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

Item/Frequency	Drug (the number of subjects)			
	Anti-allergic drugs	Lipid-lowering drugs	Uric acid production inhibitors	Hypotensors
Smoking				
non-smoking (n=312)	7	3	5	4
smoking (n=54)	2	1	0	0
Frequency of alcohol consumption				
<1 day/month (n=103)	3	3	1	3
>1 day/month and <1 day/week (n=92)	3	0	1	0
2-4 days/week (n=107)	0	0	2	1
>4 days/week (n=64)	3	1	1	0
Frequency of exercise				
<1 times/week (n=129)	3	2	1	1
1-3 times/week (n=165)	3	2	1	2
≥4 times/week (n=72)	3	0	3	1
Frequency of fermented milk product consumption				
<3 times/week (n=127)	1	0	0	1
≥3 times/week (n=239)	8	4	5	3
Frequency of consumption of fermented milk products containing LcS				
<3 times/week (n=201)	4	0	0	1
≥3 times/week (n=165)	5	4	5	3

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

Targeted bacterial group	Major validated species	References
Clostridium coccoides group	*Eubacterium ventriosum*	Kurakawa *et al*., 2015
	Eubacterium hallii	
	Coprococcus eutactus	
	Eubacterium eligens	
	Eubacterium rectale	
	Eubacterium ramulus	
	Blautia hydrogenotrophica	
	Blautia luti	
	Blautia obeum	
	Blautia schinkii	
	Blautia hansenii	
	Blautia producta	
	Blautia coccoides	
	Clostridium symbiosum	
	Fusicatenibacter saccharivorans	
	Clostridium asparagiforme	
	Clostridium hathewayi	
	Clostridium indolis	
	Ruminococcus gnavus	
	Anaerostipes caccae	
	Roseburia intestinalis	
	Coprococcus comes	
	Clostridium nexile	
	Dorea longicatena	
	Dorea formicigenerans	
	Clostridium scindens	
	Clostridium hylemonae	
	Ruminococcus lactaris	
	Ruminococcus torques	
Clostridium leptum subgroup	*Faecalibacterium prausnitzii*	Matsuki *et al*., 2004
	Eubacterium siraeum	
	Eubacterium desmolans	
	Ruminococcus bromii	
	Ruminococcus callidus	
	Ruminococcus albus	
	Ruminococcus flavefaciens	
	Clostridium viride	
	Clostridium leptum	
Bacteroides fragilis group	*Bacteroides fragilis*	Matsuki *et al*., 2002
	Bacteroides ovatus	
	Bacteroides thetaiotaomicron	
	Bacteroides uniformis	
	Bacteroides vulgatus	

Targeted bacterial group	Major validated species	References
Atopobium cluster	*Atopobium minutum*	Matsuki *et al.*, 2004
	Atopobium fossor	
	Atopobium rimae	
	Atopobium parvulum	
	Collinsella aerofaciens	
	Collinsella intestinalis	
	Collinsella stercoris	
	Eggerthella lenta	
	Cryptobacterium curtum	
Eubacterium cylindroides subgroup	*Eubacterium tortuosum*	This study
	Eubacterium dolichum	
	Faecalicoccus pleomorphus	
Clostridium ramosum subgroup	*Clostridium ramosum*	Matsuki *et al.*, 2007
	Clostridium cocleatum	
	Clostridium spiroforme	
Acidaminococcus group	*Phascolarctobacterium faecium*	This study
	Acidaminococcus fermentans	
	Succiniclasticum ruminis	
	Succinispira mobilis	

Table S3. Main probiotic strains consumed by subjects and frequency of consumption.[1,2]

Product	Contained strains	Yes	No	Proportion (%)	References
Brand A	*L. casei* strain Shirota	213	153	58.2	Nagao *et al*., 2000 Koebnick *et al*., 2003 Mitsuyama *et al*., 2008
Brand B	*L. delbrueckii* subsp. *Bulgaricus* 2038 & *Streptococcus thermophiles* 1131	98	268	26.8	Ohashi *et al*., 2007
Brand C	*L. gasseri* SBT2055	44	322	12.0	Kobatake *et al*., 2017 Ogawa *et al*., 2015
Brand D	*L. delbrueckii* subsp. *bulgaricus* OLL1073	32	334	8.7	Makino *et al*., 2016

[1] We surveyed the 366 subjects regarding their consumption of fermented milk products during the year prior to stool collection. Table S2 lists the top four probiotic *Lactobacillus* strains consumed and the number of subjects who consumed them.

[2] Yes: number of subjects who consumed the *Lactobacillus* strain within the previous year; No: number of subjects who did not consume the *Lactobacillus* strain within the previous year; Proportion: percentage of total (n=366).

Table S4. Difference in the impact on stool frequency and defecatory symptoms in subjects who consumed fermented milk products without LcS and with LcS.[1,2]

Item/Group	without LcS (n=56)	with LcS (n=151)	*P*-value
Stool frequency	6.11 ± 2.53	7.17 ± 3.37	0.034
Stool consistency	3.64 ± 1.02	3.93 ± 0.85	0.044
Straining at stool	0.50 ± 0.69	0.42 ± 0.68	0.475
Feeling of incomplete evacuation	0.88 ± 0.76	0.95 ± 0.82	0.533
Abdominal pain	0.18 ± 0.47	0.21 ± 0.55	0.746

[1] We surveyed the stool frequency and defecatory symptoms of the 207 subjects who drunk fermented milk products without LcS and with LcS.

[2] Values are mean ± SD; *P*-value: significant difference by *Student's* T-test, $P<0.05$.

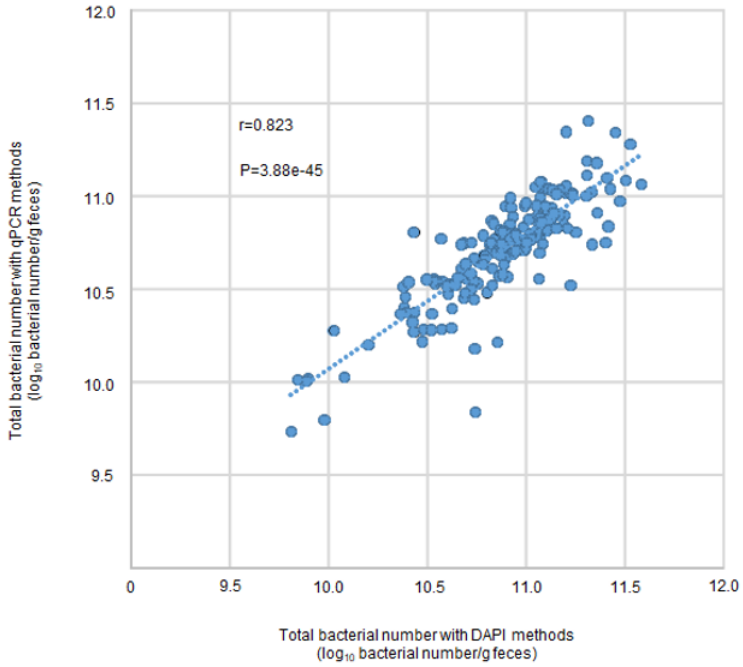

Figure S1. Correlation between total bacterial count in stool by qPCR and DAPI in healthy adults.

A very strong correlation is found between the total bacterial count in stool by qPCR and the DAPI method in healthy adults (n=178). Dotted line shows a linear approximation curve. Spearman's rank correlation coefficient (*r*) and associated *P*-value are shown.

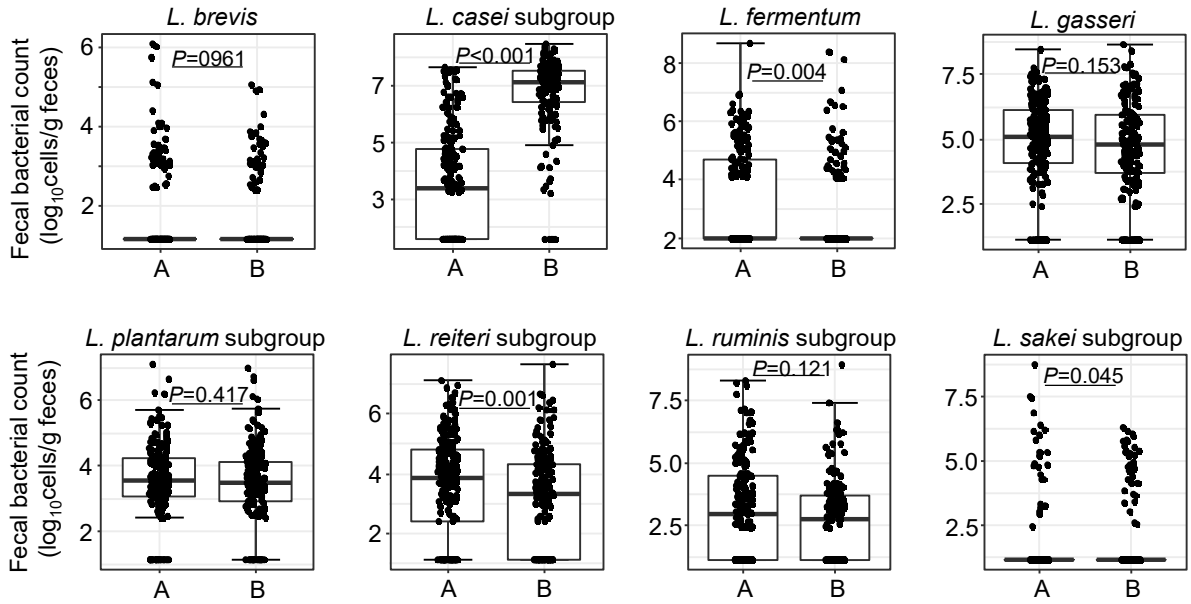

Figure S2. Effects of the frequency of consumption of LcS-containing fermented milk on *Lactobacillus* subtype counts and species in faeces.

A: <3/week (*n* = 201); B: ≥3/week (n=165). *P*-value indicates significant difference obtained by Mann Whitney U test.

Supplementary materials and methods

Subjects and stool collection

We collected stool samples from 178 healthy Japanese adults (average, 42.4 years; range, 23 to 64 years), all of whom gave written informed consent, and determined the total bacterial counts. About 0.5 g of fresh stool was collected with a collection spoon and submitted in a refrigerated condition, immediately stored at -30 °C, and then transported to Yakult Central Institute within 1 week by a sample carrier.

Stool preparation

The stool samples were prepared as described in Materials and Methods in the main text and then stored at -30 °C for DNA extraction. The thawed samples were diluted to 10 times their original volume with PBS. A 3-fold volume of 4% paraformaldehyde was added and the suspension was incubated at 4 °C overnight. The suspension was then used for the determination of the total bacterial count by the DAPI method (below) (Jansen *et al*., 1999).

Strain and culture conditions

Faecalibacterium prausnitzii ATCC27768^{T} was cultured at 37 °C for 72 h in Modified GAM broth (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) containing 1.0% (w/v) glucose (Beckton Dickinson Co., Sparks, MD, USA). The number of bacteria was determined by the DAPI method (Jansen *et al*., 1999) (below) and adjusted to 1×10^{10} cells/ml. DNA was extracted from this solution as in the main text and used to determine the total bacterial count as standard DNA.

Analysis of total bacterial count with DAPI fluorescent dye

Stool samples were diluted to about 600 times their volume with paraformaldehyde and PBS. Each diluted sample or cultured bacterial solution was placed on a microscope slide (Matsunami, Osaka, Japan), dried, and mounted and stained with DAPI stain-added mounting medium (Vectashield with DAPI, Vector Laboratories, Burlingame, CA, USA) (Jansen *et al*., 1999). A fluorescence imaging workstation (Leica Q550FW, Wetzlar, Germany) was used to observe fluorescence and obtain images (Takada *et al*., 2004). In each specimen, 10 visual fields were randomly selected to obtain images through a fluorescence filter (A4: excitation 360/40 nm, dichroic mirror 400 nm, emission 470/40 nm). The number of each bacterial group was calculated as: av. no. of bacteria in 10 visual fields × dilution factor × total number of visual

fields within 1 cm^2 in Image-Pro Plus v. 4.5 software (Media Cybernetics, Silver Spring, MD, USA).

Analysis of total bacterial count by qPCR

The total bacterial count was quantified by qPCR using reported primers (Fuller *et al.*, 2007). The reaction solution was composed of 1× PCR buffer (Takara Bio Inc., Shiga, Japan), 200 µM deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 1:75,000-diluted SYBR green I (BioWhittaker Molecular Applications), 0.02 units/µl Takara Taq (Takara Bio Inc.), 5.5 ng/µl TaqStart antibody (Takara Bio Inc.), 5 µl template DNA, and 0.2 µM UniF and UniR primers. The amplification program was 1 cycle of 95 °C for 5 min and 40 cycles of 94 °C for 20 s, 60 °C for 20 s, and 72 °C for 50 s. Real-time PCR was performed in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

The correlation of the total bacterial count in stool samples by qPCR and the DAPI method was tested by Spearman's rank correlation analysis and the significant difference test in R v. 3.3.0 software (https://cran.r-project.org/) (Ihaka and Gentleman, 1996) at $P<0.05$.

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