

Probiotics modulate gut microbiota and health status in Japanese cedar pollinosis patients during the pollen season

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Abstract

Background Japanese cedar pollinosis (JCP) is a challenging public health problem in Japan. Altered gut microbiota is associated with several diseases, including allergic diseases. However, only a few studies have focused on JCP and the underlying mechanisms for probiotic effects remain unclear. In addition, this study is the first observation of the correlation between the gut microbiota and blood lipid in JCP.

Methods Faecal samples from JCP subjects were collected before and after treatment with ($n = 14$) and without ($n = 11$) LGG–TMC0356-fermented milk for 10 weeks. Gut microbiota composition was characterized from faecal DNA using sequencing of 16S rRNA genes.

Results 16S rRNA-based operational taxonomic unit clustering of the microbiota revealed that LGG–TMC0356-fermented milk significantly altered gut microbiota after 10 weeks of milk consumption, and eight dominant genera of microbes were detected. During the JCP season, the *Bacteroidetes*/*Firmicutes* ratio, when compared to baseline, was significantly decreased in subjects at end of the study. *Bacteroidetes* showed positive correlation with LDL- and HDL-cholesterol levels, whereas *Firmicutes* showed negative correlation with total cholesterol, LDL- and HDL- cholesterol.

Conclusions The altered gut microbiota through supplementation of fermented milk containing the study probiotics may be a prospective target for protection against JCP, with beneficial effects on blood lipid levels.

Keywords JCP · Gut microbiota · Blood lipid · *Lactobacillus rhamnosus* GG · *Lactobacillus gasseri* TMC0356 · Probiotic · Allergy

Introduction

Allergic rhinitis and asthma are common chronic conditions worldwide. The World Allergy Organization [1] reports that the prevalence of allergic diseases in infants whose parents and siblings do not have allergies is approximately 10 % and reaches 20–30 % in those with an allergic first-degree relative. Allergic rhinitis is an especially serious challenge in Japan where the Japanese cedar pollen allergy has become common. The main symptoms of Japanese cedar pollen allergy include sneezing, rhinitis, nasal blockage and itching eyes with conjunctivitis symptoms. More than 16 % of the Japanese population has been estimated to be positive for the IgE antibody specific to Japanese cedar pollen. However, the percentage is higher at about 25 % in 30–49-year-old Japanese subjects, who constitute the greater part of the working force [2]. Although Japanese cedar pollen allergy and allergic rhinitis, which are caused by exposure to environmental allergens, are seasonal, even outside the pollen season allergic rhinitis is frequently accompanied by allergic conjunctivitis. Intestinal microbiota alterations have been reported to precede allergic disease development, and subjects with allergic diseases have shown differences in microbiota composition and activity [3–5].

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Probiotics, which are ‘live microorganisms, which when administered in adequate amounts, confer a health benefit on the host’, have been reported to modulate both intestinal microbiota and immune responses, and their supplementation has been proposed as a preventive intervention [6]. Probiotics are also endorsed by the World Allergy Organization, which recommends probiotic supplementation for the prevention of allergies to support families, parents and health care professionals in their decisions regarding perinatal (during pregnancy, breastfeeding, and infant feeding) use of probiotics [1]. Earlier studies have demonstrated that *Lactobacillus rhamnosus* GG (LGG) has a preventive effect on atopic eczema development in high-risk infants [3, 7]. Studies of birch pollen allergy suggested the reactions to be associated with changes in faecal microbiota composition. Specific probiotics were shown to prevent the pollen-induced infiltration of eosinophils into the nasal mucosa and indicated a trend for reduced nasal symptoms. A Japanese double-blind, placebo-controlled study using milk fermented with LGG and *L. gasseri* TMC0356 (TMC0356) reported alleviation of seasonal allergic rhinitis caused by Japanese cedar pollen [8].

Recently, several studies have been conducted to focus the possible association between metabolic abnormalities and allergic disease because both of them have the commonalities in inflammatory disorders [9–11]. For example, hypercholesterolemia has been associated a skewing of adaptive immune system towards a Th2-oriented response, which could affect the risk of diseases, such as asthma [9–11]. These lead a hypothesis that the abnormal lipid metabolism caused by intestine microbiota could be also one more pathway by which intestinal microbiota influences the allergic disease including JCP.

Therefore, this study assessed the effects of the probiotic combination on faecal microbiota composition in detail to uncover the potential preventive role of probiotics against

cedar pollen rhinitis. In addition, we assessed whether serum lipid deviations associated with cedar pollen allergy could be modified through probiotic treatment.

Materials and methods

Study design, subjects and samples

A double-blind, randomized, placebo-controlled trial was conducted to ascertain the intestinal microbiota-altering properties of *Lactobacillus rhamnosus* GG (ATCC53103) and *L. gasseri* TMC0356 (TMC0356) in Japanese cedar *Cryptomeria japonica* pollinosis (JCP) patients, as described previously [8]. All procedures in the study were in complete accordance with the Helsinki declaration and were informed about the nature of study and were asked to provide written informed consent. They were reviewed and approved by the Shinagawa East One Medical Clinic Ethics Committee. Briefly, subjects had mild symptoms of JCP and tested positive for IgE against JCP and subjects who had milk allergies or perennial allergic rhinitis, or who were receiving specific immunotherapy or any drug treatment, were excluded. The fermented milk prepared with LGG and TMC0356 was administered orally for 10 weeks to JCP patients. The numbers of LGG and TMC0356 in the fermented milk were more than 1.4×10^8 and 1×10^7 cfu/ml, respectively. The placebo was prepared by adding lactic acid to pasteurized milk, and there were no differences between the tested fermented milk and placebo milk at packaging in terms of appearance and taste. The subjects were randomly divided into two groups and were given 110 g/day of the test fermented milk or placebo for 10 weeks. The patient monitoring, medical examination and blood analysis were conducted as described previously [8] and subjects information in Table 1. Faecal

Table 1 Subjects characteristics, the lipids and IgE levels

	Test		Placebo	
	Baseline	10 weeks	Baseline	10 weeks
Sex (female/male)	9/5	9/5	7/4	7/4
Age (years)	36.9 ± 6.9	36.9 ± 6.9	36.5 ± 6.1	36.5 ± 6.1
Creatinine, mg/dL	0.7 ± 0.2	0.7 ± 0.2	0.6 ± 0.1	0.7 ± 0.2
Uric acid, mg/dL	5.1 ± 1.7	4.8 ± 1.7	4.8 ± 1.8	4.4 ± 1.6
Total cholesterol, mg/dL	194.0 ± 30.1	191.8 ± 26.7	188.3 ± 20.9	193.4 ± 27.1
LDL-cholesterol, mg/dL	109.4 ± 28.4	104.6 ± 24.9	110.0 ± 19.3	107.2 ± 19.9
HDL-cholesterol, mg/dL	74.2 ± 15.1	71.7 ± 15.6	62.2 ± 15.0	65.5 ± 18.9
Triglyceride, mg/dL	75.9 ± 39.7	80.1 ± 48.2	115.1 ± 77.9	127.1 ± 72.4
Total IgE, IU/ml	102.7 ± 116.2	130.6 ± 174.7	133.6 ± 146.9	158.4 ± 194.8
Anti-Japanese cedar pollen IgE, UA/ml	19.2 ± 19.7	25.5 ± 36.5	21.5 ± 22.5	25.3 ± 32.5

Data are given as mean ± SE (test, $n = 14$, placebo, $n = 11$)

samples from the test group ($n = 14$) and the placebo group ($n = 11$) were collected before and after administration at 0 and 10 weeks. The stool samples were stored at $-80\text{ }^{\circ}\text{C}$ until DNA extraction.

DNA extraction and Illumina library generation

DNA extraction from 200 mg of stool samples was carried out using ISOFEAL for Beads Beating kit (Nippon Gene Co., Ltd., Japan) and Mini-Beadbeater (Biospec Products, Inc., USA), based on the manufacturers' instructions. The DNA concentration and quality of purified DNA were analysed with a QUBit fluorometer (Life Technologies) and TapeStation (Agilent). 50 ng of DNA was used for PCR on a Veriti Dx 96-well Thermal Cyclor (Life Technologies). The V4-V5 region of the 16S rRNA gene was amplified using 515F and 926R fusion primers containing identification indices as well as sequences required for Illumina sequencing [12]. Each reaction mixture contained 0.3 μM of each primer, a 0.3-mM concentration of each deoxynucleoside triphosphate (dNTP), and 0.5 U KAPA HiFi DNA polymerase (KAPA Biosystems,). The optimized PCR conditions comprised an initial denaturation step at $98\text{ }^{\circ}\text{C}$ for 4 min, followed by 25 cycles of $98\text{ }^{\circ}\text{C}$ for 20 s, $65\text{ }^{\circ}\text{C}$ for 20 s, and $72\text{ }^{\circ}\text{C}$ for 35 s, and ended with an extension step at $72\text{ }^{\circ}\text{C}$ for 10 min. PCR products were purified with Agencourt AMPure XP beads (Bechman Coulter). DNA concentration and size distribution of ready libraries were analysed with QUBit fluorometer and TapeStation. Libraries with unique combinations of 8 bp-long index sequences were mixed in equimolar quantities. The sequencing was carried out at Turku Clinical Sequencing laboratory. The libraries were clonally amplified on a flow cell surface. The library clusters were sequenced with MiSeq benchtop sequencer (Illumina) using v3 chemistry ($2 \times 300\text{ bp}$). Image analysis, base calling, and error estimation were performed using Illumina Analysis Pipeline (v 2.6). For microbial sequence analysis, the low-quality sequences were filtered and chimeric sequences were removed by UCHIME software. The QIIME (v 1.8) pipeline was used with default parameters for identifying representative sequences for each operational taxonomic unit (OTU) generated from complete linkage clustering with a 97 % similarity and aligned to the GreenGenes 13_8 database. OTU tables with percent relative abundances were further processed at different taxonomic levels.

Quantitative PCR

Quantitative PCRs were conducted as previously described [13–15]. PCR amplification and detection were performed with an ABI PRISM 7300-PCR sequence detection system (Applied Biosystems, Foster City, CA). The bacterial

concentration in each sample was determined by comparing the Ct values obtained from standard curves. The standard curves were formed from serial dilutions of DNA extracted from known amounts of LGG, *L. casei*, *L. plantarum*, *L. acidophilus*, *S. aureus nuc* and *A. muciniphila* cells.

Statistical analysis

Results are presented as means $\pm$ standard errors. The Mann–Whitney *U* test was used for the comparison of differences between groups, and the paired Student's *t* test or the Wilcoxon signed-rank test was used for the measurements taken before and after treatment. Correlation analyses between bacterial relative abundance and blood biochemical measures were analysed by Spearman's rank correlation coefficient. Differences with a *p*-value below 0.05 were considered statistically significant.

Results

Impact of fermented milk on microbial composition

The intake of study fermented milk had a significant impact on microbiota composition. The evenness and richness of gut microbiota of the tested subjects as determined by the Shannon, Chao and Simpson Index suggested that Shannon, Chao and Simpson Index significantly increased in a test group after intervention (10 weeks) ($P = 0.018$, $P = 0.007$ and $P = 0.047$, respectively), while no significant changes were observed in the placebo group during intervention ($P = 0.92$, $P = 0.92$ and $P = 0.53$, respectively) (Table 2). The increase/decrease ratio of intestinal microbes during intervention was compared between groups. The Chao Index also showed an increase in the test group compared to the placebo group ($P = 0.013$), although such changes were not statistically significantly using the Shannon Index ($P = 0.06$) and the Simpson Index ($P = 0.47$). No significant difference was found in the composition of the intestinal microbes at phylum and genus level between the test group and the placebo group before intervention (Table 3). However, apparent changes were observed in several groups of intestinal microbes in the test group. At phylum level, *Firmicutes* were significantly increased ($P < 0.001$), while *Bacteroidetes* were significantly decreased in the test group after administration of probiotic fermented milk ($P < 0.001$). *Actinobacteria* were also slightly increased ($P = 0.074$). Among genera, significant decreases in *Bacteroides* ($P < 0.001$), *Parabacteroides* ($P = 0.004$), *Prevotella* ($P = 0.008$) and *Oscillospira* ($P = 0.006$) and increases in *Collinsella* ($P = 0.046$), *Lactobacillus* ($P < 0.001$), *Blautia* ($P = 0.003$) and *Ruminococcus* ($P = 0.006$) were detected after intervention. The increase/decrease ratio of intestinal

Table 2 Diversity metrics of gut microbiota

	Test		Placebo		Test	Placebo	<i>P</i> value a	<i>P</i> value b	<i>P</i> value c
	Before	After	Before	After	After–before	After–before			
Shannon	5.42 (3.91–6.21)	5.88 (4.83–6.47)	5.36 (3.21–6.07)	5.20 (3.52–6.32)	0.43 (–0.215–1.544)	0.083 (–0.571–1.063)	0.018	0.929	0.062
Chao	2225 (700–3215)	2685 (1920–3620)	2413 (513–3277)	2197 (780–3328)	560 (–593–1184)	10.32 (–946–1245)	0.007	0.929	0.042
Simpson	0.93 (0.85–0.96)	0.94 (0.88–0.97)	0.93 (0.81–0.96)	0.90 (0.84–0.96)	0.015 (–0.028–0.085)	0.015 (–0.049–0.051)	0.047	0.533	0.476

Bold values indicate $P < 0.05$

a = test, b = placebo for the p values between before and after by Wilcoxon rank-sum test

c = Comparison of increase/decrease value during season between test and placebo by Mann–Whitney test

microbes during intervention was compared between groups at genus level. Compared to the placebo group, the ratio of the test group was significantly lower in *Parabacteroides* ($P = 0.01$), *Prevotella* ($P < 0.001$), *Oscillospira* ($P = 0.029$) and *Dialister* ($P = 0.019$), but it was higher in *Lactobacillus* ($P < 0.001$). With qPCR analysis (Table 4), LGG was detected in two subjects in the placebo group and in three in the test group (18.2 and 21.4 %, respectively) before the study. At end of the study, LGG was detected in all subjects in the test group, while it was not detected in any of the subjects in the placebo group. *Akkermansia muciniphila* was detected in both the placebo and the test groups (36.4 and 35.7 %, respectively) before intervention. This bacterium was detected in both the placebo and the test groups (45.5 and 57.1 %, respectively) at the end of the study. There were no significant differences in *L. casei*, *L. acidophilus* and *S. aureus* NUC before and after intervention, although the number of *L. plantarum* was significantly higher before intervention in the placebo group ($P = 0.029$, Table 4).

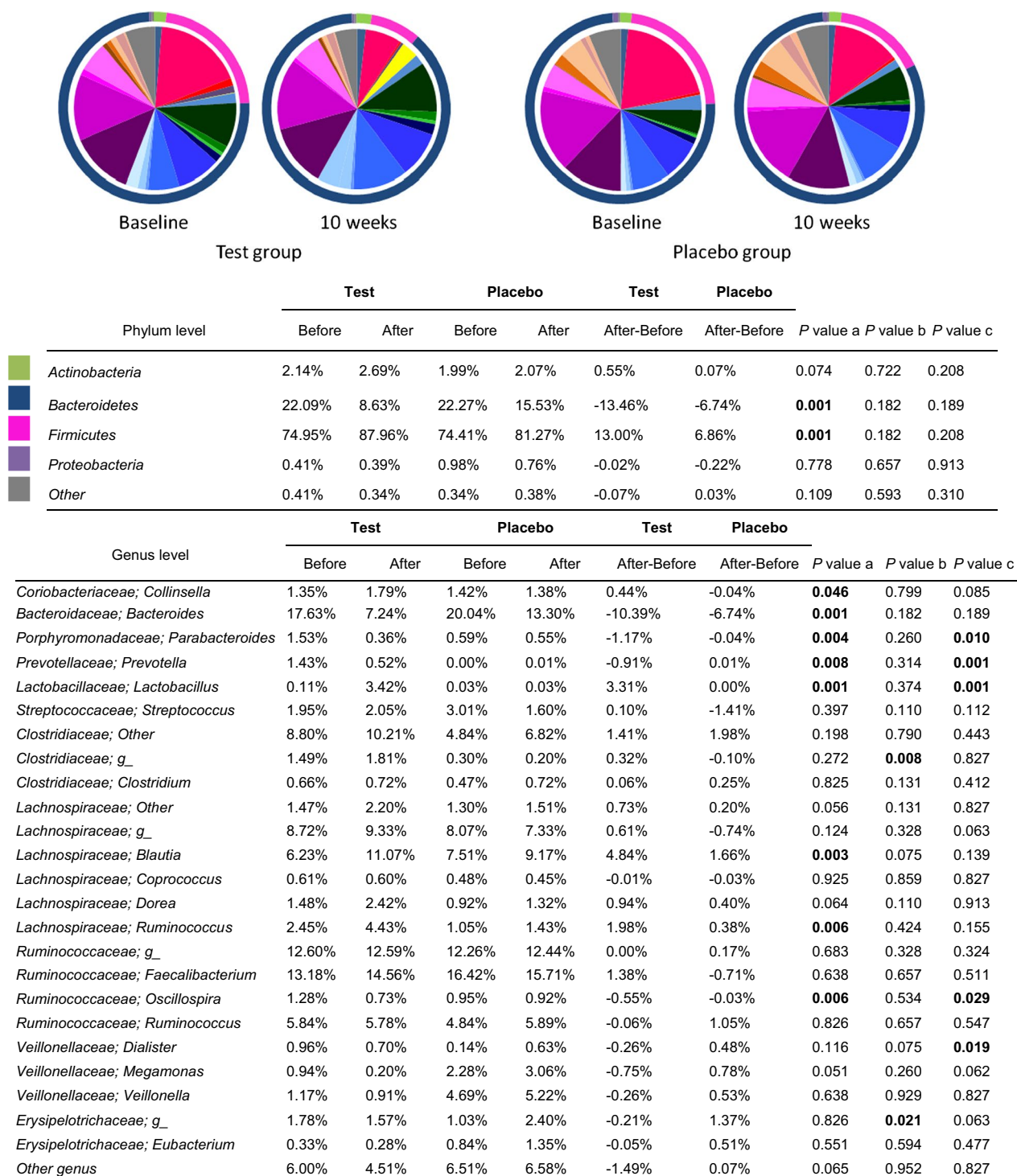
Association of microbial composition with lipid metabolites and with the JCP season

The possible association of intestinal microbiota composition with blood lipid levels, including total cholesterol, LDL-cholesterol and HDL-cholesterol and Triglyceride, was analysed. Additionally, creatinine, uric acid levels, total IgE and anti-Japanese cedar pollen IgE in blood samples were measured (Table 5). The abundance of *Firmicutes* throughout JCP season and the increase/decrease ratio were negatively correlated with total cholesterol ($\rho = -0.40$, $P = 0.049$), LDL-cholesterol ($\rho = -0.43$, $P = 0.030$) and HDL-cholesterol ($\rho = -0.61$, $P = 0.001$); the abundance of *Bacteroidetes* correlated positively with LDL-cholesterol ($\rho = 0.43$, $P = 0.032$) and HDL-cholesterol ($\rho = 0.58$, $P = 0.002$), and tended to correlate with total cholesterol ($\rho = 0.39$, $P = 0.055$).

The *Bacteroidetes/Firmicutes* ratio throughout JCP season was significantly lower in subjects at end of the study (median: 0.09; quartiles: 0.05–0.20; $P = 0.0017$) compared to that at baseline (median: 0.26; quartiles: 0.18–0.43; Fig. 1a) but was not influenced by LGG-TMC0356-fermented milk supplementation. The correlation coefficients were calculated for each increase/decrease in ratio during the study, and *Bacteroidetes* levels were negatively correlated with *Firmicutes* levels ($\rho = -0.7392$; $P = 0.0002$; Fig. 1b).

Discussion

The fermented milk prepared with LGG and TMC0356 was given orally to JCP patients in a double-blind placebo-controlled manner during the 2006 Japanese cedar pollen season. This probiotic-containing fermented milk significantly decreased the mean symptom score for nasal blockage after 9 weeks and mean symptom-medication score after 9 and 10 weeks [8]. However, the underlying mechanisms of the effects of this probiotics-fermented milk on the pollen sufferers remain unclear. The same phenomenon has also been observed with other probiotics when used in the management of seasonal allergic disorders—including JCP symptoms—and in all cases the underlying mechanisms related to clinical improvement were not well understood [16, 17]. Accumulating scientific evidence from both human and animal studies has suggested that an association may exist between intestinal microbiota and allergic disease. An abnormal composition of intestinal microbiota might be one of the key indicators of allergic diseases [18–21]. In the previous study, orally administered LGG and TMC0356 colonized the tested JCP patients following the intervention, as indicated by good faecal recovery of the probiotics. Some changes have also been observed in other intestinal microbiota composition in association with LGG and TMC0356 [22]. Therefore, an understanding of

Table 3 Relative abundance of taxa in faecal microbiota

Bold values indicate $P < 0.05$

Pie charts depict phylum (outer ring) and genus (pie) level distribution. The proportions of genus relative abundance are showed in the table. In this analysis only phyla and genera with relative abundance of more than 0.5 % were included. All OTUs with lower abundance were grouped as “other”

a = Test, b = Placebo for the P values between before and after by Wilcoxon rank sam test. c = comparison of increase/decrease ratio between Test and Placebo during study by Mann–Whitney test

Table 4 Microbial composition (prevalence and median) of faecal samples by qPCR analysis

	Test				Placebo				Test		Placebo	
	Before		After		Before		After		P value			
	Median	Prevalence (%)	Median	Prevalence (%)	Median	Prevalence (%)	Median	Prevalence (%)	a	b	c	d
LGG	5.2E + 05	21.4	2.2E + 08	100.0	2.3E + 05	18.2	N.D	0.0	0.008	0.001	N.D.	0.138
<i>L. casei</i>	2.0E + 04	50.0	1.8E + 04	35.7	N.D	0.0	N.D	0.0	0.167	0.431	0.724	1.000
<i>L. plantarum</i>	4.4E + 04	78.6	2.7E + 04	64.3	9.5E + 04	54.5	3.3E + 04	81.8	0.790	0.352	0.029	0.375
<i>L. acidophilus</i>	4.3E + 05	35.7	3.4E + 06	35.7	2.2E + 06	36.4	5.0E + 05	27.3	0.835	1.000	N.D.	0.647
<i>S. aureus</i> NUC	1.0E + 05	50.0	1.1E + 05	42.9	3.4E + 05	54.5	1.3E + 05	45.5	0.886	0.695	0.855	0.670
<i>A. muciniphila</i>	8.3E + 07	42.9	4.0E + 07	57.1	7.8E + 08	36.4	3.3E + 08	45.5	0.796	0.431	0.806	0.665

Bold values indicate $P < 0.05$

a = test, c = placebo for statistical differences were calculated using Mann–Whitney U test

b = test, d = placebo for statistical differences in bacterial prevalences between before and after were calculated using X2 test (2×2)

N.D not detected.

Table 5 Effects of the gut microbiota on lipids in the blood

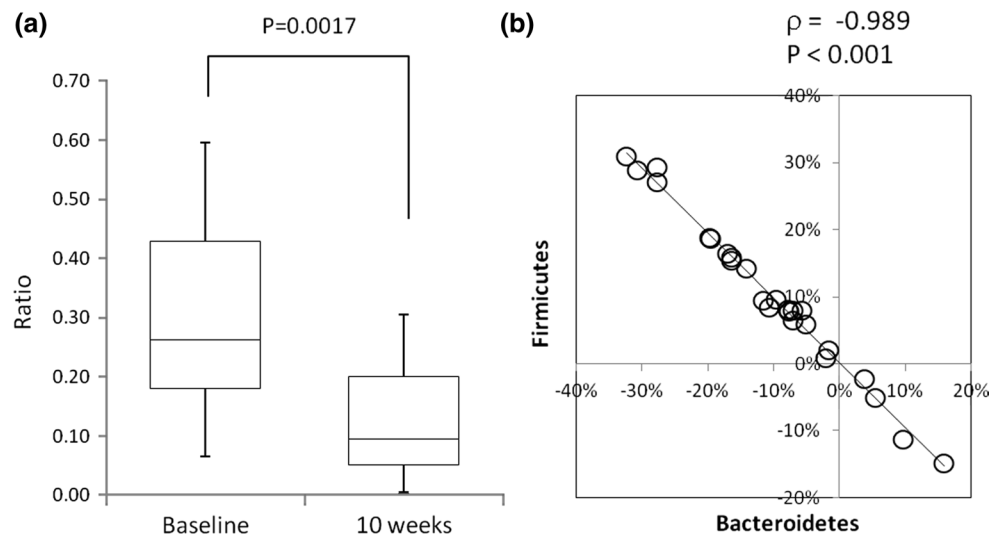
Both Test and Placebo	Creatinine		Uric acid		Total cholesterol		LDL-cholesterol		HDL-cholesterol		Triglyceride		Total IgE		Antigen-IgE	
	ρ	P value	ρ	P value	ρ	P value	ρ	P value	ρ	P value	ρ	P value	ρ	P value	ρ	P value
<i>Actinobacteria</i>	0.041	0.842	-0.168	0.410	-0.316	0.121	-0.347	0.089	-0.397	0.050	-0.038	0.851	-0.236	0.247	-0.138	0.498
<i>Bacteroidetes</i>	0.024	0.907	0.244	0.232	0.389	0.057	0.431	0.035	0.579	0.005	-0.071	0.729	0.057	0.780	-0.004	0.985
<i>Firmicutes</i>	0.023	0.910	-0.228	0.263	-0.397	0.050	-0.431	0.035	-0.609	0.003	0.114	0.576	-0.014	0.946	0.026	0.898
<i>Proteobacteria</i>	0.128	0.530	0.143	0.483	-0.174	0.394	-0.205	0.316	0.478	0.019	-0.240	0.240	-0.087	0.672	-0.028	0.889
Test	Creatinine		Uric acid		Total cholesterol		LDL-cholesterol		HDL-cholesterol		Triglyceride		Total IgE		Antigen-IgE	
	ρ	P value	ρ	P value	ρ	P value	ρ	P value	ρ	P value	ρ	P value	ρ	P value	ρ	P value
<i>Actinobacteria</i>	-0.016	0.953	-0.541	0.051	-0.329	0.236	-0.341	0.219	-0.410	0.139	-0.156	0.574	-0.282	0.309	-0.064	0.818
<i>Bacteroidetes</i>	0.034	0.902	0.620	0.025	0.405	0.144	0.490	0.077	0.524	0.059	-0.055	0.843	0.166	0.550	-0.042	0.880
<i>Firmicutes</i>	-0.021	0.940	-0.543	0.051	-0.414	0.135	-0.490	0.077	-0.520	0.061	0.077	0.782	-0.151	0.587	0.020	0.943
<i>Proteobacteria</i>	0.010	0.972	0.123	0.657	-0.016	0.953	-0.064	0.818	0.588	0.034	-0.130	0.640	-0.025	0.927	0.138	0.618
Placebo	Creatinine		Uric acid		Total cholesterol		LDL-cholesterol		HDL-cholesterol		Triglyceride		Total IgE		Antigen-IgE	
	ρ	P value	ρ	P value	ρ	P value	ρ	P value	ρ	P value	ρ	P value	ρ	P value	ρ	P value
<i>Actinobacteria</i>	0.100	0.752	0.284	0.369	-0.391	0.216	-0.391	0.216	-0.373	0.239	0.145	0.646	-0.173	0.585	-0.191	0.546
<i>Bacteroidetes</i>	0.068	0.829	0.007	0.983	0.327	0.301	0.309	0.328	0.595	0.060	-0.200	0.527	-0.045	0.886	-0.018	0.954
<i>Firmicutes</i>	-0.005	0.989	-0.002	0.994	-0.336	0.287	-0.314	0.321	-0.655	0.038	0.264	0.404	0.100	0.752	0.073	0.818
<i>Proteobacteria</i>	-0.009	0.977	0.243	0.442	-0.218	0.490	-0.182	0.565	0.427	0.177	-0.436	0.168	-0.309	0.328	-0.427	0.177

 Negative correlation, $0.05 < P < 0.1$
 Negative correlation, $P < 0.05$
 Positive correlation, $0.05 < P < 0.1$
 Positive correlation, $P < 0.05$

Bold values indicate $P < 0.05$

The correlation coefficient was calculated for each value of increase/decrease ratio during the study, and the significance of the microbial contribution is indicated as the p value for the comparison of blood lipid values. HDL indicates high-density lipoprotein; LDL, low-density lipoprotein

Fig. 1 *Bacteroidetes/Firmicutes* ratio (a), Spearman rank correlation between before/after ratio of *Bacteroidetes* and *Firmicutes* (b)



events taking place within the intestinal microbiota accompanied by the consumption of LGG and TMC0356 might explain why probiotic can express protective effects against JCP, thereby improving the level of protection offered by the product. As previous studies were not able to provide a mechanistic explanation of the alleviation of symptoms of JCP, we uncovered the changes in microbiota related to the probiotic administration [22]. 16S ribosomal RNA (rRNA) sequencing is a common amplicon sequencing method used to identify and compare bacteria present within a given sample. 16S rRNA gene sequencing is a well-established method for studying phylogeny and taxonomy of samples from complex microbiomes or environments that are difficult or impossible to study. The present study was the first attempt to analyse the intestinal microbiota of JCP patients using 16S rRNA gene sequencing. In the present study, LGG–TMC0356-containing fermented milk significantly increased the microbiome diversity characterized by increased *Collinsella*, *Lactobacillus* and the family *Lachnospiraceae* (*Blautia* and *Ruminococcus*), subsequently increasing Firmicutes. On the other hand, LGG–TMC0356-fermented dairy products significantly decreased the levels of *Bacteroides*, *Parabacteroides*, *Prevotella* and *Oscillospira* compared to the levels present at the time prior to administration. These results demonstrated that LGG–TMC0356-fermented milk modulated the composition of intestine microbiota of JCP patients. Such results were not demonstrated in previous studies using the culture methods T-RFLP and qPCR. The modified intestinal microbiota composition describes the success of microbiota modulation in the management of JCP by probiotics. These results also suggest that strain-dependent effects of different probiotics or probiotic combinations may be required for decreasing the risk of JCP symptoms in vulnerable subjects. On a microbial metabolism level, the production of

lactate by *Lactobacillus* may be the one factor impacting health. Conversion of lactate to butyrate can result in more mucin synthesis [23, 24], improving the intestinal barrier by increasing tight junction assembly [25, 26]. *Prevotella* are non-butyrate-producing bacteria and potential mucin degraders [27]. The butyrate producers, family *Ruminococcaceae* (Cluster IV) and *Lachnospiraceae* (Cluster XIVa), belong to the phylum *Firmicutes*, class *Clostridia*, which may influence colonic regulatory T cells (Treg) towards the suppression of inflammatory and allergic responses [28, 29]. Additionally, dietary intervention with LGG appears to accelerate tolerance in infants with milk allergies. This is associated with increased butyrate-producing bacteria [30] and an increase in Treg-dependent mechanisms in the murine model of asthma [31]. Therefore, the colonized LGG–TMC0356 in JCP patients might modulate both the intestinal mucosal barrier and the microbiota to normalize dysbiosis and related allergic reactions.

Bacteroidetes/Firmicutes ratio has been well suggested as one of the valuable markers to evaluate the association between abnormal intestine microbiota and gut barrier disruption and inflammation of the patients with obesity and metabolic syndromes [32]. The shift from *Bacteroidetes*-dominated microbiota towards the *Firmicutes*-dominated has been considered unfavourable in the management of metabolic disorders, although the underlying mechanisms still remain unclear [33]. Recently, the altered composition of intestine microbiota characterized with less phylum *Bacteroidetes* and more phylum *Firmicutes* has also been found to company with allergy diseases such as asthma [34, 35]. These indicate that intestine microbiota could be deeply involved in the pathology of allergy in same manner by which metabolic disorders of host are influenced. In the present study, *Bacteroidetes/Firmicutes* ratio decreased significantly among the tested subjects at end of the JCP

season, and *Bacteroidetes* levels correlated negatively with *Firmicutes*. These results have good agreements with the previous studies to support that the intestine microbiota could influence the development of metabolic disorders and allergopathology with same underlying mechanism, at least partly. Therefore, *Bacteroidetes/Firmicutes* ratio, one of the markers suggested for metabolic syndromes, might be also used to monitor/or study allergic disease including JCP.

Gut microbiome is associated blood lipids profiling which is independent of age, sex and host genetics [36]. We also observed altering the gut microbiome caused by JCP was associated with altered lipid metabolism throughout JCP season. In particular, we confirmed that *Bacteroidetes* and the LDL- and HDL-cholesterol levels had strong positive correlations, whereas *Firmicutes* had negative correlations with blood lipid levels. The altering microbiome throughout JCP season may reflect or resemble the obesity related to intestine microbiome. These results indicated that alternation of lipid metabolism might be one more pathway by which the intestine microbe could influence allergy disease. In the present study, no significant differences in lipid metabolisms were found after the probiotics intervention compared to placebo during JCP season. These results might explain in one more way why the anti-allergic effects of the reported probiotic intervention including the present study were limited. One of the important reasons might be the most probiotics used in management of allergic diseases that were screened for their potent immunoregulatory effects with less abilities to modify lipid metabolism.

However, the present was a preliminary study and the information from the present study was still limited to lead the good conclusion on the association among the serum lipid, the gut microbiota and allergic disease. Therefore, further well-designed clinical study, especially targeting patients with allergic and lipid disorders, is still necessary.

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Compliance with ethical standards

Conflict of interest G. Harata, F. He, K. Miyazawa, K. Yoda, M. Kawase, A. Kubota and M. Hiramatsu are employees of Takanashi Milk Products Co., Ltd.; H. Kumar, S. Rautava and S. Salminen declare no conflicts of interest.

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