Lactulose ingestion causes an increase in the abundance of gut-resident bifidobacteria in Japanese women: a randomised, double-blind, placebo-controlled crossover trial

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Abstract

The genus *Bifidobacterium* comprises various bacterial species, and the complement of species within the human intestinal tract differs from individual to individual. The balance of these bifidobacterial species remains poorly understood, although it is known that the abundance of bifidobacteria increases following the ingestion of prebiotics. We previously conducted a randomised, placebo-controlled, double-blind, crossover study of 2 g/day lactulose ingestion for 2 weeks in 60 Japanese women. To study the effect of lactulose ingestion on each bifidobacterial species, here, we measured the abundance of each of the principal bifidobacterial species. After lactulose ingestion, the log cell counts of the *Bifidobacterium adolescentis* group (8.97 ± 0.08 vs 9.39 ± 0.08 , $P=0.0019$), *Bifidobacterium catenulatum* group (9.45 ± 0.10 vs 9.65 ± 0.10 , $P=0.0032$) and *Bifidobacterium longum* group (9.01 ± 0.07 vs 9.29 ± 0.07 , $P=0.0012$) were significantly higher than in the placebo ingestion control group. However, the log cell counts were similar for *Bifidobacterium breve* (8.12 ± 0.12 vs 8.33 ± 0.12 , $P=0.20$), *Bifidobacterium bifidum* (9.08 ± 0.12 vs 9.42 ± 0.14 , $P=0.095$) and *Bifidobacterium animalis* subspecies *lactis* (8.65 ± 0.53 vs 8.46 ± 0.46 , $P=0.77$). Cluster analysis of the log cell count data at the bifidobacterial species level revealed three distinct clusters, but the combinations and ratios of the constituent bifidobacteria were not affected by lactulose ingestion. Furthermore, principal coordinate analysis of the intestinal microbiota in the lactulose and placebo ingestion groups using Illumina MiSeq showed no significant differences in the intestinal microbiota as a whole. These results suggest that 2 g/day lactulose ingestion for 2 weeks significantly increases the abundance of intestinal bifidobacteria, but does not affect the intestinal microbiota as a whole.

Keywords: indigestible oligosaccharide, intestinal microbiome, intestinal microbiota, diversity

1. Introduction

Bifidobacterium is one of the most well-recognised, beneficial genera of bacteria that inhabits the human intestinal tract (Yaeshima, 1996). Bifidobacteria are present in both the intestines and faeces of both humans and animals. The genus *Bifidobacterium* comprises 70 species and 10 subspecies (LPSN, <http://www.bacterio.net>). The species that live in the human gut are designated as

human-residential bifidobacteria (HRB) (Wong *et al.*, 2018), and those of animal origin are designated as non-HRB, of which *Bifidobacterium animalis* is often used as a probiotic, and is consequently detected in human faeces (Kato *et al.*, 2017). These species release vitamins and short-chain fatty acids (SCFAs), such as acetic acid, which are absorbed by the host and metabolised to provide energy. Bifidobacteria are not detected immediately after birth, but after the ingestion of breast milk and/or bottled milk they

rapidly increase in number and become one of the most abundant types of bacteria, because of the prebiotics and oligosaccharides that are present in human milk and/or the oligosaccharides in bottled milk. However, the abundances of other intestinal bacteria increase after ablactation and the number of bifidobacteria decreases with aging (Odamaki *et al.*, 2016). The abundance of bifidobacteria in Japanese adults is known to be higher than that in the inhabitants of other countries (Nishijima *et al.*, 2016), and these bacteria are thought to be important for the intestinal health of the Japanese population.

Bifidobacteria exist in a dynamic state, and both the abundance and proportions of constituent species change with aging. Bifidobacterial species, such as *Bifidobacterium longum* subspecies (ssp.) *infantis* and/or *Bifidobacterium bifidum* are present in the faeces during infancy, but disappear with aging, whereas species such as *Bifidobacterium catenulatum* and/or *Bifidobacterium adolescentis* are present in the faeces during adulthood. *Bifidobacterium longum* ssp. *longum*, which is closely related to *B. longum* ssp. *infantis*, is commonly present in the human gut from infancy to old age (Kato *et al.*, 2017).

Various types of bifidobacteria coexist within humans throughout their life, and the types of bifidobacteria present vary between individuals; however, these changes have not been investigated in detail. For example, it remains to be determined how the proportions of commensal bifidobacterial species change following prebiotic treatment, and whether all, or specific species of bifidobacteria, predominate following prebiotic treatment. If these deficiencies can be rectified, new insight into the usefulness of prebiotics may be provided.

Previously, we conducted a clinical trial to investigate how the defaecation frequency and the number of bifidobacteria in the faeces changed during the ingestion of 2 g/day lactulose for 2 weeks (Sakai *et al.*, 2019a). Defaecation frequency was found to be significantly higher during lactulose ingestion than during placebo treatment, and the number of bacteria belonging to the genus *Bifidobacterium* in faeces was significantly higher. In the present study, we analysed the samples obtained in the clinical trial to determine how lactulose intake affects bifidobacterial species, with a specific focus on its effects on the numbers of each bifidobacterial species and the composition ratios in the intestinal microbiota.

2. Materials and methods

Trial design

This study was designed as a randomised, double-blind, placebo-controlled crossover trial, consisting of a pre-observation period, then two ingestion periods, which

were separated by a washout period. The prebiotic effect of the ingestion of 2 g/day lactulose for 2 weeks was evaluated (Figure 1). The study was conducted at Showa Women's University (Japan) between May and December 2017, in accordance with the principles of the Declaration of Helsinki. The study protocol was approved by the Institutional Review Board of Showa Women's University (Approval No. 17-10, 7 June 2017). All participants provided their written informed consent. The protocol for this study is registered with the University Hospital Medical Information Network Clinical Trials Registry (No. UMIN000027305).

Participants

The participants were healthy students, staff members, and affiliates of Showa Women's University, and their families. The inclusion criteria were: (1) age 18–65 years and (2) defaecation frequency 2–4 times/week. Those over 65 years who may have been affected by other diseases and children under 18 were excluded. In addition, because the primary outcome of the original clinical trial was to investigate the effect of 2 g/day of lactulose ingestion on defecation frequency, the participants of the study were those who had a defaecation frequency of around once every two days, which is generally considered to represent a tendency toward constipation.

The exclusion criteria were: (1) a severe hepatic, renal, cardiac, gastrointestinal, cerebrovascular, endocrine, metabolic or infectious disease; (2) a history of gastrointestinal resection; (3) gastrointestinal dysfunction, such as irritable bowel syndrome or inflammatory bowel disease; (4) the use of medicines or supplements that could influence defaecation frequency (e.g. antibiotics, probiotics, prebiotics, laxatives, anti-diarrhoeal drugs and fibre); (5) milk allergy; (6) lactose intolerance; (7) participation in another study; and (8) individuals who were judged inappropriate for the study by the investigator or a physician (e.g. who could not meet the deadline for submitting their survey diary or faecal sample, or who could not be contacted during the study period).

Randomisation

Participants were randomly assigned to group A (lactulose first) or group B (placebo first) using the sealed envelope method. An assignment manager, who was independent of the trial staff, created the allocation order using the replacement block method (block size 4). The allocation ratio was 1:1. According to the allocation order, the test food number was displayed on the test food package for each participant. The correspondence chart between the test food number and the assignment group was hidden from the authors, participants and trial staff, including the intestinal microbiome analyst and the statistician, until the

Figure 1. Flow chart of the participant numbers throughout the trial.

completion of the trial. The trial staff assigned the test foods in ascending order of test food number, which corresponded to the order of participant registration.

Intervention

2 g portions of lactulose crystal anhydrate powder (MLC-97, $\geq 97\%$, Morinaga Milk Industry Co., Ltd., Tokyo, Japan) were provided in aluminium sachets. Glucose crystal anhydrate powder (Nihon Shokuhin Kako Co., Ltd., Tokyo, Japan) was used as the placebo, because this could not be visually discriminated from the lactulose. Participants in group A received lactulose during the first intervention period and the placebo during the second intervention period, for 2 weeks each, whereas participants in group B received the

placebo during the first intervention period and lactulose during the second intervention period. A 3-week washout period was included between the two intervention periods (Figure 2). The time of day for ingestion was not specified. The participants were instructed in advance to avoid the use of pharmaceuticals and supplements (e.g. antibiotics, laxatives, anti-diarrhoeal drugs, probiotics, prebiotics and fibre), which could affect defaecation during the study period.

DNA extraction

Faecal samples were collected before and at the end of each intervention period (Figure 2), and were immediately placed in storage at $\leq 18^\circ\text{C}$ until they arrived at the laboratory, and

Figure 2. Trial design. Faecal sample collections are indicated by arrows.

at -80 °C thereafter. Because the participants' defaecation frequencies were 2-4 times per week and it was difficult to designate a specific date for sample collection, this was scheduled to occur before administration and on the last day of administration ± 2 days. DNA was extracted from these samples, as described by Sugahara *et al.* (2015), and used to determine both the log cell count of *Bifidobacterium* species and their percentages within the faecal microbiome.

Log cell count of bifidobacterial

To determine the log cell count of bifidobacterial species per gram of faeces, a quantitative PCR method was used. The specific primers used in the PCR are shown in Supplementary Table S1. For the genus *Bifidobacterium*, a standard curve was prepared using dilutions of *B. longum* JCM1217^T (ATCC15707^T) cells. For the *B. adolescentis* group (comprising *B. adolescentis* genotypes A and B), a standard curve was prepared using dilutions of *B. adolescentis* JCM1275^T cells. For *B. animalis* ssp. *lactis*, a standard curve was prepared using dilutions of *B. animalis* ssp. *lactis* JCM1190^T cells. For *B. bifidum*, a standard curve was prepared using dilutions of *B. bifidum* JCM1255^T cells. For *B. breve*, a standard curve was prepared using dilutions of *B. breve* JCM1192^T cells. For the *B. catenulatum* group (*B. catenulatum* and *Bifidobacterium pseudocatenulatum*), a standard curve was prepared using dilutions of *B. pseudocatenulatum* JCM1200^T cells. For the *B. longum* group (*B. longum* ssp. *infantis*, *B. longum* ssp. *longum* and *Bifidobacterium longum* ssp. *suus*), a standard curve was prepared using dilutions of *B. longum* JCM1217^T (ATCC15707^T) cells. For *B. longum* ssp. *longum*, a standard curve was prepared using dilutions of *B. longum* JCM1217^T (ATCC15707^T) cells. The data before and at the end of intervention were logarithmically transformed and statistically analysed.

Percentages of bifidobacterial species in the faecal microbiome

To determine the percentage of each bacterial taxon of interest in the faecal microbiome, the same DNA extract that was used to determine the log cell counts of *Bifidobacterium* was sequenced using Illumina MiSeq (Illumina, Inc., San Diego, CA, USA). Amplification of 16S rRNA by PCR and sequencing using Illumina MiSeq were performed as previously reported by Kato *et al.* (2018). The V3-V4 regions of the bacterial 16S rRNA genes were then amplified by PCR using a TaKaRa Ex Taq HS Kit (TaKaRa Bio, Shiga, Japan) and the primer set Tru357F (5'-CGCTCTTCCGATCTCTGTA CGGRAGGCAGCAG-3') and Tru806R (5'-CGCTCTTCC GATCTGACGGACTACHVGGGTWTCTAAT-3'). Reads containing the bacteriophage phiX DNA sequence in the Genome Reference Consortium human build 38 (GRCh38) were identified by mapping them against Bowtie2 (version

2.2.6) and discarded. Then, the sequences were analysed using the QIIME2 software package, version 2017.10 (<https://qiime2.org/>). Potential chimeric sequences were removed using DADA2 (Callahan *et al.*, 2016), then 30 and 90 bases of the 3' region of the forward and reverse reads were trimmed, respectively. Taxonomic classification was performed in QIIME2 software using a Naive Bayes classifier trained on Greengenes 13.8, with a 99% threshold for operational taxonomic unit (OTU) full-length sequences. UniFrac distances were calculated using QIIME2 software. The data collected at the end of the intervention period were statistically analysed.

Detection rate

The detection rate was defined as the number of people in whom bifidobacteria were detected using quantitative PCR divided by the total number of participants who submitted faecal samples, for each bifidobacterial species. The detection rates were determined both prior to (the leftmost arrow in Figure 2) and following the intake of lactulose or placebo. The detection of bifidobacteria was evaluated on the basis of the detection limits of the quantitative PCR: a log cell count/g faeces of 6.

Statistical methods

On the basis of the results of a preliminary trial (Sakai *et al.*, 2019b), the number of participants required was calculated using a two-tailed significance level of 5% and a power of 90%, with the assumption that the difference in defaecation frequency between the groups would be 0.7 and the standard deviation would be 1.5. The number of participants required was calculated to be 51. Assuming that the combined withdrawal and dropout rate would be ~10%, the number of participants required was determined to be 60.

Statistical analysis was conducted per protocol set (PPS), meaning that the participants who took medicines or supplements that could affect defaecation, or those who had a disease, were excluded from the analysis.

The lactulose and placebo groups were compared using a linear mixed model, in which the test food group and timing were fixed effects, and participant identity was a variable effect. The least mean square value and standard error for each group, the difference between the groups, and the associated 95% confidence interval (CI) and *P*-value were calculated. The two-tailed significance level was set at 5%.

Samples taken before and after lactulose ingestion, and before and after placebo ingestion, were analysed using a paired *t*-test. The simple mean and standard deviation for each group, and the *P*-value were calculated. The two-tailed significance level was set at 5%.

The relationship between the overall gut microbiome composition and lactulose ingestion was assessed using unweighted (qualitative) and weighted (quantitative) UniFrac distance matrices and permutational multivariate analysis of variance (PERMANOVA). The number of permutations was set to 10,000. Principal coordinate analysis (PCoA) plots were generated using the first two principal coordinates.

Statistical analyses of the data were performed using JMP 13.2 (SAS Institute Inc., Cary, NC, USA). To construct the dendrogram, XLSTAT 2014.1.09 (Addinsoft Inc., New York, NY, USA) was used. The unweighted and weighted UniFrac distances obtained from the microbial data using the QIIME2 software package were used for PCoA. PERMANOVA was performed using the package 'vegan' in R software (version 3.6.0).

3. Results

Participants

Sixty healthy participants were enrolled in the study, of whom 28 were assigned to group A and 32 to group B (Figure 1). There were no male participants in the study and no participants dropped out. Seven participants who used antibiotics during the trial and one who had type I diabetes were excluded. Three participants were not willing to collect faeces; therefore, this additional analysis ultimately included 49 participants. There was a risk of bias due to the use of PPS analysis. The mean age the 49 participants in this additional analysis was 20.3 ± 2.4 years (range, 18–31 years).

Detection rate

The detection rate of each bifidobacterial species before the test food intervention is shown in Table 1. The genus *Bifidobacterium* was present in 100% of the participants in this trial, of which the *B. adolescentis* group was present in 49.0%, *B. animalis* ssp. *lactis* was present in 32.7%, *B. bifidum* was present in 14.3%, *B. breve* was present in 53.1%, the *B. catenulatum* group was present in 63.3%, the *B. longum* group was present in 89.8%, and *B. longum* ssp. *longum* was present in 89.8%.

Table 2 shows the detection rate for each bifidobacterial species after placebo or lactulose treatment. The genus *Bifidobacterium* was present in 100% of samples in each group. Specifically, the *B. adolescentis* group was present in 49.0 and 44.9%, *B. animalis* ssp. *lactis* was present in 20.4 and 28.6%, *B. bifidum* was present in 16.3 and 16.3%, *B. breve* was present in 51.0 and 57.1%, the *B. catenulatum* group was present in 63.3 and 61.2%, the *B. longum* group was present in 89.8 and 89.8%, and *B. longum* ssp. *longum* was present in 89.8 and 89.8% of each group, respectively.

Table 1. Detection rate of each bifidobacterial species before the interventions.¹

Bifidobacterial species	Before the test food intervention	
	Number detected	Detection rate (%)
Genus <i>Bifidobacterium</i>	49	100.0
<i>Bifidobacterium adolescentis</i> group	24	49.0
<i>Bifidobacterium animalis</i> ssp. <i>lactis</i>	16	32.7
<i>Bifidobacterium bifidum</i>	7	14.3
<i>Bifidobacterium breve</i>	26	53.1
<i>Bifidobacterium catenulatum</i> group	31	63.3
<i>Bifidobacterium longum</i> group	44	89.8
<i>Bifidobacterium longum</i> ssp. <i>longum</i>	44	89.8

¹ The presence of bifidobacteria was evaluated using a detection limit for the quantitative PCR assays of 6 log cell counts/g faeces. The total number of participants is 49.

Log cell counts for *Bifidobacterium*

The log cell counts for bifidobacterial species are shown in Table 3 and Supplementary Figure S1. In Table 3, the least square mean and standard error are used, because this result shows the comparison between after lactulose and placebo ingestion in the crossover trial (Choi *et al.*, 2014). After lactulose ingestion, the log cell counts, not only of the genus *Bifidobacterium* (9.16 ± 0.06 vs 9.53 ± 0.06 , $P < 0.0001$), but also of the *B. adolescentis* group (8.97 ± 0.08 vs 9.39 ± 0.08 , $P = 0.0019$), the *B. catenulatum* group (9.45 ± 0.10 vs 9.65 ± 0.10 , $P = 0.0032$), and the *B. longum* group (9.01 ± 0.07 vs 9.29 ± 0.07 , $P = 0.0012$), including *B. longum* ssp. *longum* (9.04 ± 0.06 vs 9.29 ± 0.07 , $P = 0.0008$) were significantly higher than in the placebo group. The log cell counts were similar for *B. breve* (8.12 ± 0.12 vs 8.33 ± 0.12 , $P = 0.20$), *B. bifidum* (9.08 ± 0.12 vs 9.42 ± 0.14 , $P = 0.095$) and *B. animalis* ssp. *lactis* (8.65 ± 0.53 vs 8.46 ± 0.46 , $P = 0.77$) in the two groups.

The abundances of *B. animalis* ssp. *lactis*, *B. bifidum* and *B. breve*, which did not show significant differences between lactulose and placebo ingestion, were also compared before and after the ingestion period, to further characterise the bifidobacterial counts within the groups. The results are shown in Table 4 and Supplementary Figure S1, in which the simple mean values and standard deviations are quoted, because they show comparisons between participants before and after lactulose and placebo ingestion. The log cell count of *B. breve* increased significantly (7.93 ± 0.87 vs 8.24 ± 0.87 , $P = 0.031$), whereas those of *B. bifidum* (9.17 ± 0.42 vs 9.34 ± 0.23 , $P = 0.10$) and *B. animalis* ssp. *lactis* (8.14 ± 1.26 vs 8.55 ± 1.40 , $P = 0.44$) did not. With respect to the other bifidobacterial species, the log cell counts for the genus

Table 2. Detection rate for each bifidobacterial species after the test food interventions.¹

Bifidobacterial species	Placebo		Lactulose	
	Number detected	Detection rate (%)	Number detected	Detection rate (%)
Genus <i>Bifidobacterium</i>	49	100.0	49	100.0
<i>Bifidobacterium adolescentis</i> group	24	49.0	22	44.9
<i>Bifidobacterium animalis</i> ssp. <i>lactis</i>	10	20.4	14	28.6
<i>Bifidobacterium bifidum</i>	8	16.3	8	16.3
<i>Bifidobacterium breve</i>	25	51.0	28	57.1
<i>Bifidobacterium catenulatum</i> group	31	63.3	30	61.2
<i>Bifidobacterium longum</i> group	44	89.8	44	89.8
<i>Bifidobacterium longum</i> ssp. <i>longum</i>	44	89.8	44	89.8

¹ The presence of bifidobacteria was evaluated using a detection limit for the quantitative PCR assays of 6 log cell counts/g faeces. The total number of participants is 49.

Table 3. Log cell counts for bifidobacterial species after the test food interventions.¹

Bifidobacterial species	n	Placebo	Lactulose	Delta (95% CI) ²	P-value
Genus <i>Bifidobacterium</i>	49	9.16±0.06	9.53±0.06	0.37 (0.23 to 0.49)	<0.0001
<i>Bifidobacterium adolescentis</i> group	22	8.97±0.08	9.39±0.08	0.42 (0.17 to 0.66)	0.0019
<i>Bifidobacterium animalis</i> ssp. <i>lactis</i>	11	8.65±0.53	8.46±0.46	0.19 (-3.27 to 2.89)	0.77
<i>Bifidobacterium bifidum</i>	8	9.08±0.12	9.42±0.14	0.34 (-0.08 to 0.75)	0.095
<i>Bifidobacterium breve</i>	26	8.12±0.12	8.33±0.12	0.21 (-0.11 to 0.55)	0.20
<i>Bifidobacterium catenulatum</i> group	30	9.45±0.10	9.65±0.10	0.20 (0.07 to 0.33)	0.0032
<i>Bifidobacterium longum</i> group	44	9.01±0.07	9.29±0.07	0.28 (0.13 to 0.43)	0.0012
<i>Bifidobacterium longum</i> ssp. <i>longum</i>	44	9.04±0.06	9.29±0.07	0.25 (0.12 to 0.38)	0.0008

¹ Values are expressed as the least square mean ± standard error (linear mixed model).

² CI = confidence interval.

Bifidobacterium (9.10±0.81 vs 9.48±0.65, $P<0.0001$), the *B. adolescentis* group (8.82±0.81 vs 9.32±0.54, $P=0.00013$), the *B. catenulatum* group (9.34±0.78 vs 9.64±0.57, $P=0.034$) and the *B. longum* group (9.04±0.55 vs 9.29±0.58, $P=0.0010$), including *B. longum* ssp. *longum* (9.08±0.53 vs 9.30±0.53, $P=0.0017$), significantly increased in the lactulose group. However, there were no significant changes in any of the bifidobacterial species in the placebo group during the study period.

Percentages of the faecal microbiome

The percentages of the top 10 most abundant species of the faecal microbiome after placebo ingestion are shown in Table 5, in which the least square mean and standard error are shown. The least square mean values for the percentages of the genus *Bifidobacterium* were 18.1±1.4 for the placebo group and 25.3±1.4 for the lactulose group. The difference between the groups was 7.2 (95% CI 2.9–11.4, $P=0.0014$). Bacteria other than those belonging to the genus *Bifidobacterium*, which included the genus *Blautia*,

and which predominated after placebo intake, did not significantly change during the study, and the abundance ranking of the other species represented in the faecal microbiome also remained largely unchanged.

Figure 3 shows the results of PCoA of the microbiota after lactulose or placebo ingestion. The differences between the groups were not significant using unweighted UniFrac distances ($P=0.99$) or weighted UniFrac distances ($P=0.085$).

Dendrogram

The dendrogram in Figure 4 shows the results of cluster analysis using the cell counts for bifidobacteria of each species. Three clusters were apparent. For almost all the participants, the clusters remained the same after lactulose or placebo ingestion. Supplementary Table S2 shows the observations that made up each cluster in the dendrogram. All the participants except S032 and S057 belonged to the same cluster both after lactulose ingestion and after placebo ingestion.

Table 4. Log cell counts of bifidobacterial species before and after the test food interventions.¹

Bifidobacterial species	n	Placebo		P-value	n	Lactulose		P-value
		before	after			before	after	
Genus <i>Bifidobacterium</i>	49	9.24±0.77	9.21±0.71	0.60	49	9.10±0.81	9.48±0.65	<0.0001
<i>Bifidobacterium adolescentis</i> group	21	8.95±0.81	9.04±0.57	0.70	22	8.82±0.81	9.32±0.54	0.00013
<i>Bifidobacterium animalis</i> ssp. <i>lactis</i>	6	9.66±0.96	9.10±1.00	0.23	10	8.14±1.26	8.55±1.40	0.44
<i>Bifidobacterium bifidum</i>	8	9.28±0.26	9.13±0.48	0.30	7	9.17±0.42	9.34±0.23	0.10
<i>Bifidobacterium breve</i>	23	8.19±0.83	8.23±0.91	0.72	25	7.93±0.87	8.24±0.87	0.031
<i>Bifidobacterium catenulatum</i> group	30	9.49±0.64	9.46±0.78	0.73	30	9.34±0.78	9.64±0.57	0.034
<i>Bifidobacterium longum</i> group	44	9.08±0.63	9.02±0.65	0.41	43	9.04±0.55	9.29±0.58	0.0010
<i>Bifidobacterium longum</i> ssp. <i>longum</i>	44	9.10±0.61	9.04±0.63	0.43	43	9.08±0.53	9.30±0.53	0.0017

¹ Values are expressed as the simple mean ± standard deviation (paired *t*-test).

Table 5. Bacterial taxa abundance, expressed as percentages of the faecal microbiome after the test food intervention.¹

Genus	n	Placebo	Lactulose	Delta (95% CI) ²	P-value
<i>Blautia</i>	49	22.8±1.1	21.7±1.1	1.1 (-2.0 to 4.2)	0.47
<i>Bifidobacterium</i>	49	18.1±1.4	25.3±1.4	7.2 (2.9 to 11.4)	0.0014
Lachnospiraceae gen.	49	7.8±0.6	6.8±0.6	1.0 (-0.5 to 2.4)	0.18
<i>Coprococcus</i>	49	6.9±0.5	7.0±0.5	0.1 (-1.2 to 1.5)	0.82
Lachnospiraceae <i>Ruminococcus</i>	49	6.6±0.5	5.3±0.5	1.3 (-0.1 to 2.7)	0.061
<i>Faecalibacterium</i>	49	5.1±0.6	4.9±0.6	0.2 (-1.8 to 2.1)	0.87
<i>Roseburia</i>	49	4.6±0.7	3.7±0.7	0.9 (-1.1 to 3.0)	0.37
<i>Streptococcus</i>	49	3.5±0.7	4.0±0.7	0.5 (-1.3 to 2.4)	0.58
<i>Ruminococcus</i>	49	3.3±0.4	3.5±0.4	0.2 (-0.9 to 1.3)	0.71
<i>Bacteroides</i>	49	2.1±0.5	2.2±0.5	0.1 (-1.3 to 1.6)	0.83

¹ Values are expressed as the least square mean ± standard error (linear mixed model).

² CI = confidence interval.

Figure 3. Principal coordinate analysis score plots generated using the first two principal coordinates (PC): PC1 and PC2. Data were analysed using PERMANOVA and were based on (A) unweighted UniFrac distances or (B) weighted UniFrac distances. The differences between groups were not significant using the unweighted UniFrac distances ($P=0.99$) or weighted UniFrac distances ($P=0.085$).

Figure 4. Dendrogram constructed using the logarithmically transformed cell counts for bifidobacterial species.

Supplementary Figure S2A shows the ratios of bifidobacterial species in each cluster separately for lactulose and placebo ingestion, which were calculated using untransformed bifidobacterial cell counts. Supplementary Figures S2B shows the cumulative untransformed cell counts for the bifidobacterial species in each cluster, separately for lactulose and placebo ingestion.

The bifidobacterial biotas of the participants belonging to cluster 1 were similar in that they contained the *B. adolescentis* group, *B. catenulatum* group, *B. longum* group and *B. bifidum*, but *B. animalis* ssp. *lactis* was uncommon, only being present on one branch of the dendrogram. All the participants possessed the *B. adolescentis* group and *B. longum* group, and 81% of the participants possessed the *B. catenulatum* group. Cluster 1 branched on the basis of the presence or absence of *B. breve*. The bifidobacterial biotas of the participants belonging to cluster 2 were similar, in that they possessed the *B. catenulatum* group and *B. longum* group, but lacked the *B. adolescentis* group. Cluster 2 also branched on the basis of the presence/absence of *B. breve*. In cluster 2, the *B. catenulatum* group showed a significantly and consistently higher log cell count than the *B. longum* group (9.98 ± 0.30 vs 9.20 ± 0.40 , $P < 0.0001$ by paired *t*-test, including both the post-lactulose ingestion and post-placebo ingestion data) (Figure 5). Furthermore, the log cell count for the *B. catenulatum* group in cluster 2 was significantly higher than that in cluster 1 (9.98 ± 0.30 vs 9.21 ± 0.57 , $P < 0.0001$, by *t*-test, including both the post-lactulose ingestion and post-placebo ingestion data).

Cluster 3 was characterised by the possession of the *B. longum* group and *B. breve*. Almost none of the participants possessed the *B. catenulatum* group and/or *B. bifidum*. In cluster 3, the branches represented the presence or absence of the *B. adolescentis* group, the *B. longum* group and *B. breve*.

Figure 5. Results of correlation analysis of the log cell counts of the *Bifidobacterium catenulatum* group and *Bifidobacterium longum* group in cluster 2. ○ after lactulose ingestion; ● after placebo ingestion. The *B. catenulatum* group showed a consistently higher log cell count than the *B. longum* group (9.98 ± 0.30 vs 9.20 ± 0.40 , $P < 0.0001$, paired *t*-test, including both after lactulose ingestion and after placebo ingestion data).

4. Discussion and conclusions

This is the first study to determine the effect of oral lactulose on the abundance of each bifidobacterial species. However, one limitation of the study was that all of the participants were young Japanese women, therefore, it may be difficult to extrapolate the results to the whole population or to populations in other geographical regions.

There have been several studies regarding the relationship between lactulose ingestion and bifidobacteria in faeces. In particular, studies conducted in healthy Japanese people have all shown a significant increase in numbers (Mizota *et al.*, 2002; Sakai *et al.*, 2019a; Tomoda *et al.*, 1991). Tomoda *et al.* (1991) reported that the number of bifidobacteria was significantly higher in participants that had consumed yogurt containing 0.65 g of lactulose than in those who had consumed yogurt that did not contain lactulose. Mizota *et al.* (2002) also reported significantly higher numbers of bifidobacteria when beverages containing 4 g of lactulose were consumed, compared with the consumption of beverages not containing lactulose. However, in these studies, only the abundance of the genus *Bifidobacterium* was assessed, and therefore it remained to be determined which bifidobacterial species contributed to the greater abundance of this genus. Similarly, in the studies by Ballongue *et al.* (1997), Tuohy *et al.* (2002), Bouhnik *et al.* (2004a), Bouhnik *et al.* (2004b) and Vanhoutte *et al.* (2006), who studied healthy non-Japanese people, the relationships between lactulose ingestion and bifidobacterial abundance in faeces were also assessed, but it also remains unknown which bifidobacterial species contributed to the significant differences in the abundance of the genus *Bifidobacterium*. In addition, it has become relatively clear that the bifidobacterial species change between generations (Kato *et al.*, 2017). Bifidobacteria do not form a majority of the microbiota in non-Japanese adults (Nishijima *et al.*, 2016), and the identity of the bifidobacterial species in non-Japanese people have not been studied in detail. In the present study, we have shown that the increase in bifidobacteria in the Japanese participants was mainly due to increases in the numbers of the species of bifidobacteria that form the majority of the bacterial population in Japanese people and in each participant, such as the *B. longum* group, *B. catenulatum* group and *B. adolescentis* group.

In previous studies of the bifidobacteria species in the Japanese population under normal conditions, the *B. longum* group was the most frequently identified species in teenagers and in people in their twenties, followed by the *B. adolescentis* group and *B. catenulatum* group (Kato *et al.*, 2017). Similar results were obtained in the present study. In addition to the comparisons at the genus level, the numbers of bacteria belonging to the *B. adolescentis* group, *B. catenulatum* group and *B. longum* group were significantly higher after lactulose ingestion than after placebo ingestion, and the log cell counts were also significantly increased by lactulose ingestion. The log cell count for *B. breve* after lactulose ingestion was not significantly higher than after placebo ingestion; however, it was significantly higher than before lactulose ingestion. For two participants, the total log cell counts of bacteria belonging to the genus *Bifidobacterium*, which were determined using the primers g-Bifid-F and g-Bifid-R, could not be fully explained by the bifidobacterial species

that were quantified in the present study. The bifidobacteria possessed by some participants may have been HRB species, such as *Bifidobacterium angulatum* or *Bifidobacterium dentium*, which were not analysed in the present study, or non-HRB species, such as *Bifidobacterium thermophilum* or *Bifidobacterium animalis* ssp. *animalis*. Non-HRB might become the predominant bifidobacterial species in the human intestine if it was continuously ingested. However, the participants were prohibited from taking probiotics in the present study period. The lack of quantification of these types of bifidobacteria should be taken into consideration when interpreting the findings of the present study.

The detection rate of *B. bifidum* was low and its abundance did not increase significantly during the trial. Because it is generally considered to be an infant-specific species of *Bifidobacterium*, its low detection rate in this trial was as expected. However, the log cell count of *B. bifidum* tended to be higher after lactulose ingestion than after placebo ingestion. The detection rate of *B. animalis* ssp. *lactis*, which generally resides in animal intestines, was similar to those of previous reports (Kato *et al.*, 2017). For example, the *B. longum* group was identified in one participant throughout the trial period, whereas *B. animalis* ssp. *lactis* was not consistently identified during the study period. This might be due to the fact that participants consumed *B. animalis* ssp. *lactis* without realising it, even though they were instructed to refrain from eating foods containing probiotics.

As described above, most of the intestinal bifidobacteria seemed to increase in abundance following lactulose ingestion. According to cluster analysis that used a dendrogram based on the log cell count of each bifidobacterial species, participants were divided into three clusters. Cluster 3 was mainly separated from clusters 1 and 2 by the presence/absence of the *B. catenulatum* group, whereas clusters 1 and 2 were separated by the presence/absence of the *B. adolescentis* group. Only S032 and S057 belonged to different clusters after lactulose ingestion and after placebo ingestion, which indicated a difference in the composition of the bifidobacteria associated with lactulose and placebo ingestion in these individuals. Further inspection revealed that the *B. longum* group and *B. adolescentis* group were detected after placebo ingestion in both participants, whereas *B. breve* and *B. animalis* ssp. *lactis* were detected after lactulose ingestion, in addition to the *B. longum* group and *B. adolescentis* group, in both participants. When lactulose had not been ingested, the log cell count of *B. breve* was below the detection limit. If *B. breve* had also been detected after placebo ingestion, it would have been presumed that this species belonged to cluster 3, both after lactulose ingestion and after placebo ingestion, which would have indicated that there was no change in the bifidobacteria between these conditions. Despite the characteristics of the cluster analysis shown

on the dendrogram, each participant's position on the dendrogram remained largely unchanged by placebo or lactulose ingestion. This indicates that the increase in bifidobacteria following lactulose intake cannot be accounted for by particular bifidobacterial species; the numbers of each bifidobacterial species increased to a similar extent. Thus, variations in the composition of the bifidobacteria within individuals do not exceed the differences between individuals during lactulose intake. The combinations of bifidobacterial species and their ratios might be of significance, but further research is needed to explore this. In particular, the *B. catenulatum* group showed a constantly higher log cell count than the *B. longum* group in cluster 2. When comparing cluster 2 with cluster 1, the *B. catenulatum* group did not predominate in the presence of the *B. adolescentis* group, but did predominate in the absence of the *B. adolescentis* group. This indicates that the abundance of the *B. catenulatum* group may be affected by the *B. adolescentis* group.

Furthermore, PCoA based on the intestinal microbiota analysis data showed no significant difference between lactulose ingestion and placebo ingestion. Analysis of the intestinal microbiota revealed that the abundance of the bifidobacteria was significantly higher following lactulose ingestion, whereas those of other intestinal bacterial taxa did not significantly change. These results suggest that lactulose ingestion significantly increases the abundance of HRB, but does not significantly affect particular intestinal microbial taxa. Because lactulose ingestion increases the amount of hydrogen gas in the breath, it is presumed that intestinal bacteria other than bifidobacteria, which do not generate hydrogen, also utilise lactulose for growth. When lactulose is metabolised by the caecal microbiota, the intestinal pH is reduced (Bown *et al.*, 1974), and this is partly due to the acetic acid produced by *Bifidobacterium*. Because acetic acid has high bacteriostatic activity, it is likely that the growth of intestinal bacteria other than bifidobacteria is suppressed, which causes bifidobacteria to predominate. In other words, the increase in the abundance of bifidobacteria that occurs following lactulose ingestion may not be attributable to the suppression of the growth of other intestinal bacteria by lactulose itself, but may simply be due to greater nutrient availability. If this is the case, the increase in bifidobacterial numbers would not greatly affect the composition of the intestinal microbiota as a whole. It is speculated that only a large amount of bifidobacteria was significantly increased as a result of the gradual decrease of many intestinal bacteria other than bifidobacteria within a range in which no statistically significant difference was obtained.

A limitation of the present study is that we were unable to clarify the roles of individual bifidobacterial species. Studies aimed at better understanding the detailed relationships between bifidobacterial species, and also between the wider

intestinal microbiota and bifidobacteria, may clarify the health benefits of bifidobacteria in the future.

The present findings show several patterns in the combinations of bifidobacterial species that are present during lactulose supplementation, which suggests that there are probably distinct effects of bifidobacterial species. Furthermore, we show that lactulose ingestion leads to an increase in HRBs in the intestines, but this greater abundance of bifidobacteria does not affect the intestinal microbiota as a whole.

Supplementary material

Supplementary material can be found online at <https://doi.org/10.3920/BM2020.0100>.

Table S1. PCR primers used to detect each bifidobacterial species.

Table S2. Observations in each cluster.

Figure S1. Numbers of bifidobacterial species.

Figure S2. Detection rate and cumulative cell counts of bifidobacterial species in each cluster of the dendrogram.

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Conflict of interest

There is no conflict of interest to disclose.

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