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Short- and long-term dynamics in the intestinal microbiota following ingestion of *Bifidobacterium animalis* subsp. *lactis* GCL2505

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Bifidobacterium animalis subsp. _lactis_ GCL2505 (_B. lactis_ GCL2505) is able to survive passage through the intestines and proliferate. The daily dynamics of the intestinal bifidobacteria following ingestion of probiotics are not yet clear. Moreover, the effects of long-term ingestion of probiotics on the intestinal microbiota have not been well studied. Two experiments were performed in the present study. In Experiment 1, 53 healthy female volunteers received _B. lactis_ GCL2505; _B. bifidum_ GCL2080, which can survive but not proliferate in the intestine; or yogurt fermented with _Lactobacillus delbrueckii_ subsp. _bulgaricus_ and _Streptococcus thermophilus_ for 2 weeks, and the daily dynamics of intestinal bifidobacteria were investigated. The number of fecal bifidobacteria significantly increased on day 1, and this was maintained until day 14 in the _B. lactis_ GCL2505 ingestion group. However, no significant change in the number of fecal bifidobacteria was observed in the other groups throughout the ingestion period. In Experiment 2, 38 constipated volunteers received either _B. lactis_ GCL2505 or a placebo for 8 weeks. Both the number of fecal bifidobacteria and the frequency of defecation significantly increased throughout the ingestion period in the _B. lactis_ GCL2505 ingestion group. These results suggested that the proliferation of ingested bifidobacteria within the intestine contributed to a rapid increase in the amount of intestinal bifidobacteria and subsequent maintenance of these levels. Moreover, _B. lactis_ GCL2505 improved the intestinal microbiota more effectively than non-proliferating bifidobacteria and lactic acid bacteria.

Key words: probiotics, _Bifidobacterium_, dynamics, intestinal microbiota, proliferation, _Bifidobacterium animalis_ subsp. _lactis_ GCL2505

INTRODUCTION

The human intestinal tract is normally inhabited by 400–500 types of bacteria, and it harbors a large, active, and complex community of microbes [1]. The intestinal microbiota play several significant roles in the digestion of food, the metabolism of endogenous and exogenous compounds, immunomodulation, and the inhibition of colonization by pathogenic bacteria, thus making them important for maintaining human health [2, 3]. Members of genus *Bifidobacterium* are among the most predominant organisms in the human intestine and are important for general health, which means that their diversity and number provides a marker for measuring the stability of human intestinal microbiota, as well as the intestinal environment [4, 5].

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Food and Agriculture Organization of the United Nations/World Health Organization 2002). Many studies have investigated the effects of probiotic consumption on intestinal microbial imbalance, on suppression of pathogens and prevention and treatment of intestinal and other disorders, and on inflammatory bowel disease, diarrhea, infection, colon cancer, constipation, and atopic diseases [6–11]. In particular, numerous attempts have been made to increase the number of intestinal bifidobacteria and improve intestinal disorders such as constipation and diarrhea through use of probiotics [12–15]. In most of these studies,

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however, the numbers of intestinal bifidobacteria were investigated using cultivation-based techniques [12–15], which are widely known to be labor-intensive and time-consuming. In addition, classification and identification based on phenotypical traits do not always provide clear-cut results and are sometimes unreliable because the recovery of bifidobacteria from feces depends on the composition of the medium and the culture conditions [16–18]. Currently, 16S rRNA-targeted oligonucleotide probes are used with fluorescent in-situ hybridization and genus- and species-specific PCR as a culture-independent method [19–23]. These methods enable rapid and specific detection of a wide range of bacterial species. Genus-specific primers or probes are expected to provide a good overall picture of the fecal bifidobacterial population, although there are few reports describing the effect of probiotic administration on bifidobacterial composition, especially those that focus on the daily dynamics of both endogenous and exogenous (ingested) bifidobacteria.

Bifidobacterium animalis subsp. *lactis* (*B. lactis*) GCL2505 is a probiotic that originates from healthy human intestines and is used in fermented milk products in the Japanese market. We previously showed that *B. lactis* GCL2505 reached the intestine in a viable form and was subsequently able to proliferate after a single ingestion, which led to an increase in the amount of intestinal bifidobacteria and more frequent defecation after 2 weeks of ingestion [24]. However, the daily dynamics of intestinal bifidobacteria at the species level following ingestion of *B. lactis* GCL2505 are not yet clear. Moreover, the effects of long-term ingestion of *B. lactis* GCL2505 on the composition of intestinal bifidobacteria and the changes in the frequency of defecation lasting over 2 weeks have not been well studied. In this study, we compared the dynamics of intestinal bifidobacteria after ingestion of *B. lactis* GCL2505 and other bifidobacteria that can survive but not proliferate in the intestine, as well as those of lactic acid bacteria used in yogurt fermentation. Quantitative real-time PCR using *Bifidobacterium* species- and subspecies-specific primers were used to elucidate the daily dynamics of endogenous and ingested strains at the species level. Moreover, we investigated the change in the intestinal microbiota and the frequency of defecation following long-term ingestion.

MATERIALS AND METHODS

Test beverages

The test beverages included a milk-like drink, a yogurt drink, or a placebo drink (100 g of each). *B. lactis* GCL2505 or *B. bifidum* GCL2080 was added to the milk-like drink. The viable cell count of *B. lactis* GCL2505 or *B. bifidum* GCL2080 in a test beverage was 1.5×10^{10} cfu or 2.6×10^{10} cfu, respectively. The yogurt drink was fermented with *L. delbrueckii* subsp. *bulgaricus* GCL1031 and *S. thermophilus* GCL1122, both of which are commonly used in the production of conventional yogurt. The viable cell count of lactic acid bacteria (*L. bulgaricus* and *S. thermophilus*) in a test beverage was 3.0×10^{10} cfu. The placebo drink was prepared with the same ingredients as the milk-like drink and had a similar flavor but it did not contain bacteria.

Subjects and study design of a short-term trial (Experiment 1)

Sixty-four healthy female subjects were recruited (age range: 18–25 years). These subjects claimed that they had a frequency of bowel movements of ≥5.0 times/week in a questionnaire performed in advance. The aim of Experiment 1 was to elucidate the daily dynamics of intestinal microbiota; thus, subjects were required to provide daily fecal samples. We therefore selected subjects who claimed in a preliminary questionnaire that they had a frequency of bowel movements of ≥ 5.0 times/week.

The study was designed as a double-blind, parallel-group comparison and consisted of two consecutive 2-week periods: a non-ingestion period and an ingestion period. Subjects were randomized and assigned into one of three groups that received a test beverage containing either *B. lactis* GCL2505 (BL group), *B. bifidum* GCL2080 (BB group), or *L. bulgaricus* GCL1031 and *S. thermophilus* GCL1122 (LBST group). During the ingestion period, the subjects consumed one test beverage daily. Each subject provided fecal samples for microbial analysis at the end of the non-ingestion period and on days 1–4, 7, and 14 of the ingestion period (Fig. 1). Subjects were instructed to avoid the intake of fermented milks, lactic acid bacteria beverages, probiotic and prebiotic products, and fermented foods such as *natto* (soybean fermented with *B. subtilis*) for the duration of the study.

Subjects and study design of a long-term trial (Experiment 2)

Forty-two mildly constipated female subjects were selected (age range: 25–59 years). These subjects claimed that they had a frequency of bowel movements of ≤5.0 times/week in a questionnaire completed in advance.

The study took the form of a double-blind, parallel-group comparison and consisted of a 2-week non-ingestion period in which initial parameters were obtained for

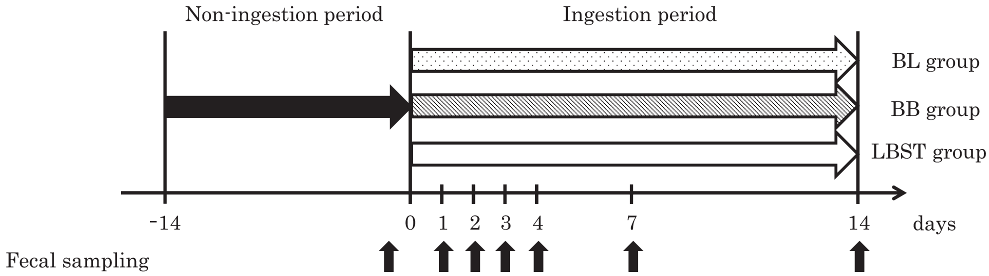

Fig. 1. Scheme of Experiment 1.

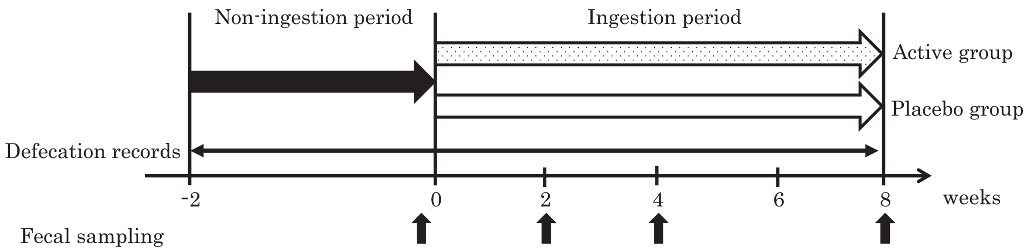

Fig. 2. Scheme of Experiment 2.

baseline measurements, followed by an 8-week ingestion period. During the ingestion period, subjects consumed 1 test beverage daily. Subjects were randomized and assigned to two groups (active or placebo group). The active group consumed a test beverage containing *B. lactis* GCL2505, and the placebo group consumed a test beverage without *B. lactis* GCL2505. Each subject provided fecal samples for microbial analysis at the end of the non-ingestion period and in weeks 2, 4, and 8 of the ingestion period and recorded their daily number of defecations (Fig. 2). As in Experiment 1, subjects were instructed to avoid the intake of other fermented milks, lactic acid bacteria beverages, probiotic and prebiotic products, and fermented foods such as *natto* (soybean fermented with *B. subtilis*) for the duration of the study.

Determination of fecal microbiota

Fecal samples were delivered in a refrigerated, anaerobic state using an AnaeroPack Kenki (Mitsubishi GAS Chemical Co., Inc., Tokyo, Japan), diluted 10-fold with phosphate-buffered saline (pH 7.4) and homogenized using a Stomacher. Suspensions were kept at −80°C until assayed.

Bacterial DNA was extracted from the 10-fold dilutions of fecal samples according to the procedure described by Matsuki et al. [25]. The number of intestinal bifidobacteria was quantified by real-time PCR using *Bifidobacterium* species- and subspecies-specific primers, according to the procedure described by Ishizuka et al. [24]. Briefly, PCR amplification and detection procedures were performed using a CFX-96 Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA). Each reaction mixture (10 µl) was composed of 5 µl of SYBR Premix Ex Taq I or II, 1 µl of each primer (Table 1, 10 pmol/µl), 2 µl of ×1, ×10, or ×100 diluted DNA template, and 2 µl of distilled water. The amplification program for *B. lactis*-specific primers consisted of 1 cycle at 95°C for 5 sec, 34 cycles at 95°C for 30 sec, and 1 cycle at 60°C for 30 sec. The amplification program for other primers consisted of 1 cycle at 94°C for 30 sec, then 35 cycles at 94°C for 20 sec, followed by cycles at 55°C, 63°C, or 65°C for 30 sec (Table 1), and then 1 final cycle at 72°C for 50 sec. Fluorescent products were detected during the final step of each cycle. Melting curve analysis was performed after amplification to distinguish the targeted and non-targeted PCR products. The melting curves were obtained by slow heating from 65 to 99°C at a rate of 0.5°C/sec

Total counts of fecal bifidobacteria were expressed as the sum of the counts of 10 species (namely, *B. bifidum, B. longum* subsp. *longum, B. adolescentis, B. breve, B. catenulatum, B. pseudocatenulatum, B. longum* subsp. *infantis, B. anglatum, B. dentium,* and *B. lactis*).

Ethics

These experiments were performed with the approval

Table 1. The 16S rRNA gene-targeted primers used in this study

Target	Primer	Sequence (5'–3')	Annealing temp (°C)	SYBR Premix Ex Taq
B. bifidum	BiBIF-1	CCACATGATCGCATGTGATTG	55	I
	BiBIF-2	CCGAAGGCTTGCTCCCAAA		
B. breve	BiBRE-1	CCGGATGCTCCATCACAC	65	II
	BiBRE-2	ACAAAGTGCCTTGCTCCCT		
B. longum	BiLON-1	TTCCAGTTGATCGCATGGTC	63	I
	BiLON-2	GGGAAGCCGTATCTCTACGA		
B. adolescentis	BiADOg-1a	CTCCAGTTGGATGCATGTC	63	I
	BiADOg-1b	TCCAGTTGACCGCATGGT		
	BiADOg-2	CGAAGGCTTGCTCCCAGT		
B. angulatum	BiANG-1	CAGTCCATCGCATGGTGGT	65	II
	BiANG-2	GAAGGCTTGCTCCCCAAC		
B. catenulatum or B. pseudocatenulatum	BiCAT-1	CGGATGCTCCGACTCCT	65	II
	BiCAT-2	CGAAGGCTTGCTCCCGAT		
B. dentium	BiDEN-1	ATCCCGGGGGTTCGCCT	65	II
	BiDEN-2	GAAGGGCTTGCTCCCGA		
B. infantis	BiINF-1	TTCCAGTTGATCGCATGGTC	63	II
	BiINF-2	GGAAACCCCATCTCTGGGAT		
B. lactis	BlactF	CCCTTTCCACGGGTCCC	60	I
	BlactR	AAGGGAAACCGTGTCTCCAC		

of the ethical committees of Fuji Women's University (Experiment 1), and Kenshokai Medical Co., (Osaka, Japan) (Experiment 2). The contents and methods were explained in full to all prospective subjects, and written informed consent was obtained according to the principals of the Declaration of Helsinki (adopted in 1964; revised in 1975, 1983, 1989, 1996, and 2000).

Statistical analysis

IBM SPSS Statistics for Windows Version 22.0 J (IBM Corp., Armonk, NY, USA) was used for statistical analyses. Within-group comparisons between the baseline and each subsequent time point were conducted using repeated-measures ANOVA followed by Dunnett's multiple comparisons. Between-group comparisons of the amount of change from baseline to each subsequent time point were conducted by one-way ANOVA followed by unpaired student's t tests. $p<0.05$ was considered statistically significant, and $0.05 \leq p<0.10$ was considered marginally significant.

RESULTS

Short-term changes in fecal bifidobacteria (Experiment 1)

Three subjects failed to complete the trial due to personal reasons. Eight subjects were excluded from analysis because of noncompliance with the study requirements, so 53 subjects were analyzed. Background characteristics for the subjects in Experiment 1 are shown in Table 2. There were no significant differences in any of the characteristics or parameters among the three groups.

Table 2. Characteristics of the subjects in Experiment 1

Group	BL group	BB group	LBST group
N	18	16	17
Age (years)	20.1 ± 1.6	20.1 ± 1.4	20.2 ± 1.9
The frequency of defecation	7.7 ± 2.0	7.5 ± 2.5	8.5 ± 2.8

Values are expressed as the mean ± SD.

Figure 3 shows the changes in the numbers of fecal bifidobacteria. In the BL group, the total number of bifidobacteria significantly increased on day 1 compared with before ingestion. The percentage of *B. lactis* reached nearly 50% of the total amount of bifidobacteria on day 2, and this level was maintained until day 14. On the other hand, no significant change was observed in the composition and number of endogenous bifidobacteria in the BL group at the species level throughout the ingestion period. No significant changes were observed in either the total number of bifidobacteria or the composition of fecal bifidobacteria in the BB and LBST groups, throughout the ingestion period.

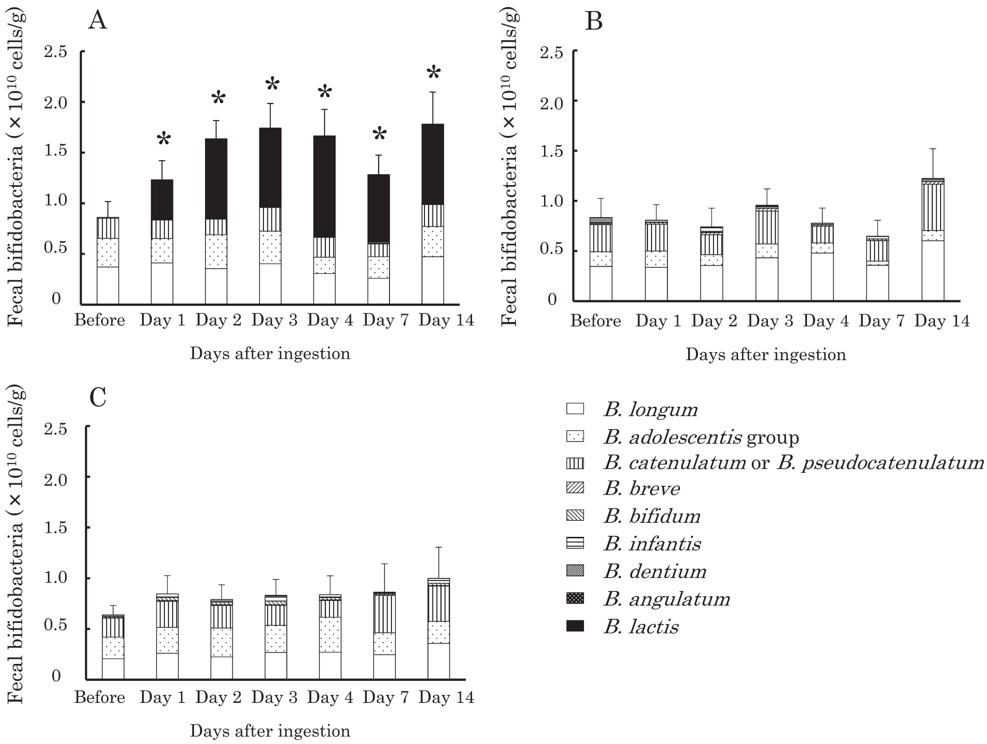

Fig. 3. The dynamics of intestinal bifidobacteria following ingestion of *B. lactis* GCL2505 (A), *B. bifidum* GCL2080 (B), or *L. bulgaricus* GCL1031 and *S. thermophilus* GCL1122 (C).
Values are expressed as the sum of the mean ± SE values of each species. *B. lactis* GCL2505 ingestion group (BL group), n = 18; *B. bifidum* GCL2080 ingestion group (BB group), n = 16; *L. bulgaricus* GCL1031 and *S. thermophilus* GCL1122 ingestion group (LBST group), n = 19. Comparisons of the total numbers of fecal bifidobacteria on each day with those before ingestion are shown. *p<0.05; †p<0.1 (Dunnett's multiple comparisons).

Long-term changes in the fecal bifidobacteria (Experiment 2)

Four subjects were excluded from analysis because of noncompliance with the study requirements. Two subjects were excluded because they had a frequency of defecation of >5.0 times a week during the non-ingestion period, so 38 subjects were analyzed. Background characteristics for the subjects in Experiment 2 are shown in Table 3. There were no significant differences in any of the characteristics or parameters between the active and placebo groups.

Figure 4 shows the changes in the number of fecal bifidobacteria. Before ingestion, there was no difference in the total amount of fecal bifidobacteria between the two groups. At 2 and 4 weeks after ingestion, the total number of bifidobacteria significantly increased in the active group compared with the placebo group. Moreover, the amounts of fecal bifidobacteria in the active group tended to increase 8 weeks after the ingestion period began compared with the placebo group. However, the total number and the number of each endogenous *Bifidobacterium* species did not significantly differ between the active and placebo groups.

Table 3. Characteristics of the subjects in Experiment 2

Group	Active	Placebo
N	18	20
Age (years)	40.8 ± 7.6	42.8 ± 7.7
The frequency of defecation	3.1 ± 0.8	3.5 ± 1.1

Values are expressed as the mean ± SD.

The changes in the frequency of defecation significantly increased in weeks 6 and 8 in the active group compared with the placebo group (Table 4).

DISCUSSION

There are many reports on the effects of probiotics on intestinal bifidobacteria [12–15, 26, 27]; however, the effects of consumption of probiotics on the daily dynamics of intestinal bifidobacteria or ingested probiotics are unclear.

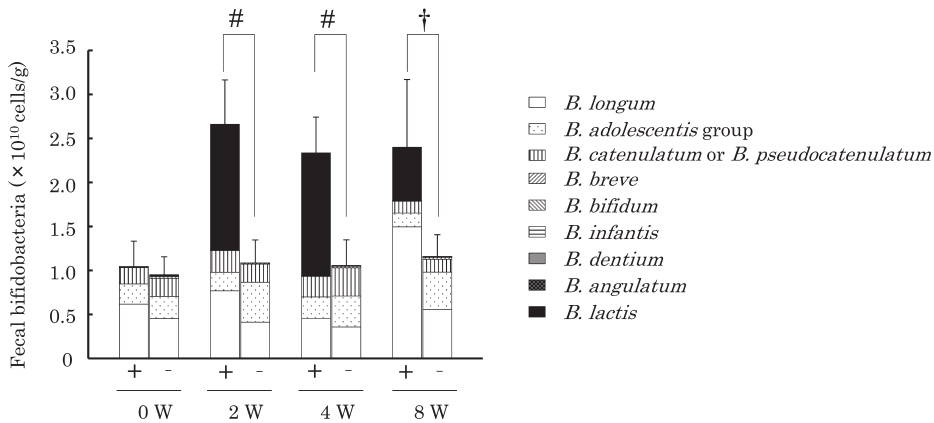

Fig. 4. The dynamics of intestinal bifidobacteria following ingestion of *B. lactis* GCL2505 (+) or placebo milk (−). Values are expressed as the sum of the mean ± SE values of each species. *B. lactis* GCL2505 ingestion group (active group, +), n = 18; placebo milk ingestion group (placebo group, −), n = 19. Comparisons between active and placebo cases at each time point are shown. #p<0.05; †p<0.1 (Student's t test).

Table 4. Change in the number of defecations

Values	Groups	0 weeks	2 weeks	4 weeks	6 weeks		8 weks	
Measured	Active	3.1 ± 0.8	4.7 ± 2.1	4.0 ± 1.3	4.9 ± 1.6		4.8 ± 1.5	
	Placebo	3.5 ± 1.1	4.2 ± 1.4	4.8 ± 1.5	4.1 ± 1.3		3.9 ± 1.4	
Change from 0 weeks	Active	—	1.6 ± 1.9	0.9 ± 1.0	1.8 ± 1.3	#	1.7 ± 1.4	#
	Placebo	—	0.8 ± 1.2	1.4 ± 1.3	0.7 ± 1.3		0.4 ± 1.3	

Values expressed as means ± SD. Comparisons between the active and placebo groups at each point are shown. #p<0.05 (Student's t test).

In Experiment 1, we elucidated the daily dynamics of intestinal bifidobacteria and ingested probiotics. Ingestion of *B. lactis* GCL2505 increased the total number of intestinal bifidobacteria on day 1 compared with before ingestion, and this level was maintained throughout the ingestion period. According to species-specific real-time PCR, the fecal cell count for *B. lactis* reached 7.9×10^9 cells/g feces and represented half the population of intestinal bifidobacteria from day 2 onward. On the other hand, ingestion of *B. bifidum* GCL2080, found to be viable on reaching the intestine in our preliminary investigation (data not shown), did not lead to significant changes in the total number of intestinal bifidobacteria, either ingested or endogenous. *B. bifidum* (including ingested *B. bifidum* GCL2080 and endogenous *B. bifidum*) reached a total of 2.8×10^8 cells/g feces and represented a small proportion of the intestinal bifidobacterial microflora (approximately 2%) following ingestion of *B. bifidum* GCL2080. In addition, no significant changes were observed in either the composition or numbers of endogenous bifidobacteria at the species level following ingestion of *L. bulgaricus* GCL1131 and *S. thermophilus* GCL1122. Nevertheless, lactic acid bacteria from yogurt cultures themselves are also considered probiotics and are believed to increase intestinal bifidobacterial levels [28–31]. These results indicated that the effects of probiotics on the total amount of intestinal bifidobacteria were strain specific and exerted within a few days of consuming fermented milk produced by *B. lactis* GCL2505. To the best of our knowledge, this is the first report describing the daily dynamics of intestinal bifidobacteria, including endogenous and ingested strains, at the species level during the short-term ingestion of probiotics.

In Experiment 1, no significant changes were observed in either the composition or numbers of endogenous bifidobacteria at the species level in any of the three groups. These results suggested that the probiotics had no effect on the number of endogenous bifidobacteria during short-term ingestion in the healthy human volunteers. In other words, the increase in the total amount of bifidobacteria was largely attributable to ingested (exogenous) probiotics. Therefore, the proliferation of ingested probiotics such as *B. lactis* GCL2505 was an important factor leading to the increase in the total amount of bifidobacteria in the intestine.

In most previous studies, the number of intestinal bifidobacteria has been investigated only at the genus level using cultivation-based techniques [12–15]. In contrast, we measured the presence of each bifidobacterium species by quantitative PCR in this study to determine accurate numbers. Moreover, we used species- or subspecies-specific primers to determine the total number of intestinal bifidobacteria as a sum of the counts of ten species because multiple copy numbers of rRNA operons and genes in different bacterial chromosomes may affect the apparent relative abundance of bacteria in the sample. For example, it is reported that *B. adolescentis* carries 5 copies of the 16S rRNA gene [32], whereas *B. longum* and *B. lactis* carry 4 and 2 copies of the 16S rRNA gene, respectively [23, 33–35]. Therefore, our method of quantifying the total amount of bifidobacteria gave a more accurate representation than previous culture-based quantification techniques or genus-specific quantitative PCR. However, the numbers determined by quantitative PCR include both viable and dead cells. Previously, we revealed that *B. lactis* GCL2505 was detected at the same level in feces on the day after ingestion by culture-based quantification techniques, followed by PCR identification and species-specific quantitative PCR [24]. Therefore, it was considered that most of the *B. lactis* GCL2505 detected in the present study was in a viable form and proliferated in the intestine.

We revealed in our previous study that ingestion of *B. lactis* GCL2505 over 2 weeks significantly increases the amount of intestinal bifidobacteria and improves the frequency of defecation [24]. However, the effects of long-term ingestion for more than 2 weeks have not yet been clarified. Thus, in this study, we investigated the effects of long-term ingestion of *B. lactis* GCL2505, using the amounts of fecal bifidobacteria and the frequency of defecation as indices of improvement in the intestinal environment, in addition to the effects resulting from short-term ingestion of probiotics. During the 8 weeks of *B. lactis* GCL2505 ingestion, the level of intestinal bifidobacteria and the frequency of defecation significantly increased compared with those in the placebo group. These results indicated that the effects of *B. lactis* GCL2505 ingestion on the intestinal microbiota were sustained for at least 8 weeks. On the other hand, the amounts of endogenous bifidobacteria were not significantly greater after 8 weeks of *B. lactis* GCL2505 ingestion, which showed that even long-term ingestion of probiotics had no effect on either the number or composition of endogenous bifidobacteria. In our previous study, ingestion of *B. lactis* GCL2505 over 2 weeks was found to significantly improve the frequency of defecation [24]. However, in the present study, improvements compared with the placebo group were observed only after 6 weeks of intervention. This is one reason why the present study was designed as a parallel-group trial, in contrast to the cross-over trial design used in the previous study. A larger sample size was required to reliably detect significant differences. It was estimated, based on the present data, that a sample size of 118 would be necessary for detecting significant differences between the active and placebo groups at 2 weeks with 80% power. Overall, however, ingestion of *B. lactis* GCL2505 increased the frequency of defecation in the active compared with placebo groups in both the previous and present studies. Therefore, it appears that ingestion of *B. lactis* GCL2505 is effective against constipation.

Our results indicated that the proliferation of *B. lactis* GCL2505 in the intestine, which may cause production of short-chain fatty acids such as acetate and stimulate smooth muscle contractions and transepithelial chloride secretion [36–43], improved the function of the large bowel throughout the long-term ingestion period.

In conclusion, we found that *B. lactis* GCL2505 increased the total number of intestinal bifidobacteria after a few days of ingestion and that long-term ingestion improved the frequency of defecation. Moreover, we showed that the proliferation of *B. lactis* GCL2505 in the intestine contributed significantly to the increase in the total number of intestinal bifidobacteria, although there was no significant change in the amounts of endogenous bifidobacteria. Based on these results, we propose that probiotics that are able to proliferate in the intestine, such as *B. lactis* GCL2505, appear to improve the intestinal microbiota more effectively than non-proliferating probiotics.

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