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Association of life habits and fermented milk intake with stool frequency, defecatory symptoms and intestinal microbiota in healthy Japanese adults

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RESEARCH ARTICLE

Abstract

Few studies have examined the effects of smoking habit, the frequency of alcohol drinking, exercise, and fermented milk consumption on defecatory symptoms and gut microbiota composition, and particularly their interactive effects. We examined the effect of these lifestyle factors on bowel movements and gut microbiota composition in 366 healthy Japanese adults by analysis of covariance. Smoking did not affect defecatory symptoms but was negatively correlated with total bacteria and *Enterococcus* counts. Drinking frequency was significantly positively correlated with a feeling of incomplete evacuation and counts of the *Bacteroides fragilis* group and *Acidaminococcus* groups. Exercise frequency tended to be negatively correlated with the Bristol Stool Form Scale score and was significantly negatively correlated with the counts of *Enterobacteriaceae* and positively correlated with the *Prevotella* counts in the faeces. The frequency of fermented milk consumption was not significant but tended to be positively correlated with stool frequency. The frequency of fermented milk consumption was significantly positively correlated with the counts of the *Atopobium* cluster, *Eubacterium cylindroides* group, *Acidaminococcus* group, *Clostridium ramosum* subgroup, and *Lactobacillus* in the faeces. The frequency of consumption of probiotic *Lactobacillus casei*-containing fermented milk was significantly positively correlated with stool frequency. The counts of probiotic *Lactobacillus casei* in the stool was positively correlated with the counts of *Bifidobacterium* and total *Lactobacillus*. These results suggest that smoking, alcohol drinking, exercise, and consumption of fermented milk, particularly containing probiotic *L. casei*, differently affect bowel movements and gut microbiota composition in healthy Japanese adults.

Keywords: microbiota, fermented milk products, stool frequency, bowel movement, life habits

1. Introduction

The human gut microbiota consists of more than 1000 species (approximately 3×10^{13} of bacterial cells) (Round and Mazmanian, 2009; Sender *et al.*, 2016) and positively induces maturation of parts of the gut, such as the mucosal immune system (Hooper *et al.*, 2012; Kundu *et al.*, 2017), the enteric nervous system and digestive function that maintains gut homeostasis and protection against infectious intestinal disease (Baumler and Sperandio, 2016; Muller *et al.*, 2014). In contrast, the gut microbiota is also associated with a variety of diseases (Round and Mazmanian, 2009; Thaiss *et al.*, 2018), including bowel movement disorders such as constipation and diarrhea (Roager *et al.*, 2016) and inflammatory bowel diseases (Chu *et al.*, 2016). In these consequences, there is thus no doubt that the gut microbiota affects human health (Lakshminarayanan *et al.*, 2013) and physiological condition. From this perspective, factors affecting it have attracted much attention. The factors most commonly studied are age (Yatsunenko *et al.*, 2012), sex (Bolnick *et al.*, 2014; Markle *et al.*, 2013), ethnic group and dietary habits (Desai *et al.*, 2016; Norman *et al.*, 2015; Sonnenburg and Backhed, 2016).

The gut microbiota in smokers differs from that in non-smokers (Opstelten *et al.*, 2016). Alcohol drinking reduces its diversity (Kosnicki *et al.*, 2019). Exercise increases its diversity in obese individuals (Allen *et al.*, 2018). However, although these factors are common lifestyle habits in healthy individuals, only one study has examined their effects on bowel movements and the gut microbiota in healthy individuals (Biedermann *et al.*, 2013). Probiotics are defined as 'Live microorganisms which when administered in adequate amounts confer a health benefit on the host' (Reid, 2005). The consumption of probiotics containing *Lactobacillus* and *Bifidobacterium* species increases stool frequency and reduces the transit time of intestinal contents in constipated adults as revealed by a meta-analysis comprising a total of 21 studies and 2,656 subjects (Miller *et al.*, 2017), and *Lactobacillus casei* strain Shirota affects the human gut microbiota and the diversity index (Kato-Kataoka *et al.*, 2016). Therefore, the consumption of probiotics is considered to be another factor that affects human physiology and the gut microbiota. However, few studies that investigated the effect of probiotic fermented milk on bowel movements and the gut microbiota have focused on interaction with other lifestyle factors (Suzuki *et al.*, 2017).

16S rRNA amplicon sequencing analysis by next-generation sequencer (NGS) is frequently used to analyse the human gut microbiota because its results are comprehensive. On the other hand, it can serve non-quantitative and abundance-based data of measure only predominant bacteria ($>10^6$ cells/g faeces) in the intestine, however, it cannot detect low-amounts bacteria that affect host health. Therefore, it can provide only limited data on the interaction between host physiological functions and bacteria. For the analysis of human intestinal bacteria, we had already developed a system, Yakult Intestinal Flora-SCAN (YIF-SCAN), based on reverse-transcription-quantitative polymerase chain reaction (RT-qPCR) targeting rRNA molecules (Matsuda *et al.*, 2007). The detection limits of NGS targeting rRNA genes and cDNA after the reverse transcription of rRNA are both about 10^6 cells/g faeces (=0.1% occupancy). In contrast, YIF-SCAN targets rRNA molecules not rRNA genes. Bacteria generally contain thousands to tens of thousands of ribosomes, which include rRNA molecules. Therefore, since the detection limit of YIF-SCAN is approximately 10^{2-3} cells/g faeces, it can be said that a YIF-SCAN analysis is 1000 times more sensitive than an NGS analysis when targeting rRNA genes and cDNA from rRNA (Matsuda *et al.*, 2007). We thought that it was very important to understand gut microbiota quantitatively.

From the above perspective, we investigated lifestyle habits and bowel movements in 366 healthy Japanese adults and quantified major intestinal bacteria by using YIF-SCAN, with the aim of determining any association of smoking, alcohol, exercise, and consumption of fermented milk with bowel movements and the gut microbiota.

2. Materials and methods

Subjects and stool collection

The KSO Corporation, Japan recruited 383 healthy Japanese adults. This study was conducted in conformity with the Helsinki Declaration, and the study plan was approved by the Human Study Ethics Committee of Nihonbashi Cardiology Clinic, Japan. All subjects gave written informed consent. Each subject was given a stool collection tube containing 2 ml of RNA*later* (Thermo Fisher Scientific KK, Waltham, MA, USA), the weight of which had been measured beforehand. RNA*later* was used to stabilise bacterial RNA (Kurakawa *et al.*, 2012; Nomoto *et al.*, 2015; Ogata *et al.*, 2015). Subjects introduced about 0.5 g of fresh stool into the collection tube with a stool collection spoon and then shook it thoroughly. The subjects completed a questionnaire about lifestyle, stool frequency, stool consistency, and medicines being taken at the same time as the faecal sampling. These tubes were triple wrapped and transported to KSO Corporation within 2-3 days of sampling. On receipt, samples were anonymised and immediately stored at 4 °C. Within a week they were transported to Yakult Central Institute, Japan and stored at 4 °C until preparation for analysis. After the exclusion of 17 subjects who met exclusion criteria (administration of antibiotics, antiflatulents, or laxatives within 1 week before stool collection), 366 subjects (average age 40.0±11.0 years, 185 men, 181 women, body mass index (BMI) 22.3±3.3) were included in the analysis.

General information on the subjects and information regarding medication can be found in Table 1 and supplementary Table S1, respectively.

Stool preparation

Stool samples were subjected to pretreatment for RNA extraction according to the method described by Kubota *et al.* (2010). Each sample was weighed and diluted 1:10 with RNA*later*. The mixture was suspended for 10 min on a Shake Master Auto v. 2.0 shaker (Bio Medical Science, Tokyo, Japan). Then 200 µl of the further 5-fold diluted suspension was introduced into a 2 ml tube containing 1 ml of phosphate-buffered saline (PBS). After centrifugation at 13,000×*g* for 5 min, the supernatant was discarded by decantation. The resulting pellet was stored at -80 °C until RNA extraction. Stool samples were subjected to pretreatment for DNA extraction also according to the method described by Kubota *et al.* (2010). Again, 200 µl of stool suspension was introduced into a 2 ml tube containing 1 ml of PBS and suspended on a vortex mixer. After centrifugation at 13,000×*g* for 5 min, 1 ml of supernatant was discarded with a pipette and then another 1 ml of PBS was added. After another suspension and centrifugation at 13,000×*g* for 5 min, the resulting 200 µl of suspension was stored at -30 °C until DNA extraction.

Table 1. General information (age, BMI and gender) on subjects in each group.[1]

Item/frequency	Age (year)	BMI	Female/male (the number of subjects)
Smoking			
non-smoking (n=312)	39.5±10.9^{a}	22.1±3.2^{b}	171/141^{c}
smoking (n=54)	42.6±11.4^{a}	23.3±3.5^{b}	10/44^{c}
Frequency of alcohol consumption			
<1 day/month (n=103)	41.7±10.3^{a}	22.4±4.0	64/39^{a}
>1 day/month and <1 day/week (n=92)	35.9±11.5^{a,b}	22.2±3.1	49/43^{b}
1-4 days/week (n=107)	38.6±11.2^{c}	22.0±2.9	50/57
>4 days/week (n=64)	45.1±8.6^{b,c}	22.8±3.0	18/46^{a,b}
Frequency of exercise			
<1 times/week (n=129)	39.5±11.2	22.0±3.3	77/52
1-3 times/week (n=165)	40.2±10.5	22.4±3.0	68/97
≥4 times/week (n=72)	40.2±12.0	22.5±4.0	36/36
Frequency of fermented milk product consumption			
<3 times/week (n=127)	38.9±11.1	22.1±3.1	56/71
≥3 times/week (n=239)	40.5±10.9	22.4±3.4	125/114
Frequency of consumption of fermented milk products containing LcS[2]			
<3 times/week (n=201)	39.5±11.2	22.0±3.0	104/97
≥3 times/week (n=165)	40.5±10.9	22.6±3.6	77/88

[1] Values are mean ± standard deviation; *P*-value (age and body mass index (BMI)): significance between same alphabet by Mann Whitney U-test and Steel-Dwass test in each group; *P*-value (female/male): significance between same alphabet by Fisher's exact test and post hoc Holm test in each group.

[2] LcS = *Lactobacillus casei* Shirota.

RNA extraction

Total RNA was extracted from samples according to the method described by Matsuda *et al.* (2007) and the RNA samples were stored at -80 °C until microbiota analysis.

Principle of YIF-SCAN

YIF-SCAN is a highly sensitive quantitative analytical method based on reverse transcription-quantitative PCR targeting rRNA molecules. Bacteria generally contain thousands to tens of thousands of ribosomes including rRNA molecules. On the other hand, only a few to a dozen copies of the rRNA genes are encoded on the bacterial chromosome. A typical property of YIF-SCAN is to provide an absolute quantification of bacteria that is 100-1000 times more sensitive (Matsuda *et al.*, 2007) than other molecular biological methods for use with human gut microbiota such as NGS targeted DNA.

Quantifying intestinal bacteria with YIF-SCAN

The total RNA was used to quantify the counts of 7 bacterial groups (*Acidaminococcus* group, *Atopobium* cluster, *Bacteroides fragilis* group, *Clostridium coccoides* group, *Clostridium leptum* subgroup, *Clostridium ramosum* subgroup, *Eubacterium cylindroides* group), 8 genera (*Bifidobacterium*, *Enterococcus*, *Fusobacterium*, *Prevotella*, *Pseudomonus*, *Staphylococcus*, *Streptococcus*, *Veillonella*), 1 family (*Enterobacteriaceae*), 6 *Lactobacillus* subgroups (*Lactobacillus casei* subgroup, *Lactobacillus gasseri* subgroup, *Lactobacillus plantarum* subgroup, *Lactobacillus reuteri* subgroup, *Lactobacillus ruminis* subgroup, *Lactobacillus sakei* subgroup), 5 species (*Akkermansia muciniphila*, *Clostridium difficile*, *Clostridium perfringens*, *Lactobacillus fermentum*, *Lactobacillus brevis*) (Table 2, Supplementary Table S2). The sum of 27 bacterial groups/genera/species counts provided by undertaken with these 27 selected primer sets can cover more than 71.3% of the total bacterial counts in stools (Matsuda *et al.*, 2009). YIF-SCAN was executed with an OneStep RT-PCR Kit (QIAGEN GmbH, Hilden, Germany). YIF-SCAN was performed in a 384-well optical plate on an ABI PRISM 7900HT Sequence Detection System (Life Technologies Japan Ltd., Minato, Tokyo, Japan). The *Lactobacillus* counts were taken as the sum of the counts of six *Lactobacillus* subgroups (*L. gasseri*, *L. casei*, *L. plantarum*, *L. reuteri*, *L. ruminis*, and *L. sakei* subgroups) and two species (*L. brevis*, *L. fermentum*).

DNA extraction

DNA extraction was performed according to the method described by Matsuki *et al.* (2004a)

Table 2. Primers and standard strains used in the study.

Target bacteria	Standard strain	Primer	Sequence (5'-3')	Reference
Clostridium coccoides group	*Blautia producta* ATCC27340^{T}	g-Ccoc-F g-Ccoc-R	AAATGACGGTACCTGACTAA CTTTGAGTTTCATTCTTGCGAA	Matsuki *et al.*, 2002
Clostridium leptum subgroup	*Faecalibacterium prausnitzii* ATCC27768^{T}	sg-Clept-F sg-Clept-R3	GCACAAGCAGTGGAGT CTTCCTCCGTTTTGTCAA	Matsuki *et al.*, 2004b
Bacteroides fragilis group	*Bacteroides vulgatus* ATCC8482^{T}	g-Bfra-F g-Bfra-R	ATAGCCTTTCGAAAGRAAGAT CCAGTATCAACTGCAATTTTA	Matsuki *et al.*, 2002
Bifidobacterium	*Bifidobacterium adolescentis* ATCC15703^{T}	g-Bifid-F g-Bifid-R	CTCCTGGAAACGGGTGG GGTGTTCTTCCCGATATCTACA	Matsuki *et al.*, 1998
Atopobium cluster	*Collinsella aerofaciens* DSM3979^{T}	c-Atopo-F c-Atopo-R	GGGTTGAGAGACCGACC CGGRGCTTCTTCTGCAGG	Matsuki *et al.*, 2004b
Prevotella	*Prevotella melaninogenica* ATCC25845^{T}	g-Prevo-F g-Prevo-R	CACRGTAAACGATGGATGCC GGTCGGGTTGCAGACC	Kikuchi *et al.*, 2002; Matsuki *et al.*, 2002
Clostridium perfringens	*Clostridium perfringens* ATCC13124^{T}	s-Clper-F ClPER-R	GGGGGTTTCAACACCTCC GCAAGGGATGTCAAGTGT	Kikuchi *et al.*, 2002 Matsuda *et al.*, 2009
Clostridium difficile	*Clostridium difficile* DSM1296^{T}	Cd-lsu-F Cd-lsu-R	GGGAGCTTCCCA TAC GGG TTG TTG ACT GCC TCA ATGCTT GGG C	Matsuda *et al.*, 2012
Enterobacteriaceae	*Escherichia coli* ATCC11775^{T}	En-lsu3F En-lsu3 R	TGCCGTAACTTCGGGAGAAGGCA TCAAGGCTCAATGTTCAGTGTC	Matsuda *et al.*, 2009
Staphylococcus	*Staphylococcus aureus* ATCC12600^{T}	g-Staph-F g-Staph-R	TTTGGGCTACACACGTGCTACAATGGAC AA AACAACTTTATGGGATTTGCWTGA	Matsuda *et al.*, 2009
Enterococcus	*Enterococcus faecalis* ATCC19433^{T}	Ec-ssu1 F Ec-ssu1R	GGATAACACTTGGAAACAGG TCCTTGTTCTTCTCTAACAA	Matsuda *et al.*, 2009
Streptococcus	*Streptococcus mutans* IFO13955^{T}	F R	GCTTAGAAGCAGCTATTCATTC GGATACACCTTTCGGTCTCTC	Sakaguchi *et al.*, 2010
Acidaminococcus group	*Acidaminococcus fermentans* ATCC25085^{T}	gAcid-1 gAcid-3	TGTCTGACRCTGAGATG TCCTCCAGGTTRTCCCT	This study
Pseudomonas	*Pseudomonas aeruginosa* IFO12689^{T}	PSD7F PSD7R	CAAAACTACTGAGCTAGAGTACG TAAGATCTCAAGGATCCCAACGGCT	Matsuda *et al.*, 2009
Akkermansia muciniphila	*Akkermansia muciniphila* ATCC BAA835^{T}	AM1 AM2	CAGCACGTGAAGGTGGGGAC CCTTGCGGTTGGCTTCAGAT	Derrien *et al.*, 2008
Clostridium ramosum subgroup	*Clostridium ramosum* JCM1298^{T}	sg-Cram171-F sg-Cram626-R	GACACTGCATGGTGACC GGTTTCTATGGCTTACTG	Matsuki, 2007
Eubacterium cylindroides group	*Clostridium innocuum* ATCC14501^{T}	gEcylin-2mF gEcylin-4R	AGTATGCACGCAAGTGTG TTATGCCACCGGCTTCGGG	This study
Fusobacterium	*Fusobacterium varium* ATCC8501^{T}	gFuso-245F gFuso-1mR	AGAGCTTTGCGTCCYATTAG GCATTTTCACATCAGAC	This study
Veillonella	*Veillonella parvula* GIFU 7884^{T}	g-Veillo68-F g-Veillo490-R	GRAGAGCGATGGAAGCTT CCGTGGCTTTCTATTCC	Matsuki, 2007
Lactobacillus gasseri subgroup	*Lactobacillus acidophilus* ATCC4356^{T}	sg-Lgas-F sg-Lgas-R	GATGCATAGCCGAGTTGAGAGACTGAT TAAAGGCCAGTTACTACCTCTATCC	Matsuda *et al.*, 2009
Lactobacillus plantarum subgroup	*Lactobacillus plantarum* ATCC14917^{T}	sg-Lpla-F sg-Lpla-R	CTCTGGTATTGATTGGTGCTTGCAT GTTCGCCACTCACTCAAATGTAAA	Matsuda *et al.*, 2009
Lactobacillus reuteri subgroup	*Lactobacillus reuteri* ATCC23272^{T}	sg-Lreu-F sg-Lreu-R	GAACGCAYTGGCCCAA TCCATTGTGGCCGATCAGT	Matsuda *et al.*, 2009
Lactobacillus casei subgroup	*Lactobacillus casei* ATCC334^{T}	sg-Lcas-F sg-Lcas-R	ACCGCATGGTTCTTGGC CCGACAACAGTTACTCTGCC	Matsuda *et al.*, 2009
Lactobacillus ruminis subgroup	*Lactobacillus ruminis* ATCC27780^{T}	sg-Lrum-F sg-Lrum-R	CACCGAATGCTTGCAYTCACC GCCGCGGGTCCATCCAAAA	Matsuda *et al.*, 2009
Lactobacillus sakei subgroup	*Lactobacillus sakei* ATCC15521^{T}	sg-Lsak-F sg-Lsak-R	CATAAAACCTAMCACCGCATGG TCAGTTACTATCAGATACRTTCTTCTC	Matsuda *et al.*, 2009

Table 2. Continued.

Target bacteria	Standard strain	Primer	Sequence (5'-3')	Reference
Lactobacillus fermentum	*Lactobacillus fermentum* ATCC14931^T	LFer-1 LFer-2	CCTGATTGATTTTGGTCGCCAAC ACGTATGAACAGTTACTCTCATACGT	Matsuda *et al.*, 2009
Lactobacillus brevis	*Lactobacillus brevis* ATCC14869^T	s-Lbre-F s-Lbre-R	ATTTTGTTTGAAAGGTGGCTTCGG ACCCTTGAACAGTTACTCTCAAAGG	Matsuda *et al.*, 2009
Lactobacillus casei strain Shirota	*Lactobacillus casei* YIT9029	LcS57F LcS-597R	CTCAAAGCCGTGACGGTC ACGTGGTGCTAATAATCCTAGTG	Fujimoto *et al.*, 2008
Total bacterial counts	*Faecalibacterium prausnitzii* ATCC27768^T	UniF UniR	GTGSTGCAYGGYYGTCGTCA ACGTCRTCCMCNCCTTCCTC	Fuller *et al.*, 2007

Quantifying *Lactobacillus casei* strain Shirota and total bacteria by qPCR

qPCR was performed according to the method described by Fujimoto *et al.* (2008) on an ABI PRISM 7900HT Sequence Detection System (Life Technologies Japan Ltd.). LcS and total bacterial counts were quantified by qPCR analysis using primer sets and standard strains listed in Table 2. To validate the accuracy of the qPCR method for counting total bacteria, the qPCR method was compared to 4,6-diamidino-2-phenylindole (DAPI) method for the enumeration of total bacteria in faecal samples collected from 178 healthy Japanese adult volunteers. As a result, the total bacterial count in stool in 178 subjects determined by qPCR was very strongly correlated with the results obtained by the DAPI method (Supplementary Figure S1, Supplemetary materials and methods).

Questionnaire survey

The subjects provided basic information (sex, age, height, and weight), the total number of defecations within 1 week before stool collection, stool consistency on defecation, defecatory symptoms (straining, feeling of incomplete evacuation, or abdominal pain), and lifestyle habits (presence or absence of smoking, weekly frequency of alcohol drinking [0-7], weekly frequency of exercise [0-7], weekly frequency of consumption of fermented milk with and without LcS [0-7]. The Bristol Stool Form Scale (BSFS) was used to evaluate stool consistency (Lewis and Heaton, 1997); the normal range was set at 3.5 to 4.4. BSFS was evaluated and entered in the questionnaire survey when subjects collected their faeces. Defecatory symptoms were evaluated on a five-point scale: none (score 0), mild (score 1), moderate (score 2), strong (score 3), to severe (score 4).

Association of smoking, alcohol, exercise and consumption of fermented milk with stool microbiota and defecation status

The analysis of covariance (ANCOVA) analysed linear trends and between-group differences in the bacterial counts in stool samples, stool frequency, stool consistency on defecation, straining, feeling of incomplete evacuation, and abdominal pain. The covariates were age, sex, BMI, smoking habit (presence or absence), drinking frequency (<1 day/month, <1 day/week, 1-4 days/week, ≥4 days/week), exercise frequency (<1/week, 1-3/week, ≥4/week), and frequency of fermented milk consumption (<3/week, ≥3/week). Frequency of consumption of LcS-containing fermented milk was divided into two groups (<3/week, ≥3/week), according to a previous study (Aoyagi *et al.*, 2017).

Statistical analysis

The ANCOVA included covariates of age, sex, BMI, smoking (presence or absence), drinking frequency, exercise frequency, frequency of fermented milk consumption, and frequency of consumption of LcS-containing fermented milk. $P<0.05$ was considered statistically significant, and $0.05 \leq P<0.1$ was considered marginally significant. Spearman's rank correlation analysis was used to assess the correlation between the number of LcS in stool and the major intestinal bacterial groups measured. Both analyses were performed in R ver.3.3.0 software (https://cran.r-project.org/) (Ihaka and Gentleman, 1996). The significance level was set at $P<0.05$. Bacterial counts of 'not detected' samples were regarded as half the detection limits (logarithm) of the corresponding primer sets.

3. Results

Association of smoking with stool frequency, stool consistency, defecatory symptoms, and gut microbiota

Smoking habit was not associated with stool frequency, stool consistency, or defecatory symptoms in the ANCOVA adjusted for age, sex, BMI, and frequency of alcohol, exercise, and fermented milk consumption (Table 3). The total bacterial counts (P=0.002) and *Enterococcus* counts (P=0.005) were significantly lower and the counts of the *Clostridium leptum* subgroup tended to be lower (P=0.074) in smokers than in non-smokers (Figure 1).

Table 3. Association of smoking and frequency of alcohol drinking, exercise, and consumption of fermented milk with stool frequency and defecatory symptoms.[1]

Items/abdominal symptom		Stool frequency	Stool consistency	Straining at stool	Feeling of incomplete evacuation	Abdominal pain
Smoking	non-smoking	6.8±3.1	3.8±1.1	0.9±0.9	0.5±0.7	0.2±0.4
	smoking	7.8±3.2	4.2±1.1	0.9±0.9	0.6±0.7	0.1±0.5
	P-value[2]	0.137	0.199	0.461	0.544	0.694
Frequency of alcohol consumption	<1 day/month	6.8±3.3	3.8±0.9	0.9±0.9	0.4±0.7	0.1±0.3
	>1 day/month and <1 day/week	6.5±2.7	3.8±1.1	0.9±0.8	0.4±0.6	0.2±0.6
	1-4 days/week	7.2±3.2	3.9±1.2	1.0±1.0	0.6±0.8	0.2±0.4
	>4 days/week	7.3±3.2	4.2±1.1	0.9±1.0	0.6±0.8	0.2±0.5
	P-value[2]	0.548	0.418	0.767	0.032	0.137
Frequency of exercise	<1 times/week	6.7±1.1	3.9±1.1	1.0±1.0	0.5±0.8	0.2±0.4
	1-3 times/week	7.1±2.8	3.9±1.1	0.9±0.8	0.5±0.7	0.2±0.5
	≥4 times/week	7.0±2.6	3.7±1.1	0.9±1.0	0.5±0.8	0.1±0.3
	P-value[2]	0.642	0.068	0.848	0.933	0.540
Frequency of fermented milk product consumption	<3 times/week	6.6±2.4	3.9±1.1	0.9±0.9	0.5±0.7	0.1±0.4
	≥3 times/week	7.1±3.4	3.9±1.1	0.9±0.9	0.5±0.7	0.2±0.5
	P-value[2]	0.059	0.466	0.714	0.603	0.713
Frequency of consumption of fermented milk products containing LcS	<3 times/week	6.6±2.8	3.8±1.1	1.0±0.8	0.5±0.7	0.2±0.5
	≥3 times/week	7.4±3.4	4.0±1.0	0.9±1.0	0.6±0.8	0.2±0.4
	P-value[2]	0.025	0.330	0.416	0.735	0.895

[1] Values are mean ± standard deviation; the analysis was adjusted for age, sex, body mass index and non-focal covariates. LcS = *Lactobacillus casei* Shirota.

[2] *P*-value, significance by ANCOVA.

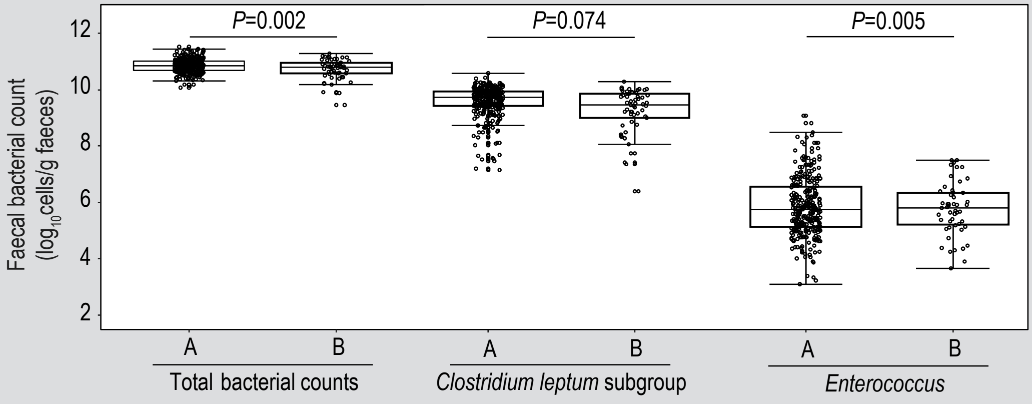

Figure 1. Association between smoking habit and gut microbiota. A: non-smokers (n=312); B: smokers (n=54). The bar indicates the overall group significance. Results with *P*<0.1 are shown.

Association of frequency of alcohol drinking with stool frequency, stool consistency, defecatory symptoms, and gut microbiota

The frequency of alcohol drinking was not associated with stool frequency or most defecatory symptoms in the ANCOVA adjusted for age, sex, BMI, smoking habit, and frequency of exercise and fermented milk consumption, but it was associated with a feeling of incomplete evacuation (Table 3). The counts of the *Bacteroides fragilis* group (*P*=0.05) and the *Acidaminococcus* group (*P*=0.029) increased significantly with frequency of alcohol drinking (Figure 2).

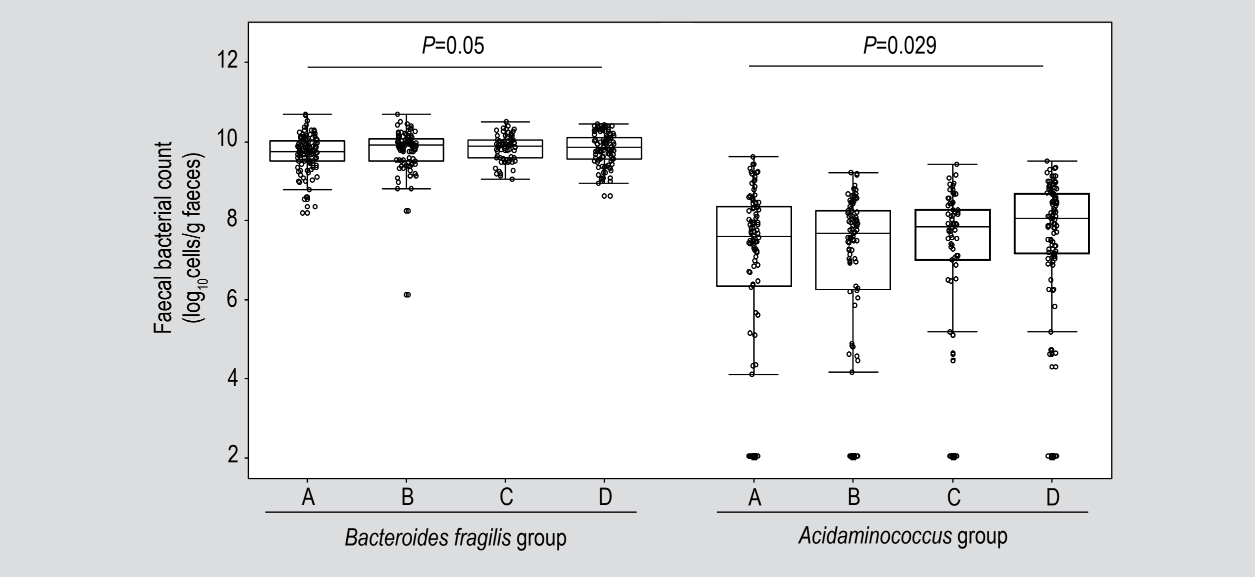

Figure 2. Association between drinking habit and gut microbiota. A: <1 day/month (n=103); B: <1 day/week (n=92); C: 1-4 days/week (n=107); D: ≥5 days/week (n=64). The bar indicates the overall group significance. Results with *P*<0.1 are shown.

Association of weekly exercise frequency with stool frequency, stool consistency, defecatory symptoms, and gut microbiota

Stool consistency tended to decrease with increasing frequency of exercise in the ANCOVA adjusted for age, sex, BMI, smoking habit, and frequency of alcohol drinking and fermented milk consumption (*P*=0.068; Table 3). Frequency of exercise was associated positively with the counts of *Prevotella* (*P*=0.021) and *Fusobacterium* (*P*=0.073; Figure 3) but negatively with the counts of the *Enterobacteriaceae* (*P*=0.043).

Association of frequency of fermented milk consumption with gut microbiota

Stool frequency tended to increase with increasing fermented milk consumption in the ANCOVA adjusted for age, sex, BMI, smoking habit, and frequency of exercise and alcohol drinking (*P*=0.059; Table 3). The counts of the *Atopobium* cluster (*P*=0.018), *Eubacterium cylindroides* group (*P*<0.001), *Acidaminococcus* group (*P*=0.018), *Clostridium ramosum* subgroup (*P*=0.021), and *Lactobacillus* (*P*<0.001) in stool increased significantly with increasing fermented milk consumption (Figure 4).

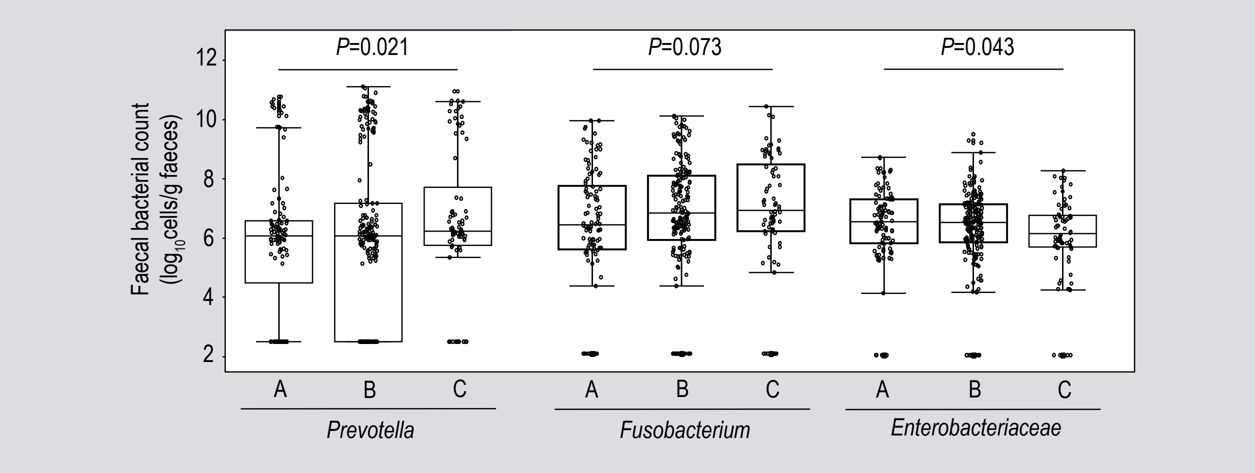

Figure 3. Association between weekly exercise frequency and gut microbiota. A: <1/week (n=129); B: 1-3/week (n=165); C: ≥4/week (n=72). The bar indicates the overall group significance. Results with *P*<0.1 are shown.

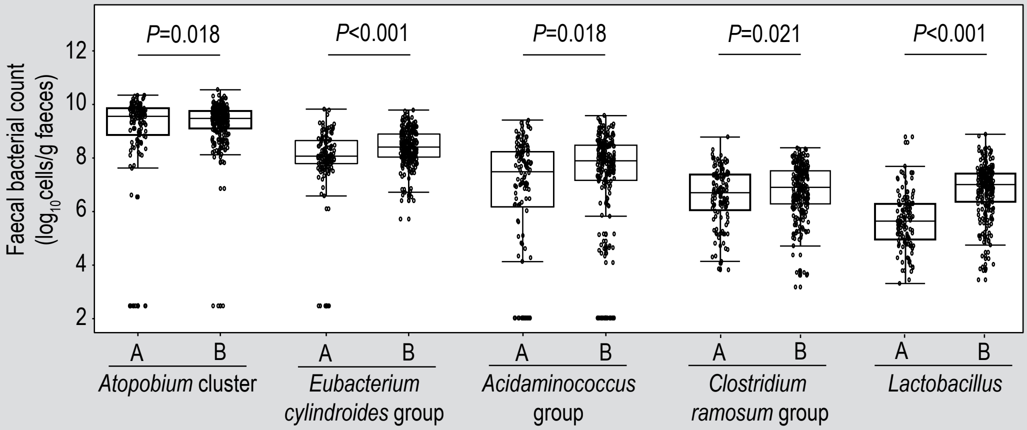

Figure 4. Association between weekly frequency of consumption of fermented milk and gut microbiota. A: <3/week (n=127); B: ≥3/week (n=239). The bar indicates the overall group significance. Results with *P*<0.1 are shown.

Association of consumption of LcS-containing fermented milk with stool frequency, stool consistency, defecatory symptoms, and gut microbiota

Stool frequency increased with increasing frequency of LcS-containing fermented milk consumption in the ANCOVA adjusted for age, sex, BMI, smoking habit, and frequency of exercise, alcohol drinking, and fermented milk consumption (*P*=0.025; Table 3). The counts of the *E. cylindroides* group (*P*=0.016), the *C. ramosum* subgroup (*P*=0.003), and *Lactobacillus* (*P*<0.001) in stool increased significantly with increasing consumption of LcS-containing fermented milk (Figure 5). The counts of the *B. fragilis* group (*P*=0.058) and the *Acidaminococcus* group (*P*=0.096) tended to increase also, but the counts of *Veillonella* tended to decrease (*P*=0.066).

Correlation between counts of *Lactobacillus casei* Shirota and other intestinal bacteria in stool samples

Spearman's rank correlation analysis found a significant strong positive correlation between the counts of LcS and *Lactobacillus* in stool (r=0.77, *P*<0.001), and significant weak correlations between the counts of LcS and each of *Bifidobacterium* (r=0.17, *P*=0.02) and *Streptococcus* (r=0.15, *P*=0.04; Figure 6).

4. Discussion

16S rRNA gene amplicon sequencing by NGS is now commonly used for the analysis of gut microbiota (Berglund *et al.*, 2011). This method can analyse the composition of the microbiota, including unculturable bacteria. However,

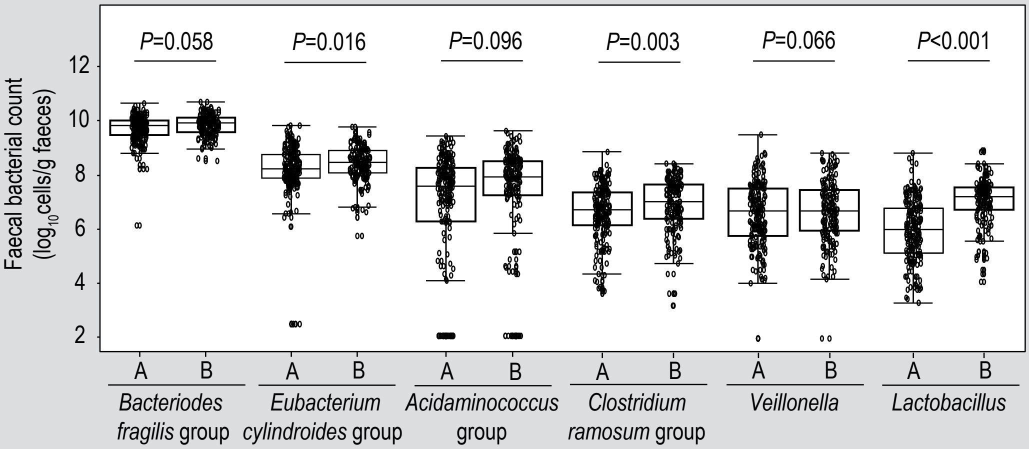

Figure 5. Association between weekly frequency of consumption of *Lactobacillus casei* Shirota-containing fermented milk and gut microbiota. A: <3/week (n=201); B: ≥3/week (n=165). The bar indicates the overall group significance. Results with *P*<0.1 are shown.

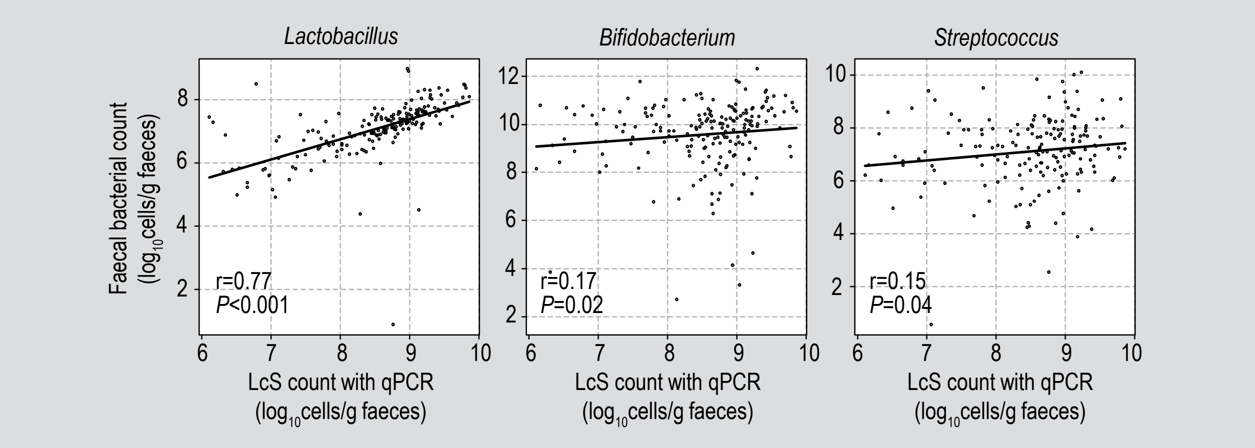

Figure 6. Correlation between numbers of *Lactobacillus casei* Shirota (LcS) and intestinal bacteria. Green line: linear approximation curve; Spearman's rank correlation coefficient (r) and associated *P*-values are shown. Results with *P*<0.05 are shown.

the resultant bacterial composition should be evaluated on the assumption that there are no inter- or intra-independent differences in the total bacterial counts in the intestine. In addition, actual bacterial counts are not taken into consideration, whereas more than 10-fold differences are observed between the total bacterial counts of healthy individuals (Vandeputte *et al.*, 2017). Therefore, 16S rRNA gene amplicon sequencing analysis is limited in its ability to accurately evaluate inter- and intra-independent differences in the gut microbiota (Vandeputte *et al.*, 2017). On the other hand, YIF-SCAN based on RT-qPCR targeted bacterial rRNA molecules can provide highly sensitive absolute quantification of as many as about 70% of major intestinal bacterial species based on RT-qPCR targeted bacterial rRNA molecules (Tsuji *et al.*, 2018). Using YIF-SCAN to quantify major human intestinal bacteria, we are the first to employ YIF-SCAN to investigate the effects of life habits such as smoking, alcohol drinking, and exercise on gut microbiota.

Although constipation increased after patients stopping smoking (Hajek *et al.*, 2003), as far as we know, no study has analysed the effect of smoking on bowel movements in healthy individuals. The lower counts of total bacteria and *Enterococcus* that we found in smokers (Figure 1) are consistent with the results of a previous study (Savin *et al.*, 2018). Nicotine, the primary active substance in tobacco, affects the activity of the central nervous system and gut microbiome community composition (Chi *et al.*, 2017). Smoking is assumed to influence gut microbiota possibly via nicotine or some other mechanism. The counts of the *C. leptum* subgroup, which decreases in several diseases (Hasegawa *et al.*, 2015; Morita *et al.*, 2015; Ohigashi *et al.*, 2013; Takaishi *et al.*, 2008) and increases by smoking cessation (Biedermann *et al.*, 2013), also tended to be lower in smokers (Figure 1). The relationship between the smoking-induced decrease in the *C. leptum* subgroup and the occurrence of various diseases would be an interesting research topic.

Alcohol and its metabolites have a diuretic effect, leading to an increase in water absorption from the lumen into the mucosa in the large intestine, affecting osmotic pressure (Elamin *et al.*, 2013). However, we found no relationship between frequency of alcohol consumption and stool consistency (Table 3). Water content in the faeces may be due to several factors such as alcohol intake, alcohol content, diet and infectious disease. On the other hand, the feeling of incomplete evacuation on defecation increased with increasing frequency of alcohol consumption (Table 3). Alcohol affects the motility of the gut (Berenson and Avner, 1981), and drinking alcohol twice a week therefore directly affected peristalsis. With increasing alcohol drinking frequency, the counts of the *B. fragilis* group and *Acidaminococcus* group increased (Figure 2). *Bacteroides* are less abundant in alcoholic patients than in the intestine of healthy individuals as determined by 16S rRNA gene amplicon analysis (Tsuruya *et al.*, 2016). As the total bacterial counts is less in patients with alcoholic liver cirrhosis than in healthy individuals (Koga *et al.*, 2013), the abundance of *Bacteroides* in the intestine may not reflect the actual counts of this bacterial genera. In this study, we did not confirm whether or not the subjects drank alcohol on the day before sampling. This information may be needed to clarify the relationship between intestinal bacteria and alcohol.

In our cohort, stool frequency did not change with an increase in exercise frequency, but stool consistency tended to decrease (Table 3). There has been no study of the relationship between exercise frequency and stool consistency in healthy individuals, so this is the first report showing the relationship. The relationship between exercise and stool consistency is likely to be affected by subject and by intensity of exercise; therefore, further study is needed to examine the relationship between exercise and stool consistency to take account of not only the frequency, but also the intensity, the kinds of exercise and the duration of each session. Exercise frequency was correlated positively with the counts of *Prevotella* and negatively with the *Enterobacteriaceae* in the intestine (Figure 3). Although exercise is reported to affect gut microbiota composition (O'Sullivan *et al.*, 2015; Zhao *et al.*, 2018), the impact of *Prevotella* and the *Enterobacteriaceae* by exercise has not previously been shown.

LcS-containing fermented milk is very popular both in Japan and around the world. In the 2016 fiscal year, 9.7 million drinks were consumed daily in Japan and 40 million throughout the world (www.yakult.co.jp/english/). In fact, a fermented milk product containing LcS was the milk product most frequently consumed by the subjects in this study (Supplementary Table S3). The results of our ANCOVA showed that with increasing frequency of consumption of LcS-containing fermented milk, stool frequency increased significantly to close to once a day (Table 3). On the other hand, the frequency of consumption of all fermented milk products with LcS and fermented milk products without LcS surveyed in this study only *tended* to be associated with stool frequency (Table 3). The stool frequency and stool consistency of the subjects who consumed fermented milk products containing LcS were significantly higher than for those who consumed fermented milk products without LcS (Student's t-test, $P<0.05$) (Supplementary Table S4). These results suggest that the consumption of LcS-containing fermented milk contributes greatly to stool frequency, as previously found in patients with chronic constipation, in whom it also improved stool consistency (Koebnick *et al.*, 2003), and it normalised stool frequency in healthy individuals with soft stools (Matsumoto *et al.*, 2010). These results, together with our findings, suggest that LcS normalises stool consistency and frequency. With the increase in the frequency of consumption of all fermented milk products surveyed in this study or LcS containing fermented milk, the counts of *E. cylindroides* group, *C. ramosum* group, and *Lactobacillus* increased in common (Figures 5, 6). *E. cylindroides* and *C. ramosum* groups are phylogenetically belonged to the *Erysipelotrichaceae*, whose role in the intestine is currently unclear, further researches are needed. Continuous consumption of LcS-containing fermented milk for 4 weeks significantly increased the counts of *Lactobacillus* (Nagata *et al.*, 2011), consistent with our results (Figures 5, 6). With increasing frequency of LcS-containing fermented milk consumption, the counts of the *L. sakei* subgroup, the *L. reuteri* subgroup, and *L. fermentum* decreased significantly, and only the counts of the *L. casei* subgroup, including LcS, increased significantly (Supplementary Figure S2). This suggests that LcS consumed replaces the indigenous lactobacilli to adapt to the niche in the intestine. The counts of LcS in stool was positively correlated with the counts of *Bifidobacterium*. Long-term consumption of LcS-containing fermented milk increases the number of indigenous bifidobacteria in the intestine and the concentrations of organic acids, including acetic acid, in the intestine of healthy school-age children and elderly people (Bien *et al.*, 2011; Nagata *et al.*, 2011; Wang *et al.*, 2015). We have now shown that consumption of LcS-containing fermented milk could increase indigenous bifidobacteria in healthy adults aged from 20 to 60. These results suggest that LcS increases the counts of *Bifidobacterium* in individuals across a very broad range of age groups. Further investigations are required to elucidate the casual associations between the consumption of LcS and increasing the indigenous bifidobacteria in the gut.

Limitations

In this study, we did not survey dietary components in the questionnaire, one of major factor affecting the gut microbiota composition, as a confounding factor. However, the impact of variation in dietary components consumed by the subjects on the results was minimised by the large number of subjects. In addition, we investigated only the frequency of confounders (smoking, alcohol, and exercise habits); investigation of the types and amounts of confounding factors as well will enable a more detailed analysis of factors affecting the gut microbiota. As mentioned above, although YIF-SCAN can cover major intestinal bacteria, it cannot analyse intestinal bacteria for which specific primer sets have not been prepared. However, it can allow absolute quantification of the majority of predominant human gut bacteria clades and low-abundance pathobionts and opportunistic pathogens in stool samples. Thus, our results have provided an important information of gut microbiota on different aspects from those of 16S rRNA gene amplicon sequencing analysis by NGS.

5. Conclusions

Our results clearly have demonstrated that smoking, the frequency of drinking, exercise, and consumption of fermented milk differently affect stool frequency, defecatory symptoms, and gut microbiota composition. We hope that these finding may contribute to elucidate how various lifestyle factors affect the gut microbiota composition and establish a strategy to contribute a host health by controlling the gut microbiota.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/BM2019.0057.

Table S1. The number of subjects taking major drugs in each group.

Table S2. List of major validated species in each targeted bacterial group.

Table S3. Main probiotic strains consumed by subjects and frequency of consumption.

Table S4. Difference in the impact on stool frequency and defecatory symptoms in subjects who consumed fermented milk products without LcS and with LcS.

Figure S1. Correlation between total bacterial count in stool by qPCR and DAPI in healthy adults.

Figure S2. Effects of the frequency of consumption of LcS-containing fermented milk on *Lactobacillus* subtype counts and species in faeces.

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Conflict of interest

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