Characteristics of gut microbiome, organic acid profiles and viral antibody indexes of healthy Japanese with live *Lactocaseibacillus* detected in stool

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RESEARCH ARTICLE

Abstract

To estimate the health-promoting effects of *Lactocaseibacillus paracasei* (previously *Lactobacillus casei*) strain Shirota (LcS) that reached the lower gastrointestinal tract alive, we investigated the characteristics of gut microbiome, organic acid profiles, defecatory symptoms and serum viral antibody indexes of healthy Japanese adults between the group in whom live LcS was detected or not from stool. The β -diversity index of the gut microbiome constituted a significant difference between the live-LcS-detected-group (LLD) and the live-LcS-not-detected-group (LLnD). In the LLD, the *Bifidobacteriaceae*, *Lactobacillaceae*, and *Coriobacteriaceae* counts were significantly higher, and the succinate concentration was significantly lower than that in the LLnD. The serum herpes simplex virus (HSV) immunoglobulin (Ig)M antibody index in the LLD tended to be lower than that of the LLnD in HSV IgG-positive subjects. Of the LLD, those in the fermented milk products containing LcS (FML)-high-frequency-group (FML-HF) and those in the FML-low-frequency-group (FML-LF) had different gut microbiome and organic acid profiles. However, the pattern of differences between FML-HF and FML-LF was dissimilar those between LLD and LLnD. In contrast, among subjects with FML-LF, those in the group with LLD in stool (LF-LLD) and those in the LLnD in stool (LF-LLnD) showed a similar pattern of differences in their gut microbiome and organic acid profiles as those in the LLnD and LLD. The LLD and LF-LLD commonly had lower caloric and carbohydrate intakes from the diet than their respective control groups. In this study, we found that the presence of live LcS in stool is associated with a healthy gut environment and inhibition of the reactivation of latently infected viruses in the host. However, these health-promoting effects on the host were not related to the frequency of FML intake. Furthermore, dysbiosis of the gut microbiome and diet including caloric intake was related to the viability of ingested LcS in the gut.

Keywords: microbiome, live LcS, organic acid profiles, herpes simplex virus, defecatory symptoms

1. Introduction

Probiotics are defined as ‘Live microorganisms which when administered in adequate amounts confer a health benefit on the host’ (Hill *et al.*, 2014) and there have been numerous reports on the effects of probiotics, such as modulation of the gut-brain axis (Westfall *et al.*, 2017; Zhang *et al.*, 2020), host immunity including innate and acquired immune responses (Azad *et al.*, 2018; Giorgetti *et al.*, 2015), the competitive exclusion of pathogens (Kristensen *et al.*, 2016; Rao *et al.*, 2016) and the improvement of defecatory symptoms (Miller *et al.*, 2017). *Lactocaseibacillus paracasei*

(the previous taxonomic nomenclature was *Lactobacillus casei*) YIT 9029 (strain Shirota: LcS) is a popular probiotic strain, and 40 million fermented milk products containing LcS (FML) are consumed daily throughout the world (www.yakult.co.jp/english/). Several double-blind, placebo-controlled trials have shown the health-promoting effects of LcS, including the preservation of the diversity of the gut microbiome in the stool (Kato-Kataoka *et al.*, 2016), improved sleep quality (Takada *et al.*, 2017), reduced fever duration in elderly people (Kushiro *et al.*, 2019), modified interleukin-12 involved in the enhancement of natural killer cell activity (Takeda *et al.*, 2006), decreased salivary

levels of secretory immunoglobulin (Ig)A and antimicrobial peptides (Vaisberg *et al.*, 2019) and decreased serum viral antibody indexes (Gleeson *et al.*, 2016). Moreover, several epidemiological studies have also suggested that frequent FML consumption contributes to the normalisation of bowel habits (Aoyagi *et al.*, 2019; Shima *et al.*, 2019) and reduces the risk of hypertension in the elderly (Aoyagi *et al.*, 2017). In most of those reports, the faecal LcS counts were obtained with a quantitative PCR method using an LcS-specific primer set, and the relationships between the faecal LcS counts and these health-promoting effects were discussed. However, since this quantitative PCR cannot distinguish between live and dead LcS, it is unclear whether LcS need to reach the lower gastrointestinal tract alive to have above mentioned health-promoting effects. In these contexts, our aim was to estimate the health-promoting effects of LcS that reached the lower gastrointestinal tract alive in healthy Japanese adults, and we attempted to characterise the gut microbiome, organic acid profiles, defecatory symptoms and serum viral antibody indexes in subjects in which live LcS was detected with the PMA-qPCR method.

2. Materials and methods

Study design

The KSO Corporation (Tokyo, Japan) recruited 80 healthy Japanese adults by employing a questionnaire (Supplementary Table S1). 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 (Approval number NJI-019-01-01, 11 January 2019). All subjects provided written informed consent before they participated in this study. The subjects responded to the questionnaire and had blood taken only once, and the serum fractionated from the blood was used to analyse the viral antibody titer. In addition, subjects also submitted a portion of their stool on the same morning as the blood collection. The stool samples were transported to Yakult Central Institute and used to analyse the gut microbiome and organic acid profiles. Abdominal symptoms, fermented milk products intake, and food intake frequency were investigated by using a questionnaire. Finally, of the 80 subjects that had been recruited, 72 subjects (average age 38.3 ± 9.0 years, 52 men, 20 women) were included in the analysis, after the exclusion of 4 subjects who met the exclusion criteria (administration of antibiotics, antifoatulents, or laxatives within 1 week of stool collection), 3 subjects whose questionnaire responses were incorrect and 1 subject for whom insufficient 16S rRNA gene reads were detected in subsequent 16S rRNA gene amplicon sequencing.

Sample collection

Each subject was given a stool collection tube containing 2 ml of RNAlater (Thermo Fisher Scientific KK, Waltham, MA, USA) and two empty stool collection tubes whose weight had been measured beforehand. Subjects introduced about 0.5 g of fresh stool into the collection tube with a stool collection spoon and then shook this tube vigorously. On receipt, the stool samples were anonymised and transported to Yakult Central Institute (Tokyo, Japan), and stored at 4 °C until their preparation for analysis. Thirteen ml of blood was collected from subjects on the same day as the stool submission. Eight ml of blood was transported to BML Inc. (Tokyo, Japan) and herpes simplex virus (HSV), varicella zoster virus and cytomegalovirus antibody titers were measured using the isolated serum. The remaining 5 ml of serum was transferred to Yakult Central Institute, of which 2 ml of serum was transferred to LSI Medience Corporation (Tokyo, Japan) and the diphtheria antibody titer was measured. The HSV IgG titer was measured at Yakult Central Institute using HSV type 1 IgG enzyme-linked immunosorbent assay (ELISA) kit (Phoenix Pharmaceuticals, Burlingame, CA, USA) according to the manufacturer's instructions included in the kit.

Stool sample preparation

Stool samples were subjected to pretreatment for DNA and RNA extraction according to the method described by Kubota *et al.* (2010). Each sample without RNAlater was weighed and diluted with phosphate buffered saline (PBS) at a ratio of 1:9. After faecal homogenate (100 mg faeces/ml) containing $\phi 5.0$ mm zirconia beads was suspended by ShakeMaster (Biomedical Science, Tokyo, Japan) for 10 min at room temperature, the faecal homogenate was then divided into three new 2 ml tubes (200, 200, 450 μ l). One was washed with PBS and stored at -80 °C until DNA extraction. Again, one sample was mixed with 800 μ l PBS and underwent centrifugation at $13,000 \times g$ for 5 min. The supernatant was then discarded and pellets were stored at -30 °C until being treated with propidium monoazide. The 450 μ l of the remaining faecal homogenate was mixed with 50 μ l of 10% (v/v) perchloric acid and incubated for 12 h at 4 °C for deproteinization. The deproteinized stool diluent was stored at -80 °C prior to measurement of the concentrations of faecal organic acids. On the other hand, the faecal homogenate that was mixed with RNAlater at the time of stool submission was suspended in 9 volumes of RNAlater and mixed with a ShakeMaster for 10 min. Then 200 μ l of the further 5-fold diluted suspension was introduced into a 2 ml tube containing 1 ml of PBS. After centrifugation at $13,000 \times g$ for 5 min, the supernatant was discarded, and the remaining pellet was stored at -80 °C until RNA extraction.

Propidium monoazide treatment

Propidium monoazide (PMA), a DNA-intercalating dye that modifies double-stranded DNA, is highly selective in that it penetrates the cytoplasm of dead bacteria but not that of live bacteria. Quantitative PCR with PMA (PMA-PCR) is utilised as a method for detecting live bacteria based on the PMA properties (Nocker *et al.*, 2006). Fujimoto *et al.* (2011) also reported that only live LcS can be detected selectively by PMA-PCR. 2 µl of 5 nM PMA (Cosmo Bio Co. Ltd., Tokyo, Japan) was added to a 200 µl faecal suspension stored at -30 °C to achieve a final concentration of 50 µM. After being stirred with a Vortex mixer, the faecal suspension was incubated on ice in the dark for 5 min and was exposed to visible light for 10 min by using an LED Crosslinker (Takara Bio Inc., Shiga, Japan) (Fujimoto *et al.*, 2008). PMA-treated samples were stored at -30 °C until the DNA was extracted.

DNA extraction and measurement of total bacterial counts by quantitative PCR

DNA extraction and qPCR were performed according to the method described by Matsuki *et al.* (2004) using an ABI PRISM 7900HT Sequence Detection System (Life Technologies Japan Ltd., Tokyo, Japan). The total bacterial counts were quantified by employing qPCR analysis using primer sets and standard strains according to the method described by Shima *et al.* (2019). The DNA of faecal samples treated with PMA was extracted with the above methods, and live LcS was measured by qPCR as described by Fujimoto *et al.* (2008). The detection limit of the PMA-qPCR was about 1×10^6 cells/g faeces in this study.

Amplification of 16S rRNA gene and sequencing

DNA was prepared for 16S rRNA gene amplicon sequencing according to the Earth Microbiome Project (EMP) protocol (Caporaso *et al.*, 2011, 2012). The concentrations of DNA extracted from stool samples were measured with a NanoDrop 2000c (Thermo Fisher Scientific) and diluted to 10 ng/µl. The V1-V2 region of the 16S rRNA gene in the diluted DNA samples was amplified using the 27Fmod2 forward primer and the 338R reverse primer, which contains Golay barcode and illumine adapter sequences (Nagara *et al.*, 2017). PCR was performed on an ABI 7500 Real-Time PCR System (Thermo Fischer Scientific). The PCR reaction solution (50 µl) contained 2×TB Green Premix ExTaq (Tli RnaseH plus) (Takara Bio Inc.) (25 µl), Nuclease-Free Water (22 µl), 100 nM each primer (1 µl), and template DNA (1 µl). The PCR conditions were an initial 95 °C for 30 s, followed by 95 °C for 5 s, 55 °C for 30 s, and 72 °C for 40 s. In accordance with previous studies (Matsuki *et al.*, 2016; Nagara *et al.*, 2017), PCR was monitored by using the SYBR signal, and stopped before signal saturation

to minimise the bias and erroneous product. The PCR products were purified with an AMPure XP Kit (Beckman Coulter, Brea, CA, USA) and quantified with a Quant-iT PicoGreen dsDNA Kit (Invitrogen, Leek, the Netherlands). The library was constructed by mixing equal amounts of DNA for every sample, and sequenced on a MiSeq System (Illumina, San Diego, CA, USA) using a paired-end 2×250 bp cycle run and MiSeq Reagent Kits v2 (Illumina). As a result, 6,092,422 amplicon sequence reads were obtained (from 32,149 to 123,527 reads per sample).

Processing of 16S rRNA gene sequence data

The amplicon sequence reads were processed using QIIME 2 (ver. 2019.01, <https://qiime2.org/>) (Bolyen *et al.*, 2019). Absolute count data obtained from taxonomic assignment was normalised into relative abundances at the phylum and genus levels using the taxa plugin. The α-diversity of the samples was analysed at a sampling depth of 10,000, allowing the retention of 80 samples. Shannon diversity, observed amplicon sequence variants (ASVs), and Faith's phylogenetic diversity indexes were calculated with QIIME2 using the diversity plugin. The number of gut bacteria at the genus level in each subject was calculated by multiplying the total bacterial count determined by quantitative PCR by the compositional data at the genus level obtained by 16S rRNA gene amplicon analysis.

RNA extraction and the measurement of the number of gut microbiome in the faeces by RT-qPCR

Total RNA was extracted from the samples according to the methods described by Matsuda *et al.* (2007). The extracted RNA samples were stored at -80 °C until the gut microbiome were analysed. The counts of 2 genera (*Prevotella*, *Pseudomonas*) and 2 species (*Clostridium difficile*, *Clostridium perfringens*) were quantified with specific primer sets and total RNA as a template according to the method described by Matsuda *et al.* (2009). A OneStep RT-PCR Kit (Qiagen GmbH, Hilden, Germany) was used for reverse transcription-quantitative PCR. RT-qPCR was performed in a 384-well optical plate on an ABI PRISM 7900HT Sequence Detection System (Thermo Fisher Scientific).

Measurement of HSV antibody titer

HSV type 1 IgG ELISA kit (Phoenix Pharmaceuticals) was used to measure antibody titers against herpes virus type 1 in the serum. Briefly, the ELISA was performed according to the manufacturer's instructions, and the absorbance of each sample measured at OD 520 nm. The antibody titer of each sample was assessed as HSV-1 IgG index by dividing the mean absorbance of two wells by the absorbance of the calibrator. Absorbance was measured using a SpectraMax 190 Microplate Reader (Molecular Devices, Tokyo, Japan),

and the index was considered negative at less than 0.9, pseudo-positive at 0.9 to 1.0, and positive at 1.0 and above. HSV IgM antibody titers, varicella zoster IgM antibody titers and cytomegalovirus IgM antibody titers were sent to BML Inc. for measurement. For each antibody index analysed with an enzyme immunoassay method (Serena *et al.*, 2018), antibody titers below 0.80 were considered negative and those above 0.80 were considered positive. Serum diphtheria IgG antibody titers (IU/ml) were judged positive at 0.01 IU/ml or higher.

Measurement of the concentration of faecal organic acids

The organic acid concentrations in faeces were measured as described by Matsumoto *et al.* (2010). After the deproteinized stool diluent stored at -80 °C was filtered with 0.45 µm Centricut Ultramini (Kurabo Ltd., Osaka, Japan), the filtrate was subjected to high performance liquid chromatography (Waters, Milford, MA, USA) to measure the concentrations of lactate, acetate, propionate, butyrate, succinate, valerate and isovalerate. The total organic acid concentration in the faeces was calculated by adding together the concentrations of each organic acid.

Questionnaire survey

The subjects provided basic information (sex, age, height, and weight), the total number of defecations during the week before stool collection, stool consistency on defecation, defecatory symptoms (straining, feeling of incomplete evacuation, or abdominal pain), and lifestyle habits (smoker or non-smoker, weekly frequency of alcohol use/consumption [0-7], weekly frequency of exercise [0-7], and the weekly frequency of consumption of fermented milk with and without LcS for the previous month [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. The BSFS was evaluated and entered in the questionnaire survey when the 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), severe (score 4). The medication status for one week before the start of the study and the time of stool submission was provided in the questionnaire survey, and if the subjects were taking antibiotics, intestinal regulators, or laxatives during that week, they were excluded from the analysis.

Subgroup analysis

Based on the questionnaire, subjects who ingested LcS-containing fermented milk products 6 times or more per week were categorised as the high-frequency intake group, and subjects who ingested LcS-containing fermented milk products 1 or more and less than 6 times per week were categorised as the low-frequency intake group.

Furthermore, the group for whom live LcS was detected by the PMA-PCR method was categorised as the live LcS detected group, and the group for whom live LcS was not detected was categorised as the live LcS undetected group.

Statistical analysis

All analyses were performed using R ver.3.6.1 software (<https://cran.r-project.org/>) (Ihaka and Gentleman, 1996). The unpaired Student's *t*-test or Mann-Whitney *U*-test were used to assess the statistical significance between the two groups. The bacterial counts of the 'not detected' samples were regarded as half the detection limits (logarithm) of the corresponding primer sets. $P < 0.05$ was considered statistically significant, and $0.05 \leq P < 0.1$ was considered marginally significant.

3. Results

Difference of life habits, gut microbiome, defecatory symptoms and viral antibody indexes between subjects

Age, frequency of FML consumption, and the amount of LcS ingested in the LLD ($n=60$) were significantly higher than those in the LLnD ($n=12$) (Table 1). Gender, height, weight, body mass index, smoking, total energy expenditure through exercise, and sleeping hours were not associated with the detection of Live-LcS in the stool. The weekly frequency of alcohol drinking tended to be lower in the LLD than in the LLnD ($P=0.073$) (Table 1). The calorie intake and the amount of carbohydrates in the LLD were significantly lower than those in the LLnD from an analysis of dietary habits (calorie intake, $P=0.035$, amount of carbohydrate, $P=0.016$) (Table 1). The β -diversity index of the gut microbiome was a statistically significant difference between LLD and LLnD (Bray-Curtis, $P=0.023$; Jaccard, $P=0.009$) (Supplementary Figure S1A,B). Faith's PD and observed ASVs as the α -diversity indicators were significantly higher in the LLD than in the LLnD (Faith's PD, $P=0.021$; observed ASVs, $P=0.020$). (Figure 1A,B). The *Lactobacillaceae*, *Bifidobacteriaceae* and *Coriobacteriaceae* counts in the LLD were significantly higher than those in the LLnD (*Lactobacillaceae*, $P<0.001$; *Bifidobacteriaceae*, $P=0.009$; *Coriobacteriaceae*, $P=0.044$) (Figure 1C). Further, the total bacterial counts and the *Rikenellaceae* count tended to be higher in the LLD than in the LLnD (total bacterial count, $P=0.088$; *Rikenellaceae*, $P=0.062$), conversely, the *Veillonellaceae* count tended to be lower in the LLD ($P=0.089$) (Figure 1C). The *Prevotella*, *Clostridium difficile*, *Clostridium perfringens*, and *Pseudomonas* counts were not associated with the detection of Live LcS in the stool (Supplementary Table S2). The concentration of succinate was significantly lower in the LLnD than in the LLD ($P=0.039$) (Figure 1D), and conversely the concentrations of butyrate and valerate tended to be higher (butyrate: $P=0.073$, valerate:

$P=0.092$) than in the LLD (Figure 1E,F). No significant differences were observed between the concentrations of total organic acid, acetate, propionate, lactate, isovalerate, and formate in the two groups (Supplementary Table S2). Serum HSV IgG antibody index was not associated with the detection of Live-LcS in the stool (Supplementary Table S2), but HSV IgM antibody index was significantly lower in the LLD than in the LLnD ($P=0.01$) (Figure 2A). Furthermore, the HSV IgM antibody index in HSV IgG-positive subjects tended to be lower in the LLD ($n=28$) than in the LLnD ($P=0.063$) (Figure 2B). The feeling of incomplete evacuation tended to be lower in the LLD than in the LLnD ($P=0.089$) (Figure 3). No association was found between the number of defecations per week, the interval of defecation, stool consistency, straining at defecation and abdominal pain and Live-LcS detection (Supplementary Table S2).

Impact of life habits, gut microbiome, defecatory symptoms, and viral antibody index in LLD subjects

To investigate the impact of the frequency of consumption of FML on the gut microbiome and immunoglobulin index against various viruses on the host, various parameters in the Live-LcS-detected subjects ($n=60$) were compared for the subjects who consumed FML at low ($n=13$) and

high frequencies ($n=47$) (Supplementary Figure S2). The amount of ingested LcS per day and the frequency of the consumption of FML were significantly higher in the subjects who consumed FML at a high frequency (FML-HF) than in the subjects who consumed FML at a low frequency (FML-LF) ($P<0.001$) (Table 2). The age, gender, height, weight, Live-LcS counts in the stool, smoking status, frequency of alcohol use/consumption, and hours of sleep per day were not associated with the frequency of consumption of fermented milk products containing LcS (Table 2). The amount of carbohydrate intake per day tended to be higher in the FML-HF than in the low-frequency intake group ($P=0.074$). No differences between the calorie, protein and fat intake per day were found for the FML-HF and FML-LF (Table 2). The total bacterial counts, *Alcaligenaceae*, *Clostridiaceae*, *Lachnospiraceae*, and *Ruminococcaceae* were significantly lower in the high-frequency intake group than in the low-frequency intake group (total bacterial counts, $P=0.029$; *Alcaligenaceae*, $P=0.024$; *Clostridiaceae*, $P=0.037$; *Lachnospiraceae*, $P=0.006$; *Ruminococcaceae*, $P=0.038$) and *Streptococcaceae* in the high-frequency intake group tended to be lower than those in the low-frequency intake group ($P=0.069$) (Figure 4). The *Lactobacillaceae* counts were significantly higher in the high-frequency intake group than in the low-frequency intake group ($P=0.031$). *Prevotella*, *C. difficile*,

Table 1. Comparison of background, life habits and dietary habits live-LcS-detected group (LLD) and live-LcS-not detected group (LLnD).¹

Parameter	LLnD	LLD	P-value
Background			
Number of subjects (n)	12	60	
Age (years)	33.58±6.78	39.23±9.19	0.048
Gender (male/female)	9/3	43/17	1.000
Height (cm)	166.62±9.03	167.47±8.62	0.758
Weight (kg)	66.17±10.45	65.36±14.03	0.851
BMI	23.76±2.70	23.13±3.66	0.576
Frequency of consumption of fermented milk products containing LcS (times/week)	2.50 [0.00, 12.50]	12.50 [1.50, 21.00]	<0.001
Live LcS dose ($\times 10^8$ cfu/day)	127.42±154.69	415.37±257.63	<0.001
Life habits			
Smoking status (yes/no)	2/10	9/51	1.000
Total energy expenditure by exercise (kcal/month)	1,775.1±1,397.5	3,758.3±6,134.5	0.272
Frequency of alcohol use/consumption (times/week)	4.5±2.6	2.9±2.7	0.073
Sleeping time (h/day)	6.0±0.9	6.0±0.9	0.976
Dietary habits			
Calorie intake (kcal/day)	2,244.8±197.6	2,087.8±236.4	0.035
Carbohydrate (g/day)	300.2±42.4	271.3±36.0	0.016
Protein (g/day)	86.9±4.9	83.0±8.6	0.134
Lipid (g/day)	64.9±3.9	62.6±4.8	0.126

¹ Values for age, height, weight, body mass index (BMI), life habits and diet habits are mean ± standard deviation; P-value was calculated by Student's *t*-test, $P<0.05$. Values for frequency are median [minimum, maximum]; P-value was calculated by Mann-Whitney *U*-test, $P<0.05$. Fisher's exact test was used to assess the statistical analysis for gender and smoking status.

Figure 1. Comparison of gut microbiome and organic acid profiles in subjects between with and without live-LcS detection. (A) Faith's PD index as α -diversity, (B) observed ASVs as α -diversity, (C) gut microbiome, (D) succinate, (E) butyrate, (F) valerate. Box plots showing differences between LLnD and LLD (A-C). Mann-Whitney U -test was used for statistical analysis of (A-C). Student's t -test was used for statistical analysis of (D-F) with $P < 0.1$. Total number of subjects $n=72$, LLD = live-LcS-detected group ($n=60$), LLnD = live-LcS-not detected group ($n=12$). The difference between gut microbiome and organic acid profiles in LLnD and LLD with $P < 0.1$ are shown.

C. perfringens, and *Pseudomonas* were not associated with the frequency of consumption of FML (Supplementary Table S3). Furthermore, there was no association between the FML intake frequency and the number of defecations per week, intervals between defecation, stool consistency, feeling of incomplete evacuation, straining at defecation and abdominal pain (Supplementary Table S3). Serum HSV IgG and IgM antibody index in HSV IgG-positive subjects, and the concentration of each organic acid in the stool were not associated with the FML intake frequency (Supplementary Table S3).

Association between detection of live-LcS in the stool, life habits, gut microbiome, defecatory symptoms and viral antibody index in LF-FML subjects

Two subgroups were set up to exclude interference from the frequency of intake of FML: the LF-LLD ($n=13$) and the LF-LLnD ($n=6$) subgroups containing subjects with a low frequency of FML intake (<6 times per week, $n=19$). Several biological parameters, such as gut microbiome, concentration of organic acids, viral antibody index and life habits were compared in both groups. The Live-LcS count in the stool and the daily calorie and carbohydrate intake in the LF-LLD subgroup were significantly higher than those in the LF-LLnD subgroup ($P=0.007$ and $P=0.001$) (Table 3).

Figure 2. Comparison of serum herpes simplex virus (HSV) antibody index in subjects between with and without live-LcS detection. (A) HSV IgM antibody index, (B) HSV IgM antibody index in HSV IgG positive subjects (total subjects (n=28), LLD = live-LcS-detected group (n=23), LLnD = live-LcS-not detected group (n=5)). Box plots showing differences between LLnD and LLD. Mann-Whitney *U*-test was used for statistical analysis of (A) and (B) with $P<0.1$.

Figure 3. Comparison of defecatory symptoms in subjects between with and without live-LcS detection. Total number of subjects (n=72), LLD = Live-LcS-detected group (n=60), LLnD = Live-LcS-not detected group (n=12). Box plots showing differences between LLnD and LLD. Student's *t*-test was used for statistical analysis with $P<0.1$.

Table 2 Comparison of background, life habits and dietary habits between low and high frequency consumption groups of fermented milk products containing LcS (FML-LF and FML-HF).¹

Parameter	FML-LF	FML-HF	P-value
General information			
Number of Live LcS detected subjects in each group (n)	13	47	
Number of Live LcS-not detected subjects in each group (n)	12	3	
Age (years)	39.8±9.7	39.1±9.2	0.815
Gender (male/female)	9/4	34/13	1.000
Height (cm)	167.3±8.4	167.5±8.8	0.930
Weight (kg)	60.6±10.8	66.7±14.6	0.170
BMI	21.5±2.3	23.6±3.9	0.069
Frequency of consumption of fermented milk products containing LcS (times/week)	3.50 [1.50, 5.00]	14.00 [11.00, 21.00]	<0.001
Live LcS dose (×10 ⁸ cfu/day)	121.5±116.4	496.6±224.6	<0.001
Life habits			
Smoking status (yes/no)	1/12	8/39	0.668
Total energy expenditure by exercise (kcal/month)	6,804.7±9,917.2	2,915.6±4,388.8	0.272
Frequency of alcohol use/consumption (times/week)	3.2±2.8	2.9±2.7	0.073
Sleeping time (h/day)	6.0±0.8	6.0±0.9	0.976
Dietary habits			
Calorie intake (kcal/day)	2,014.4±203.0	2,108.1±242.8	0.208
Carbohydrate (g/day)	255.6±19.6	275.7±38.4	0.074
Protein (g/day)	81.9±8.2	83.3±8.8	0.598
Lipid (g/day)	62.6±5.1	62.6±4.7	0.995

¹ Values for age, height, weight, body mass index (BMI), life habits and diet habits are mean ± standard deviation; *P*-value was calculated by Student's *t*-test, $P<0.05$. Values for frequency are median [minimum, maximum]; *P*-value was calculated by Mann-Whitney *U*-test, $P<0.05$. Fisher's exact test was used to assess the statistical analysis for gender and smoking status.

Figure 4. Difference between gut microbiome with low and high frequency consumption of fermented milk products containing LcS in subjects with LcS detected in their stools (FML-LF and FML-HF). Live-LcS detected subjects (n=60), FML-LF (n=13), FML-HF (n=47). The faecal bacterial counts (total bacterial counts, *Alcaligenaceae*, *Clostridiaceae*, *Lachnospiraceae*, *Lactobacillaceae*, *Ruminococcaceae*, *Streptococcaceae*) for FML-LF and FML-HF are shown by box plots with $P < 0.1$. The Mann-Whitney *U*-test was used for statistical analysis.

Table 3. Comparison of background, life habits and dietary habits between low frequency consumers of LcS fermented milk live-LcS-detected (LF-LLD) and live-LcS-not detected (LF-LLnD) groups.¹

Parameter	LF-LLnD	LF-LLD	P-value
General information			
Number of subjects (n)	6	13	
Age (years)	37.3±6.9	39.8±9.7	0.588
Gender (male/female)	5/1	9/4	1.000
Height (cm)	168.1±8.8	167.3±8.4	0.851
Weight (kg)	67.3±11.9	60.6±10.8	0.237
BMI	23.8±3.5	21.5±2.3	0.105
Frequency of consumption of fermented milk products containing LcS (times/week)	2.50 [1.50, 3.50]	3.50 [1.50, 5.00]	0.138
Live LcS dose (×10 ⁸ cfu/day)	89.5±76.3	121.5±116.4	0.550
Life habits			
Smoking status (yes/no)	1/5	1/12	1.000
Total energy expenditure by exercise (kcal/month)	1,831.0±1,320.8	6,804.7±9,917.2	0.153
Frequency of alcohol use/consumption (times/week)	4.8±2.6	3.2±2.8	0.198
Sleeping time (h/day)	5.9±1.1	6.0±0.8	0.893
Dietary habits			
Calorie intake (kcal/day)	2,281.1±207.6	2,014.4±203.0	0.007
Carbohydrate (g/day)	308.7±46.0	255.6±19.6	0.001
Protein (g/day)	87.3±5.3	81.9±8.2	0.096
Lipid (g/day)	65.2±4.0	62.6±5.1	0.223

¹ Values for age, height, weight, body mass index (BMI), life habits and diet habits are mean ± standard deviation; *P*-value was calculated by Student's *t*-test, $P < 0.05$. Values for frequency are median [minimum, maximum]; *P*-value was calculated by Mann-Whitney *U*-test, $P < 0.05$. Fisher's exact test was used to assess the statistical analysis for gender and smoking status.

Age, gender, height, weight, BMI, smoking status, total energy expended through exercise, frequency of alcohol drinking, and sleeping hours were not associated with the Live-LcS detection in the stool among low-frequency consumers of FML (n=19) (Table 3). Faith's PD as the α -diversity indicators tended to be higher in the LF-LLD than in the LF-LLnD (Faith's PD, $P=0.096$) (Figure 5A). The total bacterial counts, and the *Streptococcaceae* and *Lactobacillaceae* counts in the stool in the LF-LLD were significantly higher than those in the LF-LLnD (total bacteria, $P=0.035$; *Streptococcaceae*, $P=0.018$; *Lactobacillaceae*, $P=0.028$) (Figure 5B). No significant association was found between Live-LcS detection and *Prevotella*, *C. difficile*, *C. perfringens* and *Pseudomonas* in the subjects who were low-frequency consumers of FML (Supplementary Table S4). The number of defecations per week, the defecation interval, stool consistency, straining at defecation, feeling of incomplete evacuation, and abdominal pain were not associated with Live-LcS detection (Supplementary Table S4) in the subjects who were low-frequency consumers of FML. In addition, serum HSV IgG and IgM antibody, varicella zoster IgG antibody index, diphtheria IgG antibody and cytomegalovirus IgG antibody were not associated with Live-LcS detection in the subjects who were low-frequency consumers of FML (Supplementary Table S4). The concentration of succinic acid tended to be higher in LF-LLD than in LF-LLnD ($P=0.062$) (Figure 5C), but the other species of organic acids were not associated with Live-LcS detection in the subjects who were low-frequency consumers of FML (Supplementary Table S4).

4. Discussion

In this study, we have estimated the effects of LcS that reached the lower gastrointestinal tract alive on intestinal microbiome, organic acid profiles, defecatory symptoms,

and viral antibody indexes in healthy Japanese adults. Our results may provide evidence that live LcS reaching the lower intestinal tract can influence the gut microbiome and viral reactivation in the host.

Characteristic of gut microbiome and organic acid profiles in LLD

In this study we found higher α -diversity of the gut microbiome in the LLD than in the LLnD (Figure 1). Moreover, the *Bifidobacteriaceae* and *Coriobacteriaceae* counts in the LLD were significantly higher than in the LLnD (Figure 1). Kato-Kataoka *et al.* (2016) reported that consumption of FML (1×10^{11} cfu/day, 8 weeks) inhibited the stress-induced reduction in α -diversity of the gut microbiome in a placebo-controlled double-blind study in healthy Japanese adults. An increase in the *Bifidobacterium* count has been reported in subjects with a wide range of ages (Bian *et al.*, 2011; Nagata *et al.*, 2011; Wang *et al.*, 2015). These results indicate that the sum of the counts of dead and live LcS determined by qPCR is associated with the *Bifidobacterium* count. Interestingly, in this study, the live LcS count measured by PMA-qPCR was also found to be associated with the indigenous bifidobacterial count. In the future, we would like to try to elucidate the mechanism by which live LcS affects the *Bifidobacteriaceae* count. It has also been reported that the counts of *Atopobium* clusters, which is a major *Coriobacteriaceae* group in the human faecal microbiome, is higher in subjects who consume FML more frequently (Aoyagi *et al.*, 2019). Therefore, it is possible that the presence of live LcS in lower gastrointestinal tracts has the ability to increase the α -diversity of the gut microbiome and several genera of gut microbiome, such as *Bifidobacteriaceae* and *Coriobacteriaceae* in the intestine.

Figure 5. Comparison of gut microbiome and organic acid profiles in subjects with and without live LcS-detected in their stools with a low-frequency consumption of fermented milk products containing LcS. The subjects drank fermented milk products containing LcS low frequency (n=19), LF-LLD = Live-LcS-detected group (n=13), LF-LLnD = Live-LcS-not detected group (n=6). (A) Faith's PD, (B) Total bacteria counts, *Lactobacillaceae*, *Streptococcaceae*. The differences between the gut microbiome (A, B) of LF-LLnD and LF-LLD are shown by box plots with $P<0.1$. (C) Succinate is shown by bar charts with $P<0.1$. A Mann-Whitney U-test was used for the statistical analysis of (A) and (B). A Student's *t*-test was used for the statistical analysis of (C).

The concentrations of the major organic acids in the LLD and LLnD were comparable, however, the concentration of succinate was significantly lower in the LLD than in the LLnD ($P=0.039$) (Figure 1D-F). The counts of *Veillonellaceae*, which are succinate-producing bacteria (Serena *et al.*, 2018), was also lower in the LLD than in the LLnD (Figure 1C). Accordingly, the lower *Veillonellaceae* counts in the LLD may contribute to the lower succinate concentration in the intestine. It has been reported that high concentrations of succinate are associated with the dysbiosis of gut microbiome, associated with inflammatory bowel disease (IBD), and to induce hypoxia-inducible factor 1 α in host immune cells, which promotes inflammation (Connors *et al.*, 2018; Tannahill *et al.*, 2013). The causal relationship between *Veillonellaceae* and the dysbiosis of the gut microbiome in healthy individuals is unclear. However, the *Veillonellaceae* count, as in IBD patients, may be one of indicators allowing us to assess gut dysbiosis in healthy people. Furthermore, it was reported that the *Veillonella* count was high in the patients who experienced Crohn's disease complications, children with a high risk of asthma, and children with food allergies (Inoue *et al.*, 2017; Kugathasan *et al.*, 2017; Stokholm *et al.*, 2018). Therefore, the association between gut *Veillonella* and diseases involving immune abnormalities is very interesting. Further investigations are required into the causal associations between the high colonisation of *Veillonellaceae* and gut dysbiosis.

From a bird's eye view, the differences in the family level gut bacterial count and faecal organic acid concentration observed when we compared LF-LLnD and LF-LLD showed a very similar pattern to that observed when we compared LLnD and LLD (Supplementary Figure S3). On the other hand, the differences in the patterns of gut microbiome and organic acid profiles observed in the comparison of FML-HF and FML-LF were very different from those observed in the comparison of LLnD and LLD (Supplementary Figure S3). These results suggest that the characteristics of the gut microbiome and organic acid profiles in LLD (Figure 1) are not directly influenced by the high intake of FML but are characteristic of subjects for whom live LcS is detected in the faeces.

The faecal *Lactobacillaceae* counts were dependent on the frequency of intake of FML (Supplementary Figure S4). Furthermore, the live LcS count in the faeces was significantly positively correlated with the *Lactobacillaceae* count in the faeces (Spearman's rank correlation coefficient = 0.624; $P<0.001$, Supplementary Figure S5). This result suggests that the *Lactobacillaceae* count is strongly dependent on the frequency of FML intake. This result supported the findings described in our previous report (Shima *et al.*, 2019).

Impact on serum viral antibody index of live LcS

It is known that HSV infection results in a persistently high concentration of HSV specific IgG in the blood and HSV specific IgM increase when persistently latent HSV is reactivated in the host (Whitley and Roizman, 2001). In HSV infection experiments using T cell defective mice such as nude mice and SCID mice, it has been reported that cellular immunity via T cells is essential for the elimination of HSV-infected cells (Minagawa and Yanagi, 2000), and an increase in the counts of CD8-positive T cells invading the ganglia inhibits the reactivation of latently infected HSV (Hoshino *et al.*, 2007; Khanna *et al.*, 2003; Liu *et al.*, 2001), suggesting that the immune response in the host may play an important role in maintaining latent HSV infection. LcS has been reported to activate innate immunological effects and T cells in a live LcS-specific manner (Dong *et al.*, 2010; Salas-Lais *et al.*, 2020).

Therefore, in the subjects of this study where HSV IgG was detected in the blood, cellular immunity activated by live LcS may be involved in that the HSV IgM antibody index in the LLD showed a trend lower than those in the LLnD (Figure 2). Although in this study the impact of the HSV antibody titer suggested a characteristic of live LcS, it was already reported that the ingestion of FML has an effect on the cytomegalovirus IgG antibody titer (Gleeson *et al.*, 2016) and the prevention of early infection with influenza A virus (Jung *et al.*, 2017). It has also been suggested that low cytomegalovirus IgG antibody titers and HSV IgG antibody titers may help to maintain a higher level of immune capacity in the host (Gleeson *et al.*, 2016). Therefore, these results raise the possibility that live LcS reaching the gut alive could help to enhance protection against infection by pathogenic viruses and bacteria.

Factors affecting live LcS survive in lower gastrointestinal tracts

In healthy Singaporeans ($n=21$) and Australians ($n=25$), live LcS has been detected by culture methods in all subjects after 2 weeks of LcS intake ($1 \times 10^{10-11}$ cfu/day) (Cox *et al.*, 2019; Khine *et al.*, 2019). In contrast, live LcS was not detected in 3 of 50 subjects in the FML-HF in this study (Table 2). Of these three subjects, *Clostridiaceae* and *Rikenellaceae*, which are generally predominant intestinal bacteria, were not detected in one subject. In the remaining two subjects, the total bacterial counts were very low (approximately 1×10^9 cells/g stool), suggesting that all three subjects were suffering from dysbiosis of the gut microbiome. The calorie and carbohydrate intake of the LLD was significantly lower than that of the LLnD (Table 1). In addition, the calorie and carbohydrate intakes were lower in the group in which live LcS was detected than in LF-LLD and LF-LLnD (Table 3), suggesting a relationship between the detection of live LcS and nutrition. However,

the causal relationship among these factors is not clear, and further investigation is needed.

Limitations

This study had several limitations. First, the number of subjects was small and the power was insufficient to perform subgroup analysis. The large difference between the number of subjects and the ratio of males to females for LLD and LLnD may have affected the results. The results obtained in this study may be considered as a preliminary observation. Second, we obtained the gut bacteria count by multiplying the total bacterial count calculated with quantitative PCR by the bacterial composition data at the family level obtained from 16S rRNA gene amplicon analysis. The obtained gut bacteria count may be estimated at less or more than the actual bacteria count, because the copy number of the 16S ribosomal RNA gene differs among bacterial species. However, our method provided quantitative data on intestinal bacteria that could not be obtained with the 16S-ribosomal RNA gene amplicon analysis. Finally, in this study, membrane permeability was used as an indicator to distinguish between viable and dead bacteria, but since there are many indicators with which to define viable bacteria, such as culture, residual RNA and enzyme activity, the results may differ depending on which indicator is used.

5. Conclusions

The present study has shown that live LcS, one of the most popular probiotics, can reach the lower gastrointestinal tract alive and may affect the gut microbiome, organic acid profiles and inhibit viral reactivation in the host. Moreover, it was shown that the survival of live LcS in the gastrointestinal tract was related to the gut microbiome and diet. These results not only characterise the impacts of live LcS but will also help to advance research on the maintenance and promotion of health by using the characteristics of LcS.

Supplementary material

Supplementary material can be found online at <https://doi.org/10.3920/BM2021.0101>.

Figure S1. Bray-Curtis index and Jaccard index as β -diversity by PCoA clustering analysis of LLnD and LLD characteristics.

Figure S2. Frequency histogram of intake of FML intake of FML-LF and FML-HF in live LcS detected subjects.

Figure S3. Heat map for gut microbiome for LLD and LLnD, LF-LLD and LF-LLnD, or FML-LF and FML-HF.

Figure S4. Correlation between the frequency of consumption of LcS containing fermented milk products and faecal *Lactobacillaceae* counts by 16S rRNA genes amplified products analysis.

Figure S5. Correlation between the faecal LcS counts by the PMA-PCR and faecal *Lactobacillaceae* counts by 16S rRNA genes amplified products analysis.

Table S1. Exclusion criteria for subject recruitment.

Table S2. Comparison of gut microbiome, organic acids profiles, stool frequency, defecatory symptoms and viral antibody indexes in subjects between with and without live LcS detected in stool.

Table S3. Effect of frequency of FML consumption on gut microbiome, organic acids profiles, stool frequency, defecatory symptoms and viral antibody indexes in subjects with live LcS detected in stool.

Table S4. Effect of live LcS detection in stool on gut microbiome, organic acids profiles, stool frequency, defecatory symptoms and viral antibody indexes among subjects with low FML intake.

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Conflict of interest

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