glu is an essential nutrient for bifidobacteria [20]. Based on these reports, we hypothesized that the combination of traditional prebiotics and Glu could be a more effective strategy for increasing intestinal bifidobacteria. However, there are no reports on their combined effect on the gut microbiota in humans, and therefore, the minimum effective dose for them is not clear. Orally administered Glu is rapidly absorbed via its transporters or receptors present in the upper gastrointestinal tract [21–25]. Thus, free Glu is poorly delivered to the lower gastrointestinal tract, which is the habitat of bifidobacteria.

Poly-γ-glutamic acid (γ-PGA) is a naturally produced polymer consisting of a large number of glutamic acid molecules combined with γ-linkages and is known to be abundant in natto, a Japanese traditional fermented food [26]. Poly-γ-glutamic acid is degraded by γ-glutamyl transferase (GGT), which is widely expressed in bacteria and humans [27]. It has also been reported that human GGT is highly expressed in the kidney and liver but weakly expressed in the intestine [28, 29]. In addition, intestinal bacteria such as *Escherichia coli* and *Bacillus subtilis* have large amounts of GGT [30–32]. Therefore, we hypothesized that orally administered γ-PGA reaches the large intestine, where it is degraded into Glu by GGT derived from the intestinal bacteria. Hence, for the current human trial, we selected γ-PGA as a carrier of Glu to facilitate its delivery to the lower part of the digestive tract.

In the present study, we investigated the effects of orally ingested GOS and γ-PGA on the intestinal environment by analyzing the relative abundance of bifidobacteria. In addition, the effects on health condition, including intestinal regulation, mood status, gastrointestinal condition, skin condition, and sleep quality, were verified. The results of the present study highlight the potential health benefits of the combined intake of the prebiotics γ-PGA and GOS.

MATERIALS AND METHODS

Study design

The study had a randomized, double-blind, parallel-group, dose-comparison design and was conducted between August 2020 and January 2021 in Tokyo, Japan. An outline of the study is shown in Fig. 1. The study contained two administration periods (two weeks each) separated by a two-week washout period. In the first period, the control group was administered a low dose (1.0 g) of GOS, and we verified the effect of four γ-PGA doses (0.1, 0.3, 0.5, or 1.0 g) supplemented with GOS. After the washout period, in the second period, we investigated the supplemental effect of the same four γ-PGA doses (0.1, 0.3, 0.5, or 1.0 g) in combination with a high dose (2.0 g) of GOS control. At the time of screening subjects for participation in the study, doctors conducted face-to-face interviews; biological blood tests and urinalysis were conducted; and height, weight, body mass index, and blood pressure were recorded. Subjects were assigned to five groups in each period using blocked randomization based on defecation frequency (per week), age, gender, and composition of their bifidobacteria at the time of screening. The block size was five. The allocations of the subjects to the test supplement groups were concealed from the subjects, investigators, technicians, data analysts, evaluators, and the medical doctor until the study was completed. The statistical analysis manager at a contract research organization (CRO) generated a random allocation sequence, and the principal investigator enrolled the participants and assigned them to the interventions. During the test period, the CRO conducted regular telephone interviews to check for adverse events or side effects and reported to them the doctors. Stool samples were collected at four time points during the study (before and after the intervention in each period). Defecation status, gastrointestinal conditions, and appetite were recorded daily. Profile of Mood States 2nd Edition (POMS 2) questionnaires and visual analog scales (VAS) for sleep conditions and skin were administered at four time points during the study (before and after the intervention in each period). This study was conducted in compliance with the Declaration of Helsinki and with the approval of the ethics review committee of Nihonbashi

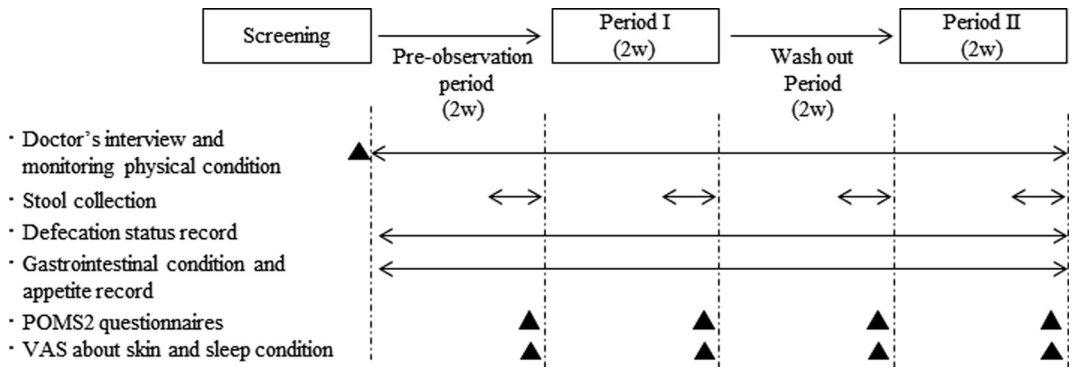

Fig. 1. Outline of this human trial.
POMS 2: Profile of Mood States 2nd Edition; VAS: visual analog scale.

Cardiology Clinic (approval code: NJI-020-2-01) and the ethics committee of Ajinomoto Co., Inc. (approval code: 2019-013). The study was also registered in the UMIN Clinical Trials Registry as UMIN000040039.

Outcomes

The primary outcome of this trial was the composition of intestinal bacteria. The key secondary outcome was information gathered with respect to defecation status, gastrointestinal condition, appetite, mood status, skin condition, and sleep quality.

Subjects

Prior to the study, participants were recruited by the CRO (KSO Co., Ltd., Tokyo, Japan). The subjects of this study were healthy Japanese males and females aged 20–60 years with defecation frequencies of 3–5 times a week and low levels of bifidobacteria (the relative abundance was less than approximately 5%) at the time of screening. In addition, subjects received a sufficient explanation of the purpose and contents of this study, and they usually consumed three meals a day. All subjects understood that participation was by consent, confirmed that they understood the explanation provided, volunteered to participate, and provided written informed consent. The exclusion criteria were as follows: (1) regular intake of intestinal drugs or laxatives; (2) regular consumption of healthy foods such as yogurt and fermented pickles, which could have beneficial effects on constipation during the test period; (3) frequent intake of health supplements; (4) regular consumption of foods containing lactic acid bacteria, bifidobacteria, oligosaccharides (probiotics and prebiotics), and natto (at least once a day); (5) gastrointestinal disorders or related surgical history; (6) diagnosis of irritable bowel syndrome or inflammatory bowel disease; (7) liver disease, kidney disease, and diabetes mellitus; (8) food allergy; (9) pollinosis or intake of drugs that affect the gut microbiome; (10) pregnancy, breast-feeding, or plan to be pregnant during the study period; (11) participation in other clinical trials (food, pharmaceuticals, and cosmetics) within one month of obtaining consent, or (12) unsuitableness for the study as judged by the principal investigators. Since the present study was conducted as an exploratory study, the sample size was not calculated, but the number of participants in this study was determined based on previous reports investigating the effect of GOS on the intestinal environment [18, 33]. A total of 388 participants were screened, and 100 subjects were included in the study. After the intervention, one subject who took laxatives in the first period and two subjects whose test supplement consumption rates were <80% in the second period were excluded from the analysis. Figure 2 shows a study flow diagram and the numbers of subjects in each period of the study.

Test supplement

The source of GOS for the study was Cup Oligo® purchased from Nissin Sugar Co., Ltd. (Tokyo, Japan), and the source of γ-PGA (average molecular weight, approximately 26,000) was CALTAKE® (Ajinomoto Co., Inc., Tokyo, Japan). The test supplement doses are shown in Table 1. We verified that there was no difference between the flavor and appearance of the test supplements. The subjects ingested their daily doses of the test supplement dissolved in water after dinner.

Intestinal microbiota analysis

Stool samples were collected in a brush-type container with guanidine thiocyanate as a preservation solution (Cat. No. FS-0006, TechnoSuruga Laboratory, Shizuoka, Japan). Each subject collected stool samples at home at room temperature, and these samples were immediately submitted to KSO Co., Ltd. and stored at 4°C under refrigerated conditions. All samples were transported from KSO Co., Ltd. to Ajinomoto Co., Inc. and stored in a refrigerator before DNA extraction.

Total DNA was extracted from stool samples using an ISOSPIN Fecal DNA kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Library preparation for 16S rRNA amplicon sequencing was conducted according to the technical notes of Illumina (16S Metagenomic Sequencing Library Preparation). The V3–V4 region of the bacterial 16S rRNA gene was PCR amplified using the primers recommended by Illumina. After preparing the library, the average fragment length was determined using an Agilent DNA 1000 Kit (Agilent Technologies, Santa Clara, CA, USA), and the concentration was measured using a Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Each amplicon library was diluted to 10 nM with PCR Grade Water (Roche, Basel, Switzerland) and mixed in equal amounts of 5 µL each. The mixed library was quantified using a Light Cycler® 96 System (Roche, Basel, Switzerland) with a GenNext NGS Library Quantification Kit (Toyobo, Osaka, Japan) and diluted to 4 nM. Finally, the library, which had been denatured to a final concentration of 4 pM, was paired-end sequenced at 2 × 250 bp using MiSeq Reagent Kit v2 (500 cycles) on an Illumina MiSeq platform (Illumina, San Diego, CA, USA).

The raw paired-end reads were primer trimmed using Cutadapt v1.8.1 [34]. Paired and unpaired reads in the processed data were divided using cmpfastq (http://compbio.brc.iop.kcl.ac.uk/software/cmpfastq_pe.php). Paired-end reads were processed with fastq-join [35] using the default parameters to combine each read pair. The remaining reads were further filtered to remove low-quality reads, using the fastq_quality_filter of the FASTX-Toolkit 0.0.14 package with -Q33 -q20 -p 80 parameters (http://hannonlab.cshl.edu/fastx_toolkit/). Chimeric sequence removal and operational taxonomic unit (OTU) clustering were performed using QIIME pipeline ver 1.9.1 [36]. Chimeric sequences were identified using the identify_chimeric_seqs.py (using the usearch61 option that runs the UCHIME algorithm) with reference-based methods against Greengenes database version 13.8 [37, 38] and subsequently removed using the filter_fasta.py. OTU clustering was performed using the pick_open_reference_otus.py, utilizing the default UCLUST algorithm with a similarity threshold of 97%. EzBioCloud 16S DB ver. 2018.05 [39] was used as the reference for OTU assignments. Low-abundance (0.005%) OTUs were removed from the OTU tables [40].

Defecation status

Defecation status was recorded by the subjects in a diary. Defecation frequency (per week), days of defecation (per week), amount of defecation (equal to a medium-sized hen's egg), Bristol stool form scale (ranging from 1 for separate hard lumps, like nuts, to 7 for watery, with no solid pieces and entirely liquid) [41], and the feeling of remaining feces (0, never; 1, sometimes; 2, always) were evaluated.

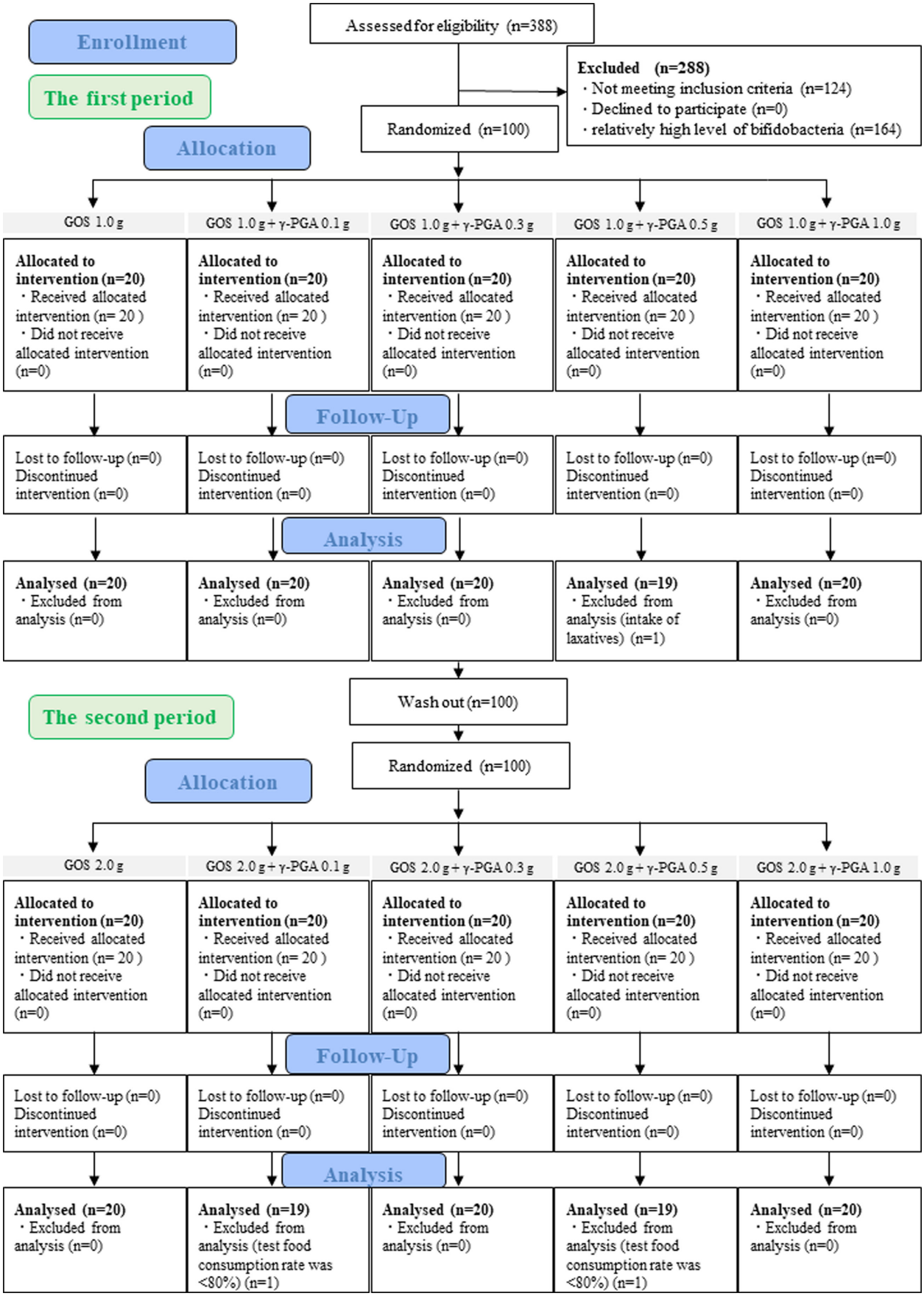

Fig. 2. Flow and number of subjects in this study.
GOS: galactooligosaccharides; γ-PGA: poly-γ-glutamic acid.

Table 1. Composition of GOS and γ-PGA in the test supplement

Test period	Supplement	GOS		γ-PGA
The first period	Test food 1	1.0 g	+	—
	Test food 2			0.1 g
	Test food 3			0.3 g
	Test food 4			0.5 g
	Test food 5			1.0 g
The second period	Test food 1'	2.0 g	+	—
	Test food 2'			0.1 g
	Test food 3'			0.3 g
	Test food 4'			0.5 g
	Test food 5'			1.0 g

GOS: galactooligosaccharides; γ-PGA: poly-γ-glutamic acid.

Gastrointestinal condition and appetite

Gastrointestinal condition and appetite status were evaluated daily using an original questionnaire (Supplementary Table 1) and averaged per week. The questionnaire was based on the Izumo Scale questionnaire for the assessment of quality of life of patients with gastrointestinal symptoms [42] and the Japanese version of the constipation assessment scale (CAS) [43, 44]. The question items were as follows: feeling of fullness (0, never; 1, sometimes; 2, always), heavy stomach feeling after meals (0, never; 1, sometimes; 2: always), early satiation (0, never; 1, sometimes; 2, always), and appetite (1, never; 2, rarely; 3, sometimes; 4, often; 5, always).

Mood status

Mood status was measured using the short Japanese version of POMS 2 before and after the intervention in each test period. The reliability and validity of POMS 2 have been confirmed, and POMS 2 is widely used in the medical and industrial fields [45]. The 35 questions in this self-reported instrument were classified into seven mood subscales: (1) Anger/Hostility, (2) Confusion/Bewilderment, (3) Depression/Dejection, (4) Fatigue/Inertia, (5) Tension/Anxiety, (6) Vigor/Activity, and (7) Friendliness. Each question was rated on a 5-point scale (from 0 for not at all to 4 for extremely), and the total mood disturbance was calculated from these subscales [46]. T-scores were calculated from the 5-point scores using a T-score conversion table (Kaneko Shobo Co., Ltd., Tokyo, Japan).

Skin condition and sleep quality

Skin condition and sleep quality were measured based on a subjective evaluation using the original VAS on a 10-cm horizontal line [47], where the far-left side was 0 and indicated bad skin condition/light sleep and far right side was 10 and indicated good skin condition/deep sleep.

Statistical analyses

In each group, the average and standard deviation or median values for the composition of the intestinal microbiota, defecation status, gastrointestinal condition, appetite, mood status, skin condition, and sleep quality were calculated. A Wilcoxon signed rank test or paired t-test was used to compare the data before and 2 weeks after the intervention. Steel's or Dunnett's test was used to compare the amount of change between each test supplement group (γ-PGA–added groups) and the control group (GOS-only group). The amount of change was obtained by subtracting the value before the intervention from the value at 2 weeks after the intervention. The data for the composition of the intestinal bacteria were highly biased due to there being a large number of bacteria with relative abundances of 0, and therefore, we used nonparametric statistics. We also used nonparametric statistics for ordinal scales, such as those for gastrointestinal condition, appetite, and mood status. Statistical analysis was performed using R version 3.6.2 (https://cran.r-project.org/bin/windows/base/old/3.6.2/). A p-value less than 0.05 was considered statistically significant.

RESULTS

Participants

The backgrounds of the participants are shown in Supplementary Table 2. No significant differences were found in any items. During the test period, regular interviews by the CRO and the doctor's judgment based on that interview reports revealed no reports of serious adverse events.

Intestinal bifidobacteria

The relative abundances of bifidobacteria at the genus level are shown in Table 2. Administration of 1.0 g GOS did not significantly increase the relative abundances of bifidobacteria. On the other hand, administration of 1.0 g GOS together with 0.3 g or more γ-PGA significantly increased or tended to increase bifidobacteria between before and after ingestion. However, administration of 2.0 g GOS significantly increased or tended to increase the relative abundances of bifidobacteria regardless of the addition or absence of γ-PGA. Meanwhile, there was no significant difference between the GOS group and γ-PGA-added groups in terms of the changes in the relative abundances of bifidobacteria. In addition to the genus-level analysis of bifidobacteria described above, we also performed a species-level analysis of the bifidobacteria (Supplementary Table 3). The relative abundance of *B. longum* was significantly higher in the 2.0 g GOS plus 1.0 g γ-PGA group after ingestion than before ingestion, and the increase was significantly different from that in the 2.0 g GOS-only group (Supplementary Table 3 and Fig. 3).

Intestinal flora other than bifidobacteria

The results of the intestinal flora (genus level) analysis, excluding those for bifidobacteria, are shown in Supplementary Table 4. Comparison of the GOS-only group with the γ-PGA-added groups revealed that there were some intestinal bacteria for which the change in relative abundance was significant (Supplementary Table 4A). In the γ-PGA-added groups, the relative abundances for the genus *Lactobacillus* were significantly higher, and those for the genera *Fusobacterium* and *Parasutterella* were significantly lower (Supplementary Table 4B).

Intestinal regulation index

We evaluated the days of defecation (per week), defecation frequency (per week), amount of defecation, Bristol stool form scale, and feeling of remaining feces (Table 3). There were no significant differences in the amounts of changes in any of the indices between the GOS-only group and γ-PGA-added groups. All the administration groups, excluding the 1.0 g GOS plus 1.0 g γ-PGA group in the first period and 2.0 g GOS plus 0.3 g

Table 2. Composition of bifidobacteria at the genus level

Group		Relative abundance of bifidobacteria			Change in relative abundance		
		Average ± SD (%)	Median (%)	Compared with before intake (p-value)	Average ± SD (%)	Median (%)	Compared with GOS group (p-value)
GOS 1.0 g	before	3.8 ± 3.2	2.8	0.154	1.4 ± 4.9	1.9	—
	after	5.2 ± 3.5	5.0				
GOS 1.0 g + γ-PGA 0.1 g	before	4.2 ± 4.2	2.9	0.784	−0.2 ± 3.9	−0.6	0.444
	after	3.9 ± 3.0	3.1				
GOS 1.0 g + γ-PGA 0.3 g	before	4.1 ± 3.0	3.4	0.058#	3.0 ± 6.3	2.9	0.984
	after	7.1 ± 5.8	5.7				
GOS 1.0 g + γ-PGA 0.5 g	before	4.2 ± 5.1	2.0	0.036*	2.1 ± 5.8	2.6	0.892
	after	6.3 ± 4.4	5.4				
GOS 1.0 g + γ-PGA 1.0 g	before	3.1 ± 2.5	2.7	0.058#	1.9 ± 4.2	1.7	0.998
	after	5.0 ± 4.3	3.8				
GOS 2.0 g	before	4.8 ± 5.5	3.8	0.005**	3.1 ± 4.1	1.3	—
	after	7.9 ± 6.7	7.6				
GOS 2.0 g + γ-PGA 0.1 g	before	3.3 ± 2.4	3.1	0.003**	2.1 ± 2.4	2.4	0.994
	after	5.4 ± 3.8	5.0				
GOS 2.0 g + γ-PGA 0.3 g	before	4.3 ± 2.6	4.7	0.014*	2.2 ± 3.9	2.1	1.000
	after	6.5 ± 4.3	7.0				
GOS 2.0 g + γ-PGA 0.5 g	before	4.6 ± 3.3	4.2	0.087#	1.5 ± 3.7	1.5	0.799
	after	6.1 ± 4.2	6.2				
GOS 2.0 g + γ-PGA 1.0 g	before	3.7 ± 3.4	2.7	<0.001***	5.4 ± 4.1	5.2	0.211
	after	9.2 ± 6.0	8.7				

The results for the composition of bifidobacteria before and after the intervention are shown. For each test supplement, the average, standard deviation (SD), and median were calculated. The medians were compared using the Wilcoxon signed rank test. For the changes in relative abundance, the average, SD, and median were calculated for each test supplement. For the between-group comparison analysis, the changes in medians were compared between the control (GOS-only group) and each test supplement group using Steel's test. #p<0.1. *p<0.05. **p<0.01. ***p<0.001. GOS: galactooligosaccharides; γ-PGA: poly-γ-glutamic acid.

or 1.0 g γ-PGA group in the second period, showed significant increases in days of defecation (per week). As for the defecation frequency (per week), all of the administration groups in the first period and the groups administered 2.0 g GOS plus 0.3 g or more γ-PGA in the second period showed significant increases or tendencies to increase. With respect to the amount of defecation, all of the administration groups in the first period and all of the administration groups except the 2.0g GOS-only group in the second period showed increases. Only the 1.0 g GOS plus 1.0 g γ-PGA administration group showed an increase in the stool form scale, and 1.0 g GOS plus 0.1 g or 0.5 g γ-PGA in the first period and 2.0 g GOS plus 0.3 g or 0.5 g γ-PGA in the second period showed an improvement in the feeling of remaining feces.

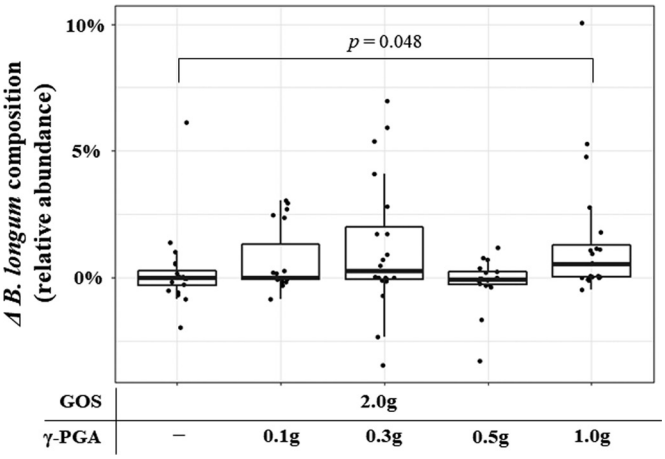

Fig. 3. Change in *B. longum* composition.

The change in the relative abundance of *B. longum* was obtained by subtracting the value before the intervention from the value at 2 weeks after the intervention. For the between-group comparison analysis, the changes in relative abundance were compared between the control (GOS-only group) and each test supplement group using Steel's test. There was a significant difference between the 2.0 g GOS group and the 2.0 g GOS plus 1.0 g γ-PGA group (p<0.048). GOS: galactooligosaccharides; γ-PGA: poly-γ-glutamic acid.

Mood status

The T-score results for the short version of POMS 2 are shown in Table 4. There were no significant differences between the GOS and γ-PGA-added groups in any of the mood indices. In the first period, administration of 1.0 g GOS significantly improved the Anger/Hostility and Tension/Anxiety scores. In the second period, administration of 2.0 g GOS plus 0.3 g or 1.0 g γ-PGA significantly improved or tended to improve the Fatigue/Inertia score, and administration of 2.0 g GOS plus 0.3 g or more γ-PGA significantly improved or tended to improve the Tension/Anxiety score.

Gastrointestinal condition, skin, and sleep quality

The results of the gastrointestinal condition, skin, and sleep quality observations are shown in Supplementary Table 5. No improvement in gastrointestinal condition was observed as a result of test supplement intake. The condition of the skin was significantly improved in the 1.0 g GOS plus 0.1 g γ-PGA group,

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and sleep quality was significantly improved in the 2.0 g GOS plus 0.3 g γ-PGA group.

DISCUSSION

This study was a randomized, double-blind, parallel-group, dose-response study performed to clarify the combined effects of orally administered GOS and γ-PGA on the intestinal environment and health conditions, including intestinal regulation, mood status, gastrointestinal condition, skin, and sleep quality. We also estimated the minimum effective dose of this test supplement.

In this study, no adverse effects due to any dose of GOS or γ-PGA were observed, as assessed by a clinical doctor. Therefore, this result provides evidence of the safety of the combination of GOS and γ-PGA.

Table 3. Intestinal regulation index

(A)

Outcome	Group		Observed value		Amount of change	
			Average ± SD	Compared with before intake (p-value)	Average ± SD	Compared with GOS group (p-value)
Days of defecation (/week)	GOS 1.0 g	before	3.5 ± 0.9	<0.001***	1.2 ± 1.1	—
		after	4.7 ± 1.0			
	GOS 1.0 g + γ-PGA 0.1 g	before	4.0 ± 0.8	0.044*	0.7 ± 1.4	0.447
		after	4.7 ± 1.3			
	GOS 1.0 g + γ-PGA 0.3 g	before	3.8 ± 0.7	0.002**	0.8 ± 0.9	0.597
		after	4.5 ± 1.0			
	GOS 1.0 g + γ-PGA 0.5 g	before	3.6 ± 0.7	0.012*	0.6 ± 1.0	0.381
		after	4.3 ± 1.2			
	GOS 1.0 g + γ-PGA 1.0 g	before	4.0 ± 1.2	0.192	0.3 ± 1.0	0.051#
		after	4.3 ± 1.4			
	GOS 2.0 g	before	4.4 ± 1.3	0.101	0.4 ± 1.0	—
		after	4.7 ± 1.3			
	GOS 2.0 g + γ-PGA 0.1 g	before	4.4 ± 1.1	0.163	0.2 ± 0.6	0.960
		after	4.6 ± 1.3			
	GOS 2.0 g + γ-PGA 0.3 g	before	4.4 ± 1.0	<0.001***	0.7 ± 0.7	0.739
		after	5.1 ± 0.9			
	GOS 2.0 g + γ-PGA 0.5 g	before	4.4 ± 0.9	0.172	0.4 ± 1.1	1.000
		after	4.7 ± 1.0			
	GOS 2.0 g + γ-PGA 1.0 g	before	4.2 ± 1.0	0.034*	0.7 ± 1.3	0.739
		after	4.8 ± 1.4			
Defecation frequency (/week)	GOS 1.0 g	before	3.7 ± 0.8	<0.001***	1.8 ± 1.9	—
		after	5.5 ± 1.9			
	GOS 1.0 g + γ-PGA 0.1 g	before	4.5 ± 1.3	0.041*	1.1 ± 2.3	0.658
		after	5.6 ± 2.1			
	GOS 1.0 g + γ-PGA 0.3 g	before	4.0 ± 0.8	0.003**	1.1 ± 1.4	0.596
		after	5.1 ± 1.6			
	GOS 1.0 g + γ-PGA 0.5 g	before	4.0 ± 0.9	0.010*	1.2 ± 1.8	0.767
		after	5.2 ± 2.1			
	GOS 1.0 g + γ-PGA 1.0 g	before	4.2 ± 1.4	0.065#	0.7 ± 1.6	0.218
		after	4.9 ± 2.1			
	GOS 2.0 g	before	5.5 ± 2.5	0.569	0.2 ± 1.5	—
		after	5.7 ± 2.1			
	GOS 2.0 g + γ-PGA 0.1 g	before	5.2 ± 1.9	0.600	0.1 ± 0.9	0.999
		after	5.3 ± 1.8			
	GOS 2.0 g + γ-PGA 0.3 g	before	4.9 ± 1.3	<0.001***	1.0 ± 1.1	0.255
		after	5.9 ± 1.6			
	GOS 2.0 g + γ-PGA 0.5 g	before	5.1 ± 1.4	0.075#	0.8 ± 1.8	0.572
		after	5.8 ± 1.9			
	GOS 2.0 g + γ-PGA 1.0 g	before	4.7 ± 1.5	0.067#	0.8 ± 1.8	0.543
		after	5.5 ± 2.0			

The results for days of defecation (per week), defecation frequency (per week), amount of defecation (A), Bristol stool form scale, and feeling of remaining feces (B) before and after the intervention are shown. Observed value: for each test supplement, the average, standard deviation (SD), and/or median were calculated. A: averages were compared using the paired t-test. B: medians were compared using the Wilcoxon signed rank test. Amount of change: for each test supplement, the average, SD, and/or median were calculated. A: average amounts of changes were compared between the control (GOS-only group) and each test supplement group using Dunnett's test. B: median amounts of changes were compared between the control (GOS-only group) and each test supplement group using Steel's test. #p<0.1. *p<0.05. **p<0.01. ***p<0.001. GOS: galactooligosaccharides; γ-PGA: poly-γ-glutamic acid.

Table 3. Continued

(A)

Outcome	Group		Observed value		Amount of change	
			Average ± SD	Compared with before intake (p-value)	Average ± SD	Compared with GOS group (p-value)
Amount of defecation (equal to hen's egg-M size)	GOS 1.0 g	before	8.1 ± 4.9	0.001**	3.0 ± 3.5	—
		after	11.1 ± 6.3			
	GOS 1.0 g + γ-PGA 0.1 g	before	9.5 ± 5.8	0.032*	2.9 ± 5.6	1.000
		after	12.4 ± 9.1			
	GOS 1.0 g + γ-PGA 0.3 g	before	7.9 ± 3.9	0.014*	1.9 ± 3.1	0.766
		after	9.7 ± 5.2			
	GOS 1.0 g + γ-PGA 0.5 g	before	9.7 ± 7.7	0.055#	1.8 ± 3.9	0.749
		after	11.6 ± 7.1			
	GOS 1.0 g + γ-PGA 1.0 g	before	10.0 ± 5.9	0.067#	1.5 ± 3.4	0.560
		after	11.5 ± 6.7			
	GOS 2.0 g	before	9.8 ± 6.4	0.231	0.8 ± 3.0	—
		after	10.6 ± 5.7			
	GOS 2.0 g + γ-PGA 0.1 g	before	10.5 ± 5.4	0.020*	1.2 ± 2.0	0.995
		after	11.7 ± 5.8			
	GOS 2.0 g + γ-PGA 0.3 g	before	10.5 ± 5.9	0.050*	1.4 ± 3.0	0.970
		after	11.9 ± 7.3			
	GOS 2.0 g + γ-PGA 0.5 g	before	12.1 ± 8.5	0.004**	2.2 ± 3.0	0.588
		after	14.3 ± 9.9			
	GOS 2.0 g + γ-PGA 1.0 g	before	10.6 ± 5.6	0.077#	2.6 ± 6.3	0.366
		after	13.2 ± 10.1			

The beneficial effects of GOS on the intestinal environment have previously been reported, and oral intake of 1.0 to 10.0 g per day increases bifidobacteria and decreases the putrefactive products produced by other intestinal bacteria [48–52]. In this study, there were no significant differences in the changes in bifidobacteria at the genus level in terms of relative abundance in the γ-PGA-added groups compared to the GOS group, as shown in Table 2. In the first period, the relative abundances of bifidobacteria were not significantly changed in the 1.0 g GOS group, even though intake of 1.0 g of GOS has been reported to increase bifidobacterial colony counts [51]. Although the method of intestinal microbiota analysis differed between our study and the previous report, a critical reason for the difference may be the shorter period of test supplement intake in this study (two weeks) than the previous report (three weeks). The intervention with 1.0 g GOS plus 0.3 g or more γ-PGA groups resulted in a significant increase in or a tendency to increase the relative abundances of bifidobacteria, suggesting that the addition of γ-PGA to 1.0 g GOS may result in an early increase in bifidobacteria. A significant increase or tendency towards an increase in the relative abundances of bifidobacteria was observed in all groups administered 2.0 g GOS, regardless of the presence or absence of γ-PGA. It is possible that the significant effects of γ-PGA addition to 2.0 g GOS on the increase in bifidobacteria were masked by the significant effect of 2.0 g GOS only.

We also analyzed the bifidobacteria at the species level (Supplementary Table 3) and found that there was a significant difference in the change in the relative abundance of *Bifidobacterium longum* after the intervention with 1.0 g γ-PGA plus 2.0 g GOS compared to the intervention with 2.0 g GOS only (Supplementary Table 3, Fig. 3). In an in vitro evaluation system, it has been reported that GOS alone increased *Bifidobacterium adolescentis*, *Bifidobacterium catenulatum, Bifidobacterium pseudocatenulatum,* and *B. longum* subsp. *infantis* but did not increase *Bifidobacterium breve, B. longum* subsp. *longum*, and *Bifidobacterium bifidum* [53]. In this study, only *B. breve* was increased in the intervention using 2.0 g GOS alone; however, the changes in other bifidobacterial species were consistent with those of the previous study [53]. It has been reported that *B. longum* inhabits the large intestine in a wide range of age groups [54], and it is known to play important roles in protection against infection and improvement of blood lipids, in addition to improvement of intestinal condition [10, 55, 56]. The combination of 2.0 g GOS and 1.0 g of γ-PGA may be effective for increasing the abundance of *B. longum* in people belonging to a wide age range and bring many kinds of health benefits.

In addition to the assessment of bifidobacteria, analysis of other intestinal bacteria indicated that some minor changes occurred in other intestinal bacteria (Supplementary Table 4). On the other hand, the amounts of the changes in the intestinal bacteria with significant changes, excluding bifidobacteria, were less than 1% when their medians were compared (data not shown). These results suggest that the effect of combined intake of GOS and γ-PGA is mainly on bifidobacteria.

In previous reports, intestinal regulation and an increased defecation frequency were demonstrated after the intake of 4.0 g or more of GOS per day [48, 57, 58]; on the other hand, bifidobacteria are known to be increased after the intake of 1.0 g GOS, as described above. In the first period of this study, intake of 1.0 g GOS resulted in improvement of intestinal regulation, even though no increases in bifidobacteria were observed, which differed from the previous findings. On the other hand, the results of intake of 2.0 g GOS in the second period were consistent with previous reports [48, 51, 57, 58] (Tables 2 and 3). It is known

Table 3. Continued

(B)

Outcome	Group		Observed value			Amount of change		
			Average ± SD	Median	Compared with before intake (p-value)	Average ± SD	Median	Compared with GOS group (p-value)
Bristol Stool Form Scale (1: Separate hard lumps, like nuts –7: Watery, no solid pieces, entirely liquid)	GOS 1.0 g	before	3.7 ± 0.8	3.9	0.344	0.1 ± 0.6	0.2	—
		after	3.8 ± 0.9	4.2				
	GOS 1.0 g + γ-PGA 0.1 g	before	3.7 ± 0.6	3.8	0.571	0.0 ± 0.5	0.0	0.730
		after	3.7 ± 0.7	4.0				
	GOS 1.0 g + γ-PGA 0.3 g	before	3.4 ± 0.5	3.4	0.490	0.2 ± 0.7	0.3	0.984
		after	3.6 ± 0.7	3.5				
	GOS 1.0 g + γ-PGA 0.5 g	before	3.5 ± 0.8	3.7	0.466	0.1 ± 0.5	0.0	0.994
		after	3.7 ± 0.7	3.6				
	GOS 1.0 g + γ-PGA 1.0 g	before	3.1 ± 0.9	3.3	0.002**	0.5 ± 0.7	0.3	0.480
		after	3.5 ± 0.8	3.8				
	GOS 2.0 g	before	3.6 ± 0.7	3.9	0.459	0.1 ± 0.5	0.0	—
		after	3.7 ± 0.6	3.9				
	GOS 2.0 g + γ-PGA 0.1 g	before	3.5 ± 0.8	3.8	0.423	0.1 ± 0.6	0.2	0.992
		after	3.7 ± 0.5	3.7				
	GOS 2.0 g + γ-PGA 0.3 g	before	3.9 ± 0.5	4.0	0.580	0.0 ± 0.5	0.1	1.000
		after	3.9 ± 0.6	4.0				
	GOS 2.0 g + γ-PGA 0.5 g	before	3.5 ± 0.6	3.6	0.404	0.1 ± 0.4	0.1	0.960
		after	3.7 ± 0.6	3.8				
	GOS 2.0 g + γ-PGA 1.0 g	before	3.5 ± 0.9	3.7	0.891	0.0 ± 0.6	0.0	0.898
		after	3.5 ± 0.9	3.8				
Feeling of remaining feces (0: Never, 1: sometimes, 2: always)	GOS 1.0 g	before	0.4 ± 0.5	0.2	0.859	0.0 ± 0.4	0.0	—
		after	0.4 ± 0.5	0.3				
	GOS 1.0 g + γ-PGA 0.1 g	before	0.4 ± 0.5	0.4	0.091#	−0.1 ± 0.3	0.0	0.964
		after	0.3 ± 0.5	0.2				
	GOS 1.0 g + γ-PGA 0.3 g	before	0.4 ± 0.4	0.4	0.967	0.0 ± 0.2	0.0	0.994
		after	0.4 ± 0.4	0.3				
	GOS 1.0 g + γ-PGA 0.5 g	before	0.6 ± 0.6	0.3	0.038*	−0.2 ± 0.3	−0.1	0.630
		after	0.4 ± 0.4	0.3				
	GOS 1.0 g + γ-PGA 1.0 g	before	0.5 ± 0.4	0.3	0.951	0.0 ± 0.4	0.0	0.997
		after	0.5 ± 0.4	0.4				
	GOS 2.0 g	before	0.3 ± 0.5	0.1	0.424	−0.1 ± 0.4	0.0	—
		after	0.3 ± 0.3	0.1				
	GOS 2.0 g + γ-PGA 0.1 g	before	0.5 ± 0.6	0.4	0.950	0.0 ± 0.2	0.0	1.000
		after	0.5 ± 0.5	0.4				
	GOS 2.0 g + γ-PGA 0.3 g	before	0.3 ± 0.3	0.3	0.025*	−0.1 ± 0.2	0.0	0.357
		after	0.2 ± 0.3	0.1				
	GOS 2.0 g + γ-PGA 0.5 g	before	0.5 ± 0.4	0.4	0.049	−0.1 ± 0.3	0.0	0.691
		after	0.3 ± 0.4	0.2				
	GOS 2.0 g + γ-PGA 1.0 g	before	0.4 ± 0.3	0.3	0.696	0.0 ± 0.2	0.0	0.998
		after	0.3 ± 0.3	0.3				

that participants in human clinical studies are psychologically affected and suppressing this effect (sometimes called the "placebo effect") is key to demonstrating the benefit of a test food intervention [59, 60]. We believe that the subjects in the first period were psychologically affected by the fact that they participated in this study and that the results in the first period were biased. Therefore, we decided to discuss quantitative results, such as the composition of bifidobacteria, based on both the first and second period results and psychologically affected indices, such as the status of defecation and mood, based on the second period only. In the second period, significant increases or trends towards increases in bifidobacteria were observed in all groups, with an improvement in intestinal regulation resulting from the use of 2.0 g of GOS plus 0.3 g or more γ-PGA (Tables 2 and 3). This is consistent with previous studies showing that the use of 2.0 g GOS alone is sufficient for an increase in bifidobacteria but not for improvement of intestinal regulation [47–51, 56, 57], indicating that the addition of γ-PGA to GOS improved intestinal regulation. The minimum effective dose was around 0.3 g of γ-PGA in combination with 2.0 g GOS.

There are many factors contributing to the regulation of intestinal peristalsis. Intestinal regulation is related not only to the composition of bifidobacteria but also to the acetic acid produced by bifidobacteria and other intestinal bacteria and the amount

Table 4. POMS 2 short version

Outcome	Group		Observed value			Amount of change		
			Average ± SD	Median	Compared with before intake (p-value)	Average ± SD	Median	Compared with GOS group (p-value)
Anger-Hostility	GOS 1.0 g	before	49.4 ± 9.3	47.5	0.048*	−4.1 ± 8.2	−1.0	—
		after	45.3 ± 6.8	44.0				
	GOS 1.0 g + γ-PGA 0.1 g	before	44.4 ± 5.9	43.0	0.409	−1.1 ± 5.2	−1.0	0.830
		after	43.3 ± 5.3	42.0				
	GOS 1.0 g + γ-PGA 0.3 g	before	45.6 ± 8.3	44.5	0.423	1.5 ± 6.5	0.0	0.309
		after	47.0 ± 8.8	45.0				
	GOS 1.0 g + γ-PGA 0.5 g	before	44.5 ± 7.5	42.0	0.443	−0.1 ± 7.7	0.0	0.875
		after	44.5 ± 8.3	40.0				
	GOS 1.0 g + γ-PGA 1.0 g	before	44.1 ± 7.9	41.0	0.574	−0.1 ± 5.2	0.0	0.179
		after	44.0 ± 7.7	42.0				
	GOS 2.0 g	before	46.1 ± 9.8	42.0	0.211	1.9 ± 6.5	0.0	—
		after	48.0 ± 10.3	43.0				
	GOS 2.0 g + γ-PGA 0.1 g	before	47.6 ± 7.6	44.0	0.532	1.1 ± 7.0	0.0	1.000
		after	48.7 ± 7.9	49.0				
	GOS 2.0 g + γ-PGA 0.3 g	before	45.6 ± 8.5	44.0	0.832	−0.7 ± 5.9	0.0	0.828
		after	44.9 ± 7.7	42.0				
	GOS 2.0 g + γ-PGA 0.5 g	before	44.4 ± 7.6	42.0	0.693	−0.2 ± 4.4	0.0	0.669
		after	44.2 ± 9.0	42.0				
	GOS 2.0 g + γ-PGA 1.0 g	before	43.3 ± 7.4	42.0	0.188	2.0 ± 5.1	0.0	1.000
		after	45.3 ± 8.7	44.0				
Confusion-Bewilderment	GOS 1.0 g	before	47.1 ± 7.6	46.0	0.767	−0.4 ± 5.0	0.0	—
		after	46.7 ± 5.5	46.0				
	GOS 1.0 g + γ-PGA 0.1 g	before	44.9 ± 6.2	43.5	0.573	−0.4 ± 4.4	0.0	1.000
		after	44.5 ± 6.8	41.0				
	GOS 1.0 g + γ-PGA 0.3 g	before	43.7 ± 7.1	41.0	0.189	1.0 ± 2.7	0.0	0.673
		after	44.7 ± 6.6	43.0				
	GOS 1.0 g + γ-PGA 0.5 g	before	45.5 ± 6.8	46.0	0.804	−0.3 ± 4.3	0.0	1.000
		after	45.2 ± 6.2	43.0				
	GOS 1.0 g + γ-PGA 1.0 g	before	47.7 ± 7.9	46.0	0.082#	−1.9 ± 4.5	0.0	0.783
		after	45.9 ± 8.7	43.0				
	GOS 2.0 g	before	45.7 ± 10.1	40.0	0.851	−0.4 ± 5.0	0.0	—
		after	45.3 ± 8.1	43.0				
	GOS 2.0 g + γ-PGA 0.1 g	before	46.4 ± 6.9	43.0	0.283	1.5 ± 6.8	0.0	0.965
		after	47.9 ± 9.0	46.0				
	GOS 2.0 g + γ-PGA 0.3 g	before	47.2 ± 8.5	44.5	0.547	−0.4 ± 4.0	0.0	0.938
		after	46.8 ± 9.2	45.0				
	GOS 2.0 g + γ-PGA 0.5 g	before	44.1 ± 6.2	41.0	0.494	−0.2 ± 3.0	0.0	0.911
		after	43.9 ± 5.5	43.0				
	GOS 2.0 g + γ-PGA 1.0 g	before	44.2 ± 7.9	41.0	0.718	−0.5 ± 4.8	0.0	0.901
		after	43.7 ± 7.6	41.0				
Depression-Dejection	GOS 1.0 g	before	49.1 ± 5.8	47.0	0.154	−1.9 ± 5.3	−2.0	—
		after	47.2 ± 5.3	46.5				
	GOS 1.0 g + γ-PGA 0.1 g	before	45.7 ± 4.9	44.5	0.177	−0.9 ± 2.8	0.0	0.756
		after	44.9 ± 5.5	43.0				
	GOS 1.0 g + γ-PGA 0.3 g	before	46.5 ± 7.2	44.0	0.857	−0.4 ± 3.8	0.0	0.538
		after	46.1 ± 6.0	44.0				
	GOS 1.0 g + γ-PGA 0.5 g	before	45.9 ± 5.3	45.0	0.957	0.0 ± 3.8	0.0	0.540
		after	45.9 ± 5.3	43.0				
	GOS 1.0 g + γ-PGA 1.0 g	before	48.2 ± 9.0	44.5	0.072#	−1.6 ± 3.7	0.0	0.951
		after	46.6 ± 8.8	41.0				

The results of the POMS 2 questionnaires before and after the intervention are shown. Observed value: for each test supplement, the average, standard deviation (SD), and median were calculated. Medians were compared using the Wilcoxon signed rank test. Amount of change: for each test supplement, the average, SD, and median were calculated. For the between-group comparison analysis, the median amounts of changes were compared between the control (GOS-only group) and each test supplement group using Steel's test. #p<0.1. *p<0.05. **p<0.01. †Significant difference (p<0.05) in the non-improvement direction. $Tendency (p<0.1) in the non-improvement direction. POMS 2: Profile of Mood States 2nd Edition; GOS: galactooligosaccharides; γ-PGA: poly-γ-glutamic acid; TMD: total mood disturbance.

of water and quality of food consumed [61–63]. In addition, γ-PGA is known to have a high water-retention ability [64]. The effect of the combined intervention of GOS and γ-PGA on the improvement of intestinal regulation appears to be the result of improvement of the intestinal environment and its relevant characteristics mediated by them.

The effects of the combined intervention of GOS and γ-PGA on mood status were evaluated in this study using POMS 2

Table 4. Continued

Outcome	Group		Observed value			Amount of change		
			Average ± SD	Median	Compared with before intake (p-value)	Average ± SD	Median	Compared with GOS group (p-value)
Depression-Dejection	GOS 2.0 g	before	45.6 ± 6.0	43.0	0.697	−0.3 ± 3.8	0.0	—
		after	45.4 ± 7.0	43.0				
	GOS 2.0 g + γ-PGA 0.1 g	before	48.0 ± 8.2	45.0	0.923	−0.5 ± 5.3	0.0	0.830
		after	47.5 ± 6.5	45.0				
	GOS 2.0 g + γ-PGA 0.3 g	before	46.3 ± 6.2	45.0	0.488	1.0 ± 5.4	0.0	0.964
		after	47.2 ± 8.7	43.0				
	GOS 2.0 g + γ-PGA 0.5 g	before	46.4 ± 5.9	43.0	0.479	−1.1 ± 4.3	0.0	0.997
		after	45.3 ± 5.0	43.0				
	GOS 2.0 g + γ-PGA 1.0 g	before	46.3 ± 9.7	43.0	0.078#	−1.3 ± 2.8	0.0	0.796
		after	45.1 ± 8.7	42.0				
Fatigue-Inertia	GOS 1.0 g	before	45.6 ± 6.2	45.0	0.722	0.4 ± 4.4	0.0	—
		after	46.0 ± 5.9	47.0				
	GOS 1.0 g + γ-PGA 0.1 g	before	43.5 ± 5.7	42.5	0.538	−0.7 ± 4.8	−1.0	0.883
		after	42.8 ± 5.5	42.5				
	GOS 1.0 g + γ-PGA 0.3 g	before	43.5 ± 8.5	41.0	0.260	1.8 ± 5.4	0.0	0.896
		after	45.2 ± 9.4	43.0				
	GOS 1.0 g + γ-PGA 0.5 g	before	45.8 ± 7.1	45.0	0.874	0.3 ± 5.0	0.0	1.000
		after	46.1 ± 6.4	45.0				
	GOS 1.0 g + γ-PGA 1.0 g	before	46.4 ± 9.3	45.0	0.528	−0.8 ± 5.0	−1.0	0.876
		after	45.7 ± 10.0	43.0				
	GOS 2.0 g	before	46.0 ± 11.8	43.0	0.910	0.3 ± 4.9	0.0	—
		after	46.2 ± 10.3	45.0				
	GOS 2.0 g + γ-PGA 0.1 g	before	45.9 ± 9.1	45.0	0.518	1.0 ± 7.5	0.0	0.992
		after	46.9 ± 8.5	45.0				
	GOS 2.0 g + γ-PGA 0.3 g	before	45.1 ± 9.6	42.5	0.045*	−2.5 ± 4.8	−2.0	0.418
		after	42.6 ± 7.5	40.5				
	GOS 2.0 g + γ-PGA 0.5 g	before	44.2 ± 7.6	43.0	0.174	−1.3 ± 4.0	0.0	0.783
		after	42.8 ± 6.0	41.0				
	GOS 2.0 g + γ-PGA 1.0 g	before	46.8 ± 9.4	45.0	0.088#	−1.7 ± 6.3	−2.0	0.627
		after	45.2 ± 10.1	44.0				
Tension-Anxiety	GOS 1.0 g	before	48.2 ± 7.1	46.0	0.039*	−2.9 ± 5.4	−3.0	—
		after	45.3 ± 4.1	44.0				
	GOS 1.0 g + γ-PGA 0.1 g	before	44.8 ± 7.5	43.0	0.816	−0.4 ± 5.5	0.0	0.250
		after	44.4 ± 5.7	44.0				
	GOS 1.0 g + γ-PGA 0.3 g	before	43.6 ± 8.0	42.0	0.244	1.4 ± 4.9	0.0	0.065$
		after	45.0 ± 8.6	43.0				
	GOS 1.0 g + γ-PGA 0.5 g	before	45.4 ± 7.4	44.0	0.703	−0.6 ± 5.8	0.0	0.445
		after	44.8 ± 7.3	44.0				
	GOS 1.0 g + γ-PGA 1.0 g	before	46.8 ± 9.3	44.0	0.101	−1.6 ± 6.2	−2.0	0.761
		after	45.2 ± 9.1	44.0				
	GOS 2.0 g	before	45.1 ± 12.3	40.5	0.934	−0.1 ± 5.1	0.0	—
		after	45.1 ± 8.6	42.0				
	GOS 2.0 g + γ-PGA 0.1 g	before	45.8 ± 10.0	44.0	0.837	−0.7 ± 5.4	0.0	0.980
		after	45.1 ± 9.1	44.0				
	GOS 2.0 g + γ-PGA 0.3 g	before	45.3 ± 7.9	44.0	0.058#	−2.2 ± 4.9	−1.0	0.262
		after	43.2 ± 7.7	40.5				
	GOS 2.0 g + γ-PGA 0.5 g	before	45.1 ± 6.5	46.0	0.024*	−1.7 ± 3.3	−2.0	0.338
		after	43.4 ± 6.3	42.0				
	GOS 2.0 g + γ-PGA 1.0 g	before	44.2 ± 8.7	44.0	0.005**	−3.3 ± 5.1	−2.0	0.090#
		after	40.9 ± 6.3	39.0				

Table 4. Continued

Outcome	Group		Observed value			Amount of change		
			Average ± SD	Median	Compared with before intake (p-value)	Average ± SD	Median	Compared with GOS group (p-value)
Vigor-Activity	GOS 1.0 g	before	47.3 ± 8.3	47.0	0.752	0.2 ± 6.8	0.0	—
		after	47.5 ± 8.6	45.0				
	GOS 1.0 g + γ-PGA 0.1 g	before	51.0 ± 11.4	55.0	0.221	1.9 ± 7.8	1.0	0.859
		after	52.9 ± 9.7	55.0				
	GOS 1.0 g + γ-PGA 0.3 g	before	49.4 ± 9.3	47.0	0.629	−0.8 ± 4.3	0.0	0.939
		after	48.6 ± 8.6	47.0				
	GOS 1.0 g + γ-PGA 0.5 g	before	51.5 ± 7.2	53.0	0.958	−0.5 ± 6.4	2.0	1.000
		after	51.1 ± 7.7	49.0				
	GOS 1.0 g + γ-PGA 1.0 g	before	53.6 ± 12.4	52.0	0.079[$]	−2.9 ± 6.3	−2.5	0.324
		after	50.7 ± 13.4	49.0				
	GOS 2.0 g	before	51.7 ± 10.9	55.0	0.479	0.8 ± 6.1	0.0	—
		after	52.5 ± 11.2	56.0				
	GOS 2.0 g + γ-PGA 0.1 g	before	47.6 ± 11.2	45.0	0.298	0.4 ± 4.1	0.0	1.000
		after	48.1 ± 10.2	49.0				
	GOS 2.0 g + γ-PGA 0.3 g	before	50.2 ± 6.9	49.0	0.678	0.8 ± 5.1	0.0	1.000
		after	51.0 ± 9.3	48.0				
	GOS 2.0 g + γ-PGA 0.5 g	before	52.8 ± 10.7	54.0	0.044[†]	−2.1 ± 4.4	−2.0	0.458
		after	50.7 ± 11.3	54.0				
	GOS 2.0 g + γ-PGA 1.0 g	before	50.8 ± 12.4	45.0	0.956	0.2 ± 7.4	0.0	1.000
		after	51.0 ± 9.8	51.5				
Friendliness	GOS 1.0 g	before	48.1 ± 6.6	49.0	0.752	0.1 ± 5.8	0.0	—
		after	48.2 ± 7.8	47.5				
	GOS 1.0 g + γ-PGA 0.1 g	before	54.3 ± 10.2	55.5	0.216	−1.8 ± 6.7	−2.0	0.726
		after	52.5 ± 8.0	55.0				
	GOS 1.0 g + γ-PGA 0.3 g	before	51.3 ± 9.7	53.5	0.636	−0.7 ± 5.4	0.0	1.000
		after	50.6 ± 9.9	53.5				
	GOS 1.0 g + γ-PGA 0.5 g	before	50.6 ± 6.5	50.0	0.472	1.2 ± 5.7	0.0	0.882
		after	51.8 ± 7.5	52.0				
	GOS 1.0 g + γ-PGA 1.0 g	before	54.0 ± 11.9	52.0	<0.001[†]	−3.3 ± 2.9	−3.0	0.250
		after	50.8 ± 12.4	49.0				
	GOS 2.0 g	before	51.9 ± 11.0	52.0	0.665	−0.2 ± 6.9	1.0	—
		after	51.7 ± 8.1	50.5				
	GOS 2.0 g + γ-PGA 0.1 g	before	49.2 ± 10.6	49.0	0.490	−0.5 ± 6.1	0.0	0.758
		after	48.7 ± 12.4	47.0				
	GOS 2.0 g + γ-PGA 0.3 g	before	49.6 ± 6.9	49.0	0.267	1.8 ± 7.0	2.5	0.926
		after	51.4 ± 8.0	50.5				
	GOS 2.0 g + γ-PGA 0.5 g	before	50.9 ± 10.8	49.0	0.170	−1.7 ± 5.4	−3.0	0.459
		after	49.2 ± 11.1	53.0				
	GOS 2.0 g + γ-PGA 1.0 g	before	52.2 ± 10.5	50.5	0.277	−1.3 ± 5.5	−2.0	0.603
		after	50.9 ± 11.3	50.5				

short-form questionnaires. Some mood indices were improved in the γ-PGA-added groups but not in the GOS group. This finding therefore provides evidence that the addition of γ-PGA, at a level of 0.3 g or more, to 2.0 g GOS may improve mood status compared to the intake of 2.0 g GOS alone. There are three hypotheses regarding the mechanism for the improvement in mood status. The first hypothesis is that γ-aminobutyric acid (GABA) produced by intestinal bacteria from γ-PGA may be involved in mood status improvement. GABA is produced from Glu by glutamic acid decarboxylase, which is widely expressed in microorganisms, including lactic acid bacteria and bifidobacteria living in the intestine [65, 66]. GABA receptors are expressed in the small intestine as well as in the large intestine [67]. GABA is also known as an inhibitory neurotransmitter in animals [68], and orally administered GABA has been reported to play an effective role in reducing stress and fatigue [69–72]. In addition, GABA produced by dietary and intestinal bacteria activates the vagus nerve [73], and the vagus nerve is reported to be important for the gut-brain axis based on results obtained using vagotomized mice [74]. These previous reports suggest that orally administered γ-PGA causes an increase in GABA concentration in the large intestine, which leads to mood improvement via GABA receptors expressed in the large intestine. The second hypothesis is that the defecation status was improved by the intake of GOS and γ-PGA, resulting in mood improvement. In fact, a relationship does exist between defecation status and mood status. Irritable bowel

Table 4. Continued

Outcome	Group		Observed value			Amount of change		
			Average ± SD	Median	Compared with before intake (p-value)	Average ± SD	Median	Compared with GOS group (p-value)
TMD	GOS 1.0 g	before	48.5 ± 5.9	47.5	0.115	−2.0 ± 5.2	−2.0	—
		after	46.6 ± 4.9	46.0				
	GOS 1.0 g + γ-PGA 0.1 g	before	44.2 ± 6.2	44.0	0.328	−1.3 ± 4.4	−0.5	0.931
		after	42.9 ± 6.2	41.5				
	GOS 1.0 g + γ-PGA 0.3 g	before	44.5 ± 7.5	44.0	0.161	1.1 ± 3.3	0.5	0.144
		after	45.6 ± 7.4	42.0				
	GOS 1.0 g + γ-PGA 0.5 g	before	44.9 ± 5.6	44.0	0.988	0.0 ± 4.2	0.0	0.580
		after	44.9 ± 5.1	45.0				
	GOS 1.0 g + γ-PGA 1.0 g	before	45.7 ± 9.9	46.0	0.532	−0.7 ± 4.7	−0.5	0.799
		after	45.0 ± 10.1	42.5				
	GOS 2.0 g	before	45.1 ± 10.3	41.5	0.495	0.3 ± 4.4	0.5	—
		after	45.3 ± 8.4	44.5				
	GOS 2.0 g + γ-PGA 0.1 g	before	47.2 ± 8.3	46.0	0.534	0.4 ± 5.8	0.0	0.999
		after	47.6 ± 8.3	46.0				
	GOS 2.0 g + γ-PGA 0.3 g	before	45.7 ± 7.7	43.0	0.219	−1.3 ± 3.7	−0.5	0.495
		after	44.4 ± 8.2	43.0				
	GOS 2.0 g + γ-PGA 0.5 g	before	44.0 ± 6.9	43.0	0.882	−0.5 ± 3.3	1.0	0.854
		after	43.5 ± 6.7	40.0				
	GOS 2.0 g + γ-PGA 1.0 g	before	44.6 ± 9.4	42.5	0.363	−1.1 ± 4.2	−1.0	0.700
		after	43.5 ± 8.8	43.0				

syndrome is associated with anxiety and mood disorders [75], and interventions with bifidobacteria improve gastrointestinal symptoms and mood [76]. The third hypothesis is that changes in the intestinal environment as a result of the intake of GOS and γ-PGA transmit signals from the intestine to the brain via the vagus nerve. This results in the production of growth factors and neurotrophic factors in the brain, leading to the maintenance of brain function and mood status. Recent studies have revealed a complex communication system between the gut and brain and the gut-brain axis [77, 78]. Further studies are required to elucidate the molecular mechanisms underlying the gut-brain relationship and enhance our understanding of the importance of improving the intestinal environment for brain health.

The present study has some limitations. First, the different lifestyles of the individual subjects might have affected their intestinal environments and constipation statuses during the interventions. It is known that a person's intestinal condition is affected by several factors, such as the composition of bifidobacteria, acetic acid, and the amount of water and quality of food consumed [61–63]. In this study, the subjects were instructed to avoid major changes in their lives, but we did not strictly regulate their lifestyles. Second, the present study was an exploratory pilot study investigating the effects of the combined intake of GOS and γ-PGA, and therefore, the generalizability of the obtained findings is limited. Third, the test period was relatively short for observing effects on not only the bifidobacteria composition but also other physiological and mood conditions. Additional studies with an appropriate sample size, calculated on the basis of the present results, and sufficient intervention period are needed. Fourth, a sequence similarity of 97% was used to identify the species in this study. The database we used, the EzBioCloud 16S DB, has been reported to have higher classification accuracy at the genus and species levels [79], but this type of identification method has sometimes been pointed out as tentative. Therefore, further verification of our species level analysis, such as by quantitative PCR, is required in future studies.

DATA AVAILABILITY

The datasets presented in this study can be found in online repositories. The name of the repository and accession number are as follows: DDBJ DRA (accession number: DRA012921).

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CONFLICT OF INTEREST

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