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Effects of a probiotic drink containing *Lactobacillus casei* strain Shirota on dental plaque microbiota

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Abstract

Objective: To investigate the effects of a probiotic drink containing *Lactobacillus casei* strain Shirota on dental plaque microbiota.

Methods: Caries-free young adults were administered a probiotic drink containing *L. casei* Shirota (Yakult) three times on day 1, then once daily for 28 days. Dental plaque samples were collected and analysed by 16S rRNA sequencing before (day 1), during (day 2), and one day following intervention (day 30).

Results: Out of samples from 10 included participants, 256 814 sequences passed through quality control, clustered into 170–234 different 'species-level' phylotypes. *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria* and *Proteobacteria* were the most abundant phyla. Dental plaque microbiota composition significantly changed at different taxonomic levels following probiotic consumption. At genus level, the relative abundance of *Veillonella* and *Kingella* increased significantly following intervention, while that of *Leptotrichia* reduced significantly during intervention, but recovered to baseline level at day 30. Alpha diversity and overall structure of the dental plaque microbiota was not significantly impacted by the probiotic.

Conclusion: Yakult intake changed the abundance of some bacteria related to caries, suggesting that the change of composition may be beneficial to oral health, while the overall microbiota structure remained unaffected.

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Introduction

Dental caries, or tooth decay, is a complex biofilm disease involving a net loss of minerals from the teeth due to prolonged periods of low pH in the mouth.[1] With negative impacts on quality of life, dental caries also adversely affects children's school attendance and academic performance,[2] and is one of the main reasons for hospitalization of children in some high-income countries.[3] Efforts to prevent dental caries include water fluoridation, fluoride toothpastes, fluoride varnishes, sealants and/or interim therapeutic restorations, and silver diamine fluoride, each of which has shown varying levels of efficacy in clinical trials.[2] Although the prevalence of dental caries has declined in most industrialized countries since the 1970s,[4] dental caries remains the most common non-communicable disease worldwide.[3] Thus, new methods for caries prevention remain in urgent demand.

Probiotic therapy has been introduced for the maintenance of oral health,[5] but studies on the same probiotic strain have presented conflicting results.[6–8] *Lactobacillus casei* strain Shirota, which is contained in the commercial probiotic-fermented milk drink Yakult, has over 80 years' history of safe consumption and proven health benefits.[9] *L. casei* Shirota has been demonstrated to significantly inhibit the *in vitro* growth of *Streptococcus mutans*[6] and reduce the amount of *S. mutans* in saliva and dental plaque.[7] However, one study found no significant change in the viability of *S. mutans*, acidogenic microorganisms, total anaerobic species and Gram-negative obligate anaerobes in the saliva and tongue coating of healthy complete denture wearers following *L. casei* Shirota administration.[8] Due to these contradictory findings, the potential impacts on the oral microbiota of consuming *L. casei* Shirota remain unclear, and no specific evidence-based proposal has been stipulated for the use of probiotics to prevent oral cavity diseases. The above-mentioned studies mainly focused on the effects of *L. casei* Shirota on single bacterial species, examined by *in vitro* plate culture and colony counting, and did not observe the probiotic impact on overall oral microbiota. Sequencing of 16S rRNA enables more detailed analysis of microbial communities, allowing detection of not only the most abundant microbes, but also those of low abundance.

To date, there are no reliable reports of species and genotypic diversity in the oral cavity following consumption of Yakult. Therefore, the present study was conducted to explore the effects of *L. casei* Shirota on the oral microbiota composition and structure using 16S rRNA sequence analysis. To the best of the present authors' knowledge, this is the first study to investigate such effects by means of 16S rRNA sequencing.

Patients and methods

Study population

This self-controlled case study recruited healthy students aged 20–22 years, who were attending Sun Yat-Sen University,

between November 2016 and December 2016. Students were recruited through advertisement and inclusion criteria were self-reported healthy status and complete dentition. The oral cavity of each subject was checked by an experienced dentist (XH) and individuals with the following conditions were excluded: less than 28 teeth; clinically detectable signs of dental caries (white spot lesion or carious cavity), periodontal disease (gingival bleeding, deep periodontal pocket, attachment loss and tooth mobility) or other pathologies (such as sinus tract, blisters and swelling); presence of dental restorations, crowns or orthodontic appliances; current smokers or smoker co-habitants; current participation in another clinical study; use of antibiotics or probiotic products within 3 months prior to examination.[10] Subjects with current, or history of, diseases associated with oral microbiota, such as diabetes mellitus, rheumatoid arthritis, inflammatory bowel disease and atherosclerosis,[11,12] were also excluded.

The study was reviewed and approved by the Ethics Committee of the Guanghua School and Hospital of Stomatology, Sun Yat-sen University (KQEC-2019-01), and all participants provided written informed consent.

Study design and sampling procedure

For each subject, supragingival dental plaque was collected from the surfaces of four first molars on the morning of day 1, namely timepoint A, at least 24 h after the last tooth brushing and 12 h after the last food intake. Subjects were then instructed to drink 100 ml of Yakult (Yakult Sdn Bhd, Selangor Darul Ehsan, Malaysia) three times on day 1, one bottle immediately following sampling, one after lunch and one after dinner, by swishing the beverage in the mouth for about 20 s to prolong contact time with the teeth before swallowing. They

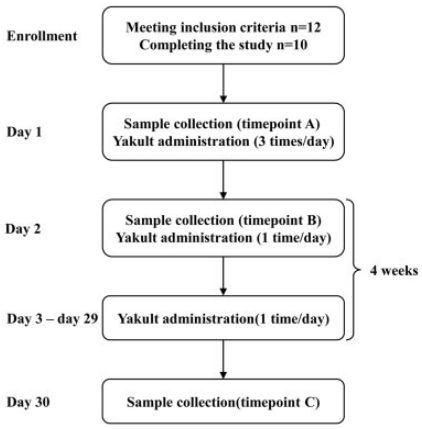

Figure 1. Flow chart showing study design.

were further instructed to drink one bottle of Yakult after lunch daily from day 2 to day 29 according to the method above. Supragingival dental plaque was collected on the morning of day 2 and day 30, namely timepoints B and C (Figure 1). During the study, subjects were instructed to maintain their regular oral hygiene regimen except for the 24 h preceding sample collections on day 1, day 2 and day 30.[10] All oral samples were collected at the Guanghua Institute of Stomatological Research, Sun Yat-Sen University, and were immersed in 4 ml of sterilized 0.9% saline solution and stored at –80°C prior to DNA extraction.

Bottles of Yakult were purchased from general stores in Guangzhou, and stored at 4–6°C prior to consumption, according to the manufacturer's instruction. Each bottle of Yakult (100 ml) was purported by the manufacturer to contain 1.2×10^{10} colony-forming units (cfu) of *L. casei* Shirota.

DNA extraction and polymerase chain reaction

DNA was extracted from plaque samples using a Powersoil® DNA isolation Kit

(MO BIO Laboratories, QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was used to generate amplicon libraries of the small-subunit (16S) ribosomal (r)RNA gene V4 and V5 hypervariable regions for each individual sample. PCR was performed using universal primers (forward, 5′-GTGACAGCAGCCGC GGTAA-3′ and reverse, 5′-CCGTCAATT TATTTAAGTTT-3′ [HonorTech Co., Beijing, China]) with 30 barcodes, which helped to assign sequences to different samples. The amplification mix contained 30 ng of DNA sample, 2 µl of forward primer (10 µM), 2 µl of reverse primer (10 µM), 4 µl dNTPs (2.5 mM), 5 µl 10 × Pyrobest™ Buffer (TaKaRa, Tokyo, Japan), 0.3 µl Pyrobest™ DNA Polymerase (2.5 U/μl; Code, DR005A; TaKaRa) and 36.7 µl ddH_2O in a total volume of 50 µl. Cycling conditions were an initial denaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and elongation at 72°C for 40 s, followed by a final extension for 10 min at 72°C, Samples were then held at 4°C. Each sample was amplified using one specific barcoded primer. To assess quality, the PCR product for each sample was subjected to electrophoresis using a 3% agarose gel and visualized using a UVP BioDoc-IT2 UV Imaging System (Analytik Jena, Upland, CA, USA). DNA fragments were excised from the gel and further purified using QIAquick Gel Extraction Kit (250) (Cat No./ID: 28706; QIAGEN GmbH). Qubit 2.0 (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the concentration of PCR products. Finally, an amplicon library was constructed and pyrosequencing of the 16S PCR-amplicons was performed using the Illumina® MiSeq System (Illumina, San Diego, CA, USA) according to the manufacturer's instructions.

Sequence processing

To minimize the effects of low-quality data, raw data generated from pyrosequencing was preprocessed before further analysis. First, raw data was split into individual samples according to the barcode. Then, paired reads obtained from dual terminal sequencing were assembled into a sequence according to their overlapping relationship using Connecting Overlapped Pair-End (COPE) software, version V1.2.3.3.[13] The length of the overlap sequence was 19 base pairs (bp) and the matching rate was set at 98%. The barcode and primer sequence were then removed by an in-house script. No mismatch was allowed for barcode while one bp of mismatch was allowed for primer. Spliced tags were pooled according to their lengths and chimeric sequences were removed. Finally, a statistical table of Operational Taxonomic Unit (OTU) classification level was obtained according to the data statistics. Raw sequences were deposited in the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) with accession numbers SRR8497113–SRR8497142.

OTU assignment and taxonomic classification

Following optimization, spliced tags were clustered into OTUs of less than 97% similarity using USEARCH software, version 8.0.1623 (https://www.drive5.com/usearch/) and the abundance of each OTU was obtained. Singleton reads, defined as reads with an abundance of 1, were removed from clustering. Sequences in the same OTU were considered to be from the same assumed taxon. Representative sequences, defined as sequences that appeared most frequently in the OTUs, were screened by UPARSE (http://www.drive5.com/uparse/) and analysed using RDP software, version 2.2 (http://pyro.cme.msu.edu/). The SILVA

database, version 123 (http://tax4fun.gobics.de/Tax4Fun/ReferenceData/SILVA123.zip) was used for taxonomic classification (with similarity threshold set at 90%) and phylogenesis. Alpha diversity was evaluated using number of observed OTUs, Chao1 Index, Shannon Diversity Index and Phylogenetic Diversity whole tree analysis.

Statistical analyses

Data are presented as *n* or % prevalence, or mean ± SD, and statistical analyses were performed using SPSS software, version 22.0 (IBM, Armonk, NY, USA) for Windows®. Normality of data was assessed using Shapiro–Wilk test, and Levene's test was used to assess homogeneity of variance, prior to selection of appropriate parametric or nonparametric tests. Differences in data were then analysed using parametric analysis of variance (ANOVA) or nonparametric Friedman test. Principal component analyses were employed to investigate differences between microbiota according to timepoints. Differences in microbiota structure according to timepoint, subject and Yakult consumption was assessed using analysis of similarities (ANOSIM) based on Bray–Curtis dissimilarity. This widely used method presents sample differences as values ranging from 0 to 1, where a difference value of 0 indicates that samples share all their species, and a value of 1 indicates that samples are totally different. An 'R' value was used to compare within- and between-group dissimilarities, where $R > 0$ indicates larger between-group distances (differences) than within-group distances. For all analyses, a P value < 0.05 was considered statistically significant.

Results

Twelve healthy students of Sun Yat-Sen University, aged 20-22 years, were recruited into the study. A total of 10 subjects (named 1–10) completed the full study and were included in the final analyses.

Thirty samples, 10 for each timepoint (A1–10, B1–10, C1–10) were collected and analysed. Barcoded 16S rDNA amplicon sequencing using the Illumina MiSeq platform yielded a total of 978 091 raw reads (OTU Sequences), resulting in a dataset of 256 814 reads (sample size). The number of reads per sample ranged from 5 891–12 571, with an average of 8 560. Richness and diversity analyses were based on OTUs, and clustering the unique sequences into OTUs at a 3% genetic distance resulted in 170–234 different 'species level' phylotypes per sample. For all of the samples analysed, the number of OTUs detected (125–234) was very close to the total number of OTUs estimated by Chao1 richness indicator (144–278). Using Good's method, the average coverage level was approximately 99.8% in all samples, indicating that approximately two new phylotypes would be expected for every 1 000 additional sequenced reads. This level of coverage suggested that the identified 16S rDNA sequences represented the majority of bacterial types present in the current study samples.

Rarefaction curves were analysed for individual samples (Figure 2a) and for samples pooled for each of the three timepoints (Figure 2b). Rarefaction curves among the three timepoint groups displayed similar richness of species, with no statistically significant between-group differences ($P > 0.05$). Using different alpha-diversity measures (number of observed OTUs, Chao1 Index, Shannon Index and Phylogenetic Diversity whole tree) consistently found similar microbial communities within the three timepoint groups ($P > 0.05$).

To determine whether microbiota from the three timepoint groups could be distinguished from each other, PCA was

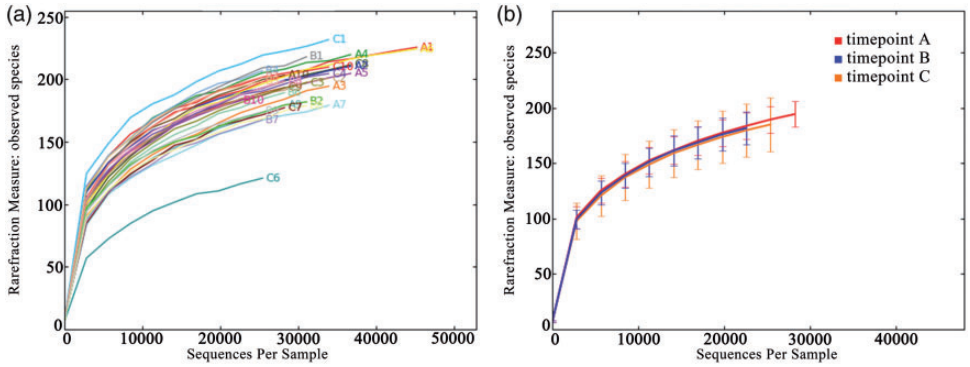

Figure 2. Rarefaction curves for all samples comprising 10 healthy subjects at three timepoints, namely A (before intervention with Yakult, day 1), B (day 2) and C (one day following end of intervention, day 30): (a) rarefaction curves for all individual samples showing number of observed species (alpha diversity) plotted against increasing subsampled sequence sets for each sample. A trend towards a plateau was first observed following approximately 20 000 reads, suggesting this was the minimum number of reads needed per sample to accurately represent microbial diversity; (b) rarefaction curves for samples pooled for each of the three timepoints showing number of observed species (alpha diversity) plotted against increasing sequences for each pooled sample. Similar patterns of increasing species diversity were observed between individual and pooled sample sets, indicating similar phylogenetic diversity.

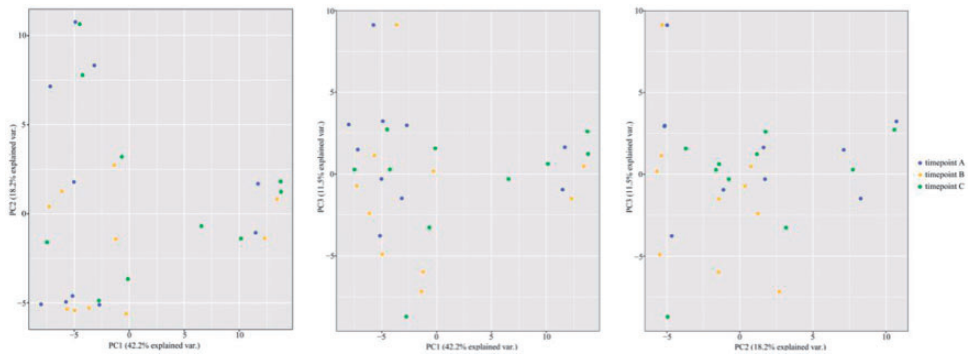

Figure 3. Principal component analysis (PCA) of dental plaque microbiota in 10 healthy subjects at the three study timepoints, namely A (before intervention with Yakult, day 1), B (day 2) and C (one day following end of intervention, day 30). No clustering trend was observed in PCA space. PC1, 2, 3, principal component 1, 2, 3; Var, variance.

employed (Figure 3). This technique extracted the important information from a multivariate dataset and visualized them in an eliminated dimension way. No apparent clustering trend was observed, indicating that the overall structure of the plaque microbiota was not affected by probiotic intake.

The effects on microbiota structure of different timepoints, subjects and Yakult consumption versus baseline control (namely 'by timepoint', 'by subject' and 'by consumption'; Figure 4) were assessed using ANOSIM, based on Bray–Curtis dissimilarity. The resulting R values were all larger than zero (Figure 4), suggesting that

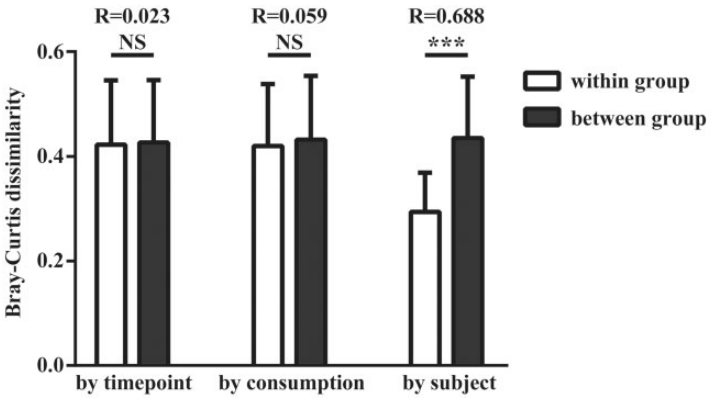

Figure 4. Analysis of similarity based on Bray–Curtis dissimilarity to investigate the effects of timepoint, Yakult consumption (versus baseline, day 1) and subject, on dental plaque microbiota in 10 healthy subjects: ***$P < 0.001$; NS, no statistically significant effect ($P > 0.05$); data presented as mean ± SD.

dissimilarities between timepoints, between consuming Yakult and baseline control, and between subjects, tended to outweigh the corresponding within-group dissimilarities. However, only the differences between and within subjects were statistically significant ($P < 0.001$).

Taxonomy-based characterization of dental plaque microbiota was performed by identifying and quantifying bacterial taxa against reference databases using mothur software (https://www.mothur.org/). Plaque microbial communities were then taxonomically profiled, summarizing the relative abundances of microbial clades at different taxonomic levels (Figure 5, showing phylum and genus levels only). All sequences were found distributed in 23 phyla, 48 classes, 82 orders, 113 families, 184 genera and 253 species. The five most abundant phyla were *Firmicutes* (7.5–53.7%), *Proteobacteria* (7.2–50.3%), *Bacteroidetes* (6.9–27.3%), *Actinobacteria* (5.6–22.2%), and *Fusobacteria* (1.9–33.2%), comprising 93.2–99.8% of the community of all samples. At the genus level, *Veillonella*, *Aggregatibacter*, *Streptococcus*, *Leptotrichia*, *Neisseria*, *Corynebacterium*, *Fusobacterium* and *Porphyromonas* made up almost 70% of the total reads. Samples from subjects 8 and 10 had obviously higher percentages of *Veillonella* (25.9–39.5%) versus other subjects (0.2–11.0%), except for samples C3 and C4. *Moraxella* presented a relatively high percentage (5.0–15.5%) in the samples of subject 7. *Capnocytophaga*, *Actinomyces*, *Prevotella* 7, *UN–f-Neisseriaceae*, *Prevotella*, *Prevotella* 2 and *Alloprevotella* were also found in all samples with uneven quantitative distributions. No detectable level of *S.mutans* was found in the study samples.

To study changes in the composition of dental plaque microbiota following Yakult intake, the plaque microbiota was compared between the three timepoints (Figure 6). Among five dominant phyla, *Proteobacteria* and *Firmicutes* showed significantly different relative abundances between the three timepoints. The relative abundance of *Proteobacteria* was significantly lower at timepoint C versus timepoint A and B ($P < 0.05$), while the relative abundance of *Firmicutes* was significantly higher at timepoints B and C compared with timepoint A ($P < 0.05$ and $P < 0.01$, respectively). At genus level, the relative abundance of *Veillonella* showed an increasing trend after consuming

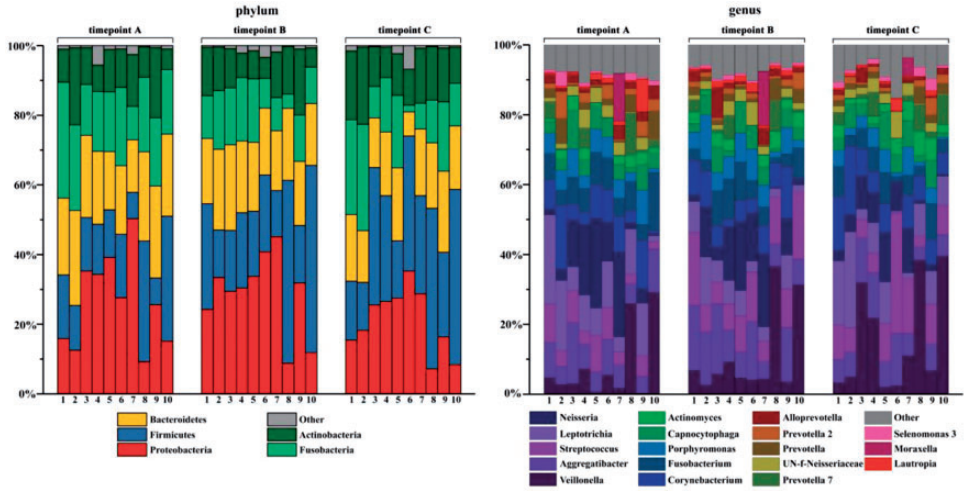

Figure 5. Taxonomic composition of dental plaque samples from 10 healthy subjects at different timepoints (A, before intervention with Yakult [day 1]; B, day 2 during intervention; and C, one day following end of intervention [day 30]), displayed as relative abundance according to phylum and genus. At phylum level, relative abundances highlighted the major roles of *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria* and *Proteobacteria* in the plaque microbiota. At genus level, *Veillonella*, *Aggregatibacter*, *Streptococcus*, *Leptotrichia*, *Neisseria*, *Corynebacterium*, *Fusobacterium* and *Porphyromonas* were shown to be the most abundant. The group 'Other' represents phyla and genera accounting for $< 1\%$ of the samples.

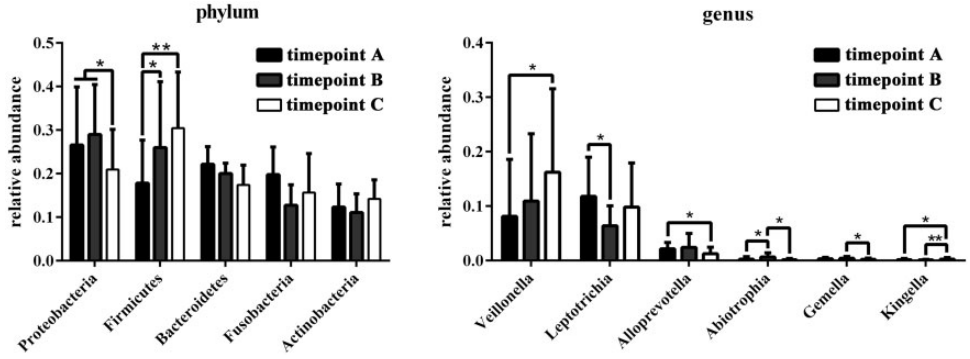

Figure 6. Comparison of bacterial phylum and genus profiles between three timepoint groups of dental plaque samples from 10 healthy subjects: Timepoint A, before intervention with Yakult (day 1); timepoint B (day 2 during intervention); and timepoint C, one day following end of intervention (day 30). Data presented as mean $\pm$ SD relative abundance of dominant taxa ($>1\%$ for phylum and $> 0.1\%$ for genus) between three timepoint groups (*$P < 0.05$; **$P < 0.01$; parametric analysis of variance or non-parametric Friedman test).

probiotic but only the difference between timepoint A and C was statistically significant ($P < 0.05$). Conversely, the relative abundance of *Leptotrichia* decreased following probiotic intake (timepoint A versus timepoint B; $P < 0.05$), but was not significantly different from baseline at timepoint C (1 day following end of Yakult

intake). *Abiotrophia* and *Gemella* displayed a significantly higher relative abundance at timepoint B versus timepoint C ($P < 0.05$), while only *Abiotrophia* presented a higher relative abundance at timepoint B versus timepoint A ($P < 0.05$). The relative abundance of *Kingella* was significantly higher at timepoint C versus timepoints A and B ($P < 0.05$ and <0.01, respectively; Figure 6). There were no significant changes in the relative abundance of *Actinomyces* and *Lactobacillus* following consumption of Yakult (data not shown).

Discussion

Modulation of resident microbial communities by consumption of probiotics has become an appealing means for host health promotion.[14] Yakult, a well-known probiotic beverage, contains a single live bacterial species, *L. casei* Shirota, at a reported concentration of 1.2×10^{10} cfu/100ml. *L. casei* Shirota has been shown to possess probiotic antimicrobial properties against common gastrointestinal pathogens *in vitro*, such as *Salmonella enterica* serovar Typhimurium,[15] *Escherichia coli*,[16] *Helicobacter pylori*.[17] In clinical studies, the effects of *L. casei* Shirota on human microbiota appear to vary depending on participant population and where samples are obtained.[18–22]

The oral cavity has also been a target site for testing various strains of probiotics.[10,14] In the present study, it was hypothesized that Yakult consumption may change the structure and composition of dental plaque microbiota, which was assessed using 16S rRNA sequencing. Data analyses revealed changes in the abundance of some bacteria that have been linked with the presence or absence of caries, while the overall microbiota structure remained unaffected. In particular, changes were noted in some genera associated with caries, including *Veillonella*, *Leptotrichia* and *Kingella*. The most dominant genus in the present study samples, *Veillonella*, significantly increased versus baseline following Yakult consumption for 4 weeks. *Veillonella* is able to utilize lactate and metabolize it into weaker acids, which may help slow down the demineralization process.[23] One study found that *Veillonella* utilized lactate produced by *S. mutans* when they were co-cultured, leading to higher yields of both organisms and lower concentrations of lactate.[24] It could be speculated that the increased abundance of *Veillonella* in the present study suggests the potential for Yakult ingestion to be associated with a reduction in caries development. The relative abundance of *Leptotrichia* reduced immediately and significantly after the one day of high-frequency Yakult intake at the beginning of the study, but recovered to baseline levels at the end of the study. As the fourth most dominant genus detected in the present study, *Leptotrichia* is highly saccharolytic,[25] and like *S. mutans*, it ferments a wide variety of mono- and disaccharides to lactic acid, alluding to its participation in tooth decay.[25] Hence, a reduction in the proportion of *Leptotrichia* in plaque, though transient, suggests that the plaque microbiota may have shifted to a less acidogenous biofilm. Another noteable genus in the present plaque samples was *Kingella*, the proportion of which increased significantly after 4 weeks of Yakult consumption compared with baseline and timepoint B, but remained low. In dental plaque studies, *Kingella* has been associated with absence of caries.[26,27] These changes might be due to the probiotics' competition for adhesion site, nutrients and growth factors.[28] Additionally, Yakult intake was found to result in a significant increase in pH and significant cariostatic effects on oral biofilm acidogenicity.[29] All these findings indicate that Yakult intake may be beneficial in promoting oral health; however, these findings require support from further studies.

Information regarding the correlation between caries and other dominant genera (*Alloprevotella*, *Abiotrophia* and *Gemella*), that were significantly changed in the present plaque samples, is lacking, and their roles in dental plaque needs further investigation.

Many studies have investigated the effects of *L. casei* Shirota on oral microbiota, particularly *S. mutans* or mutans streptococci, but data are conflicting.[7,8,29,30] In the present study, taxonomic analysis found no detectable level of *S. mutans* and no significant changes in the relative abundance of non-mutans streptococci, *Actinomyces* and *Lactobacillus* following consumption of Yakult (data not shown), though Yakult is rich in carbohydrate (17% w/w). For many years, *S. mutans* has been emphasized as having a critical role in dental caries,[31,32] but studies have revealed that it is no longer appropriate to state *S. mutans* as the principle aetiological agent of tooth decay,[33] as the microflora involved in caries is much more complex than hitherto thought.[33,34] Other bacteria, such as non-mutans streptococci, *Actinomyces*, *Lactobacillus*, *Bifidobacterium* and *Propionibacterium*, may behave in exactly the same way as *S. mutans*.[33,34] Dental plaque is currently described as a dynamic ecosystem in which non-mutans streptococci and *Actinomyces* are the key players.[32] Sugar supplement lowers the pH of the environment, which subsequently increases the number of key bacteria and leads to a microflora shift, pushing the ecosystem towards demineralization.[32,35] Only when the acidic environment is prolonged will mutans streptococci and *lactobacilli* take the place of non-mutans streptococci and *Actinomyces*, and accelerate net mineral loss.[32]

Since the majority (>90%) of microbial species cannot be readily cultured using current laboratory culture techniques,[36] conventional plate culture and colony counting are insufficient in microbiota analysis. In order to gain a deeper insight into the plaque microbiota, the present study engaged 16S rRNA sequencing. As the most common sequencing approach to analyse microbiota, it has been used to compile most of the data collated by the Human Microbiome Project.[36] The major phyla found in the present study samples were *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Fusobacteria*, which concurred with results of the Human Microbiome Project Consortium.[37] With a Good's Coverage of up to 99.8%, the alpha diversity in the present study, as a marker of species richness, did not significantly change following Yakult intake. In addition, PCA did not show apparent clustering by timepoints, consistent with the ANOSIM results, in which the between-timepoint and between-intervention differences did not significantly outweigh the within-timepoint and within-intervention differences, respectively. These results indicated that the overall microbiota structure was not significantly affected by probiotic intake, partially echoing the findings of other studies.[10,14] Oral microbiota is thought to be the most stable microbial community compared with other communities of the body,[38] and dental plaque exhibits a complex organization that remains stable with time despite changes in the oral environment.[31] Hence, intervention to change its overall structure may not be easy. Further studies, with longer-term or higher intake frequency design and larger sample sizes are required to understand the potential effects of probiotic intervention on dental plaque microbiota.

As the first study to investigate the effects of *L. casei* Shirota on dental biofilm microbiota by means of 16S rRNA sequencing, the relatively small sample size ($n = 10$) of the present study was comparable to previous reports,[10,14,39] and the results provide valuable information for

further large-scale whole metagenomic analysis in a larger population. It should be noted, however, that the results of the present study may be limited by a number of factors. Without prior knowledge of whether there would be compositional changes in the microbiome, the present study lacked functional analysis of factors such as enzymatic activity and acidogenicity of the oral biofilm, limiting the direct application of the results of this study. In future studies, for deeper understanding of the microbiota metabolic potentials, the Tax4Fun software package may be used to predict the functional capabilities of microbial communities based on 16S rRNA datasets.[40] Analyses of biofilm acidogenicity and enzymatic activities (such as glucosyltransferases, lactic dehydrogenase, fructosyltransferase and dextranases)[41,42] should be conducted in combination with 16S rRNA in further studies to reveal whether any correlations exist between them. In addition, considering some subjects with low caries risk may experience potential cariogenic/detrimental effects following consumption of a probiotic drink,[29] future studies should include a caries risk assessment as a quantitative method to precisely evaluate the participants' oral caries status prior to Yakult consumption. Although every subject in the present study acted as their own control, an additional control group receiving placebo would improve the study design. Finally, potential noncompliance of the study subjects may exist, such as inadequate Yakult contact time with the teeth or inability to refrain from brushing prior to the clinical measurements. These noncompliant factors are likely to reduce the intensity of effects observed in the present study. Therefore, the actual effects of Yakult on plaque microbiota may have been underestimated.

In conclusion, Yakult intake was found to change the abundance of some bacteria related to caries, while the overall microbiota structure remained unaffected. The compositional change may be beneficial for oral health, but further analyses of biofilm acidogenicity and other enzymatic activity changes are needed to verify whether continued intake of probiotics for longer periods may have a significant impact on dental plaque microbiota.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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