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ORIGINAL ARTICLE

Short- term effect of probiotic *Lactobacillus reuteri* consumption on the salivary microbiome profile of subjects undergoing orthodontic treatment with fixed appliances

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ABSTRACT

Objective: This prospective clinical study aim was to analyze the effect of the probiotic Lactobacillus reuteri Prodentis lozenges on salivary microbiome of subjects wearing fixed orthodontic appliances.

Methods: Saliva samples were collected prior to consumption and 14th-day post probiotic lozenges consumption (n=40, age 18–23). Oral hygiene index-score (OHI-S) and papilla bleeding index (PBI) were recorded. The salivary microbiome was profiled by next-generation sequencing using the V3-V4 region of 16S-rRNA. Microbial composition, diversity and taxonomic biomarkers were analysed in comparison to probiotic intervention and the clinical characteristics of the cohort using standard bioinformatics tools.

Results: The diversity and bacterial community structures did not change significantly in salivary microbiome of periodontally healthy subjects during short-term probiotic intervention. Probiotic consumption correlated with reduction of OHI and PBI scores (50% reduction of scores, $P<0.001$). The reduction of clinical indices was evident in conjunction with significantly reduced abundance of oral pathogens, such as Porphyromonas pasteri, Treponema sp., Fretibacterium fastidiosum, Kingella oralis and Propionibacterium acnes.

Conclusion: Short-term probiotic intervention helped maintaining good oral health in patients undergoing fixed orthodontic therapy. Although overall oral microbiome structure remained largely unchanged, a significant alteration in the abundance of health and disease-associated species highlighted the beneficial effect of probiotic.

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Probiotic; *Lactobacillus reuteri*; oral microbiome; fixed orthodontic appliance

Background

Fixed orthodontic treatment is a risk factor for the development of various oral diseases, such as dental caries, periodontal disease, and candidiasis. The presence of brackets, bands, and arch wire complicates oral hygiene practices and increases the accumulation of dental plaque. Hence, patients wearing a fixed orthodontic treatment are at higher risk for developing oral diseases [1]. Therefore, orthodontic patients need supplementary oral hygiene measures in addition to personal mechanical oral hygiene practices. Probiotics, which are defined as 'living microorganisms which, when administered in adequate amounts, confer a health benefit on the host' by the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) have been shown to confer various health benefits to humans, including improvement of oral health [2].

Lactobacillus reuteri is a probiotic bacterium known to confer various health benefits to humans [3]. It produces the antimicrobial substances reuterin and reutericyclin, which are active against a wide range of pathogenic organisms [4]. Previously, we performed a pilot study on the beneficial health effects of lozenges containing the probiotic *L. reuteri* on a few orthodontic patients [5]. We found that consumption of probiotics for 2 weeks significantly reduced the number of pathogenic bacteria in the patients' saliva. Moreover, other studies using appropriate *in vitro* and *in vivo* models have demonstrated that *L. reuteri* is able to modulate host inflammatory responses [6]. However, clinical studies that examine the effect of the probiotic *L. reuteri* on the oral microbiome are sparse in the literature.

To our knowledge, there is no information available regarding how *L. reuteri* probiotics regulate the oral microbiome in periodontally healthy subjects

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Supplemental data for this article can be accessed here.

using fixed orthodontic appliances. Considering this research gap, we aimed to analyze the short-term effect of the consumption of the probiotic *L. reuteri* Prodentis lozenges on the salivary microbiome profile of subjects wearing fixed orthodontic appliances.

Materials and methods

Cohort characteristics

The subjects undergoing fixed orthodontic therapy were included in this open-labelled prospective clinical trial. Ethical approval for the study was obtained from the Institutional Review Board, Faculty of Dentistry, Universitas Indonesia (approval number 100,701,020). The inclusion criteria included an age above 18 years, undergoing orthodontic treatment with a fixed appliance of a minimum of 1 year and no consumption of probiotics or antibiotics within the past 3 months. Exclusion criteria included subjects who had systemic diseases, such as hypertension or diabetes, or were on systemic drugs *i.e.* antihypertensives, analgetics, hormonal drugs, sedatives, anti-seizure medication and subjects with features of severe periodontal diseases and who were allergic to probiotics. The lozenges containing *L. reuteri* Prodentis were obtained from BioGaia, Stockholm, Sweden. BioGaia Prodentis lozenges (800 mg of one lozenge contained a minimum of 2×10^8 live *L. reuteri* Prodentis) were a food supplement for oral health containing the patented lactic acid bacterium *Limosilactobacillus reuteri* (formerly known as *Lactobacillus reuteri*) Prodentis. They also contained a strain combination of *L. reuteri* DSM 17938 and *L. reuteri* ATCC PTA 5289 that was expected to help maintain a good oral health.

To standardize the oral hygiene practice, each participant was given a toothpaste and a toothbrush during the study period. The participants were given oral hygiene instructions and were requested to brush their teeth twice a day. Each subject was given one lozenge per day, containing the probiotic *L. reuteri* for 14 days to be taken once a day after brushing teeth, followed by breakfast. Saliva samples were collected from the subjects on the day of the recruitment prior to the consumption of probiotic lozenges on the 14th-day post-consumption of the probiotics.

In addition, clinical data pertaining to the oral hygiene index score (OHI-S) and papilla-bleeding index (PBI) were recorded at each visit. Previously, it had been found that consumption of probiotic *L. reuteri* Prodentis over 14 day-duration is able to exert considerable impact on reducing clinical parameters (Periodontal Index-PI, OHI-S and Gingival index-GI) in healthy test subjects compared to a placebo control [7,8].

Sample collection and DNA extraction

Prior to sample collection, subjects had to take a rapid antigen test for SARS-CoV-2 detection due to the COVID-19 pandemic (as of 30 September 2020) and the result of the test had to be negative for taking part in the study. Following this confirmation, sample specimens from the subjects were collected with a cover of a complete set of personal protective equipment. A standard method was used for the saliva sample collection [9]. In brief, the subject was made to sit quietly, bending the head forward and opening the mouth to allow the saliva to drip passively from the lower lip. Saliva was collected using sterile, preweighted tubes. Subjects were requested to refrain from food for 2 h prior to the sample collection. Following the collection, the saliva samples were stored at −80°C prior to DNA extraction.

Microbiome profiling of the saliva samples was performed using a previously established protocol with some modification [10]. In brief, DNA was extracted from the samples using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) per the manufacturer's protocol. Extracted DNA was subjected to a quality check using Nanodrop™ 2000 (Thermo Fisher, Waltham, MA) for its purity and its concentration of gDNA using a Qubit 3.0 fluorometer (Thermo Fisher, Waltham, MA).

16S rRNA amplicon library preparation and sequencing

Polymerase chain reaction (PCR) amplifications had been finished for library preparation prior to sequencing as previously described [11]. Primers targeted for the V3-V4 region of 16S rRNA and modified with Illumina overhang adapters had been amplified by using PCR and the primers: F (5′ - TCGTCGGCAGCGTCAGATGTGTATAAGAGAC-AGCCTACGGGNGGC WGCAG-3′) and R (5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGA-CAGG ACTACHVGGGTATCTAATCC −3′). The PCR amplification was done as described previously [10]. The amplified library was subjected to purification using AMPure XP beads (Beckman Coulter Genomics, MA), visualized via agarose gel electrophoresis and quantified by using the Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA). The amplified library after the second purification using AMPure XP Beads was eluted, normalized, and pooled to the recommended concentration as per the manufacturer's protocol (80 pM). An internal 5% spike-in control using the Phi-X manage v3 library (Illumina, San Diego, CA) was pooled together with the normalized amplicon library. Sequencing was performed using the iSeq 100 instrument with the i1 v2 reagent (Illumina, San Diego, CA) with the

parameters of 2 × 150 cycles paired-end reads, with 10 cycles for every index, in line with the manufacturer's protocols.

Data analysis

Sequencing runs were first analysed in the Illumina's Sequencing evaluation Viewer version 2.4.7 for analytical run quality controls assessments [12–14]. Preprocessing of the raw sequencing data and the Quality Control (QC) was assessed as previously described [15]. The 16S metagenomics workflow based on the Local Run Manager v2.0 used the Illumina-curated version of the Greengenes database (greengenes.secondgenome.com/downloads/database/13_5) for taxonomic classification of 16S rRNA centred amplicon reads. It generated a total of 1,282 operational taxonomic units (OTUs) clustered at 97% identification with an open-reference OTU picking approach. The resulting accumulation curves showed reasonable sequence saturation for subsequent analysis (Supplementary Figure 1).

Alpha diversity (Chao1, Shannon, ACE), along with rarefaction curves, was evaluated and visualized using the MicrobiomeAnalystR Platform and the 'phyloseq' and 'ggplot' and 'microbiomeseq' [16,17] codes obtained from GitHub (https://github.com/xia-lab/MicrobiomeAnalystR). The microbial community structure was assessed by beta diversity with the respective algorithms implemented in the MicrobiomeAnalystR package using Principal Coordinate Analysis (PCoA) and the Bray–Curtis's dissimilarity indexes retrieved from sample pairwise comparisons based totally on weighted Unifrac distances. Significance of the community structure analysis was performed using the permutational multivariate analysis of variance (PERMANOVA) test [18].

DESeq2 [19] analysis was run under default settings and q values were calculated using the Benjamini–Hochberg procedure to control for false discovery rates applying the MicrobiomeAnalystR. Based on both significance and biological relevance, differentially abundant microbial biomarkers were characterized using the linear discriminant analysis effect size (LEfSe) method with default parameters and an LDA log score threshold set to 2 with a P value less than 0.05 considered as statistically significant [20,21]. Species with a P value <0.1 on the univariate analysis with probiotic intervention were selected for further analysis. The relative abundance differences in the top 27 bacterial species between pre- and post-probiotic cohorts were compared by Welch's t-test using STAMP (v2.1.3) [22]. Further, the random forest (RF) algorithm was applied to determine the proper number of microbial predictors in random forest classifiers by maximizing the area under the curve of the receiver operator characteristic (AUC) with the default parameters of the 'RandomForest' (RF) package in R (v. 4.6.1). To avoid over-fitting of the data in the model, 5-time and 10-fold cross-validations were made. The resulting model was subsequently used for the validation cohort [23,24].

To identify key members of the microbiome (based on relative abundance) associated with 50% reduction of the oral hygiene index score from the baseline value after probiotic intake, the Boruta algorithm [Boruta package for R (v. 5.1)] was used to perceive species with the highest predictive power [25]. The relative abundance >0.1% and presence in >50% of the samples in at least one group were used for the above analyses unless otherwise specified.

Statistical analyses

Statistical analyses were carried out using the GraphPad Prism software (version 8) and the R package (http://www.r-project.org/). The Mann–Whitney rank sum test or the Kruskal–Wallis test was used to evaluate variations among groups in the diversity indices of the oral microbiota. False discovery rate (FDR) values were used for the evaluation of the Benjamini–Hochberg method to control for multiple testing – adjusted P values lower than 0.05 were considered as statistically significant [19]. The analysis was confined to species with a prevalence greater than 10% and a maximum proportion (relative abundance) greater than 0.002. An FDR-adjusted P value (or Q-value) <5% was considered as significant.

The Boruta algorithm was used to identify the most predictive taxonomic biomarker based on the importance values determined by RF, indicated as 'confirmed' in the Boruta output [25]. The importance value pertaining to a taxon was calculated by RF based on the loss of accuracy by random permutation of the abundance profile of the taxon. To assess whether the importance was significant, the Boruta algorithm compared the observed importance to those produced by the spiked-in 'shadow' taxa, which were randomized versions of original taxa; hence, this algorithm typically gave more strength to identify taxa that jointly predicted a phenotype [26].

Results

Effect of probiotic intake on the clinical parameters

The mean age of the participants (n = 20; male – 4, female – 16) was 21 years (SD ± 1.59) across all samples (Figure 1A). Consumption of probiotic *L. reuteri* lozenges for 14 days improved the oral health parameters of the subjects as measured by the reduction of the OHI-S ($P < 0.0001$) and PBI scores ($P < 0.0056$) (Figure 1B).

Effect of probiotics on taxonomic composition and complexity of the salivary microbiome

We then taxonomically profiled each group based on the relative abundances of microbial clades at distinctive taxonomic tiers. Five phyla made up >1% of the community in at least one sample with two phyla naming, *Firmicutes* and *Proteobacteria*, dominated the groups with relative abundance (33–30% and 33–26% in the pre and post-probiotic groups, respectively) among the top 10 predominant phylum groups. *Actinobacteria* and *Bacteroidetes* were present in all samples, however to a lesser extent (16–11% and 22–10% in the pre- and post-probiotic groups, respectively) (Figure 2A). Lower-abundance phyla such as *Spirochaetes* were increased in post-probiotic organisms, and the so-called 'environmental' phyla such as SR1 were detected in similar level of consistency. In the top 15 genus level, *Streptococcus* and *Haemophilus* made up for almost 28% of the total reads, with 27% and 28% in the pre- and post-probiotic groups, respectively. *Rothia, Actinomyces, Fusobacterium, Neisseria,*

a)

Variable	Subjects (n=20)
Age (years)	21(SD± 1.59)
Gender	
Male	16
Female	4
OHIS index (Simplified Oral Hygiene index)	
Pre- Probiotic group	0.60 (SD± 0.50)
Post- Probiotic group	0.30 (SD± 0.36)
PBI index (Papillary Bleeding Index)	
Pre- Probiotic group	0.34 (SD± 0.47)
Post- Probiotic group	0.06 (SD± 0.10)
DMF-T index (Decayed, Missing, and Filled Teeth)	2.5 (SD± 2.25)
Orthodontic Treatment Usage Period (in months) at the time of recruitment	18 (SD± 4)

b)

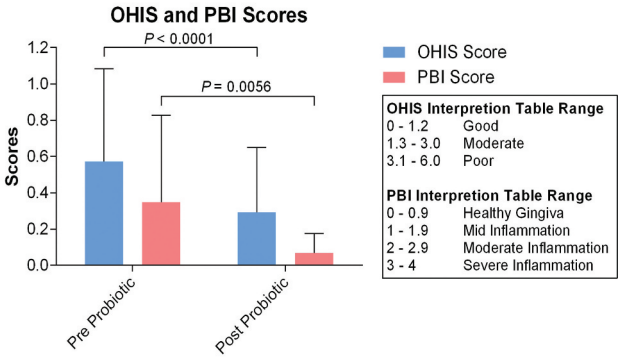

Figure 1. Demographic and clinical evaluation of individuals with good oral hygiene using orthodontic-fixed appliances prior and post-probiotic *L. reuteri* lozenges supplementation. (**A**) Baseline characteristics of the study participants in both intervention groups. (**B**) Two clinical outcomes were evaluated before and after 14 days of probiotic intervention. The Simplified Oral Hygiene index (OHIS) for determining the soft sediments classified with microbial signatures designed no plaque (0), mild [1- (1/3) covered with soft plaque], moderate [2-(1/3-2/3) covered with soft plaque], bad [3- (> 2/3) covered with soft plaque] according to the Green – Vermillion – Hirschman index. The Papillary Bleeding Index (PBI) used to determine the presence or absence of inter- dental plaque; designed no bleeding (0), only one bleeding point appearing [1], several isolated bleeding points or a small blood area appearing [2], inter dental triangle filled with blood soon after probing [3], and profuse bleeding when probing led to blood spreads towards the marginal gingiva [4]. All subjects showed significant oral health improvement, as measured by the reduction of the OHIS and PBI scores ($P < 0.001$), by the end of the probiotic supplementation.

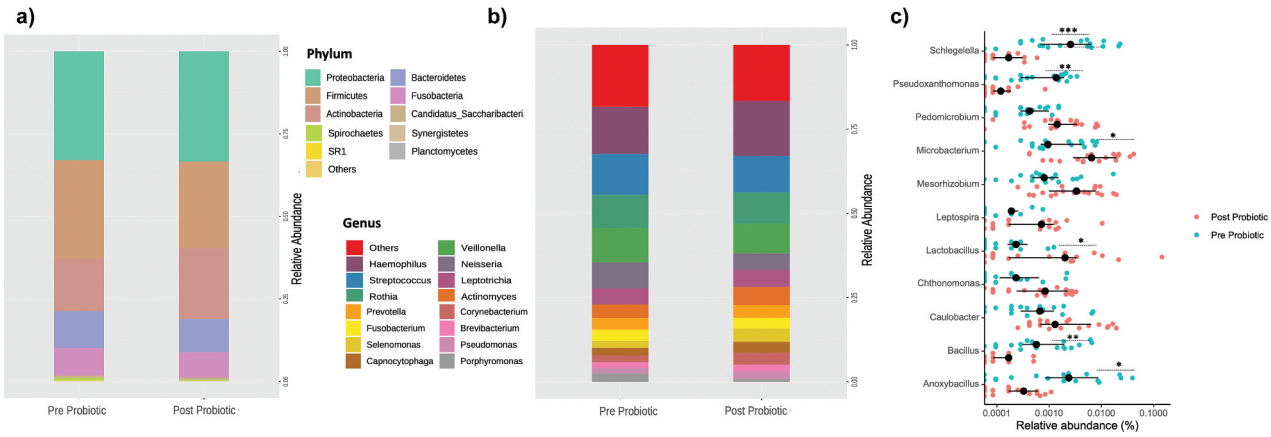

Figure 2. Taxonomic composition of the salivary microbiome before and after probiotic supplementation. (**A**) The top 10 relative abundances of the microbial phyla highlights the major role played by *Firmicutes* and *Proteobacteria* (green and brown) in the microbiome, and to a less extent by *Actinobacteria* and *Bacteroidetes* (light brown and purple). (**B**) At the top 15 genus level, *Haemophilus, Streptococcus, Veillonella, Rothia* and *Neisseria* (maroon, blue, dark green, light green and light purple) are shown to be the most abundant clades. (**C**) Out of the genera accounting for less than 1% of the samples which are grouped in the 'Other' category, *Schlegelella, Bacillus, Pseudoxanthomonas, Lactobacillus, Microbacterium* and *Anoxybacillus* were significantly increased after probiotic intake. Letters indicate statistical differences between samples are $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Veillonella, Prevotella, Selenomonas and *Leptotrichia* were also detected in all samples with uneven quantitative distributions (Figure 2B). At the species level, *Haemophilus parainfluenzae, Rothia dentocariosa, Leptotrichia hongkongensis, Haemophilus haemolyticus* and *Neisseria elongata* were the main species components in both the pre- and post-probiotic groups (relative abundance >3%). Overall, taxonomic phyla and genus abundance distributions of these dominant oral microbes (top 10 taxa) were minimally influenced by short-term probiotic intervention. Out of lesser abundant genera (relative abundance <1%), *Schlegelella* (P = 0.00043), *Bacillus* (P = 0.0011), *Pseudoxanthomonas* (P = 0.003), *Lactobacillus* (P = 0.037), *Microbacterium* (P = 0.043), and *Anoxybacillus* (P = 0.037) were significantly increased following the probiotic intervention (Figure 2C).

The microbial community composition of the saliva samples at baseline and 2-week post-consumption was analysed. There was no significant difference in the alpha diversity (Shannon – P value 0.698; [Mann–Whitney]) and evenness (Chao1 – P value 0.881; ACE – P value 0.989; Simpson – P value 0.62 [Mann–Whitney]) of the post-consumption samples compared to the baseline (Figure 3A). Post-consumption saliva samples had a slightly higher alpha diversity than pre-probiotic intake samples, although the difference was not statistically significant. To understand whether the quantitative structure of the microbiome changed, PCoA on the taxonomic profiles using the weighted UniFrac measure was performed. Ordination analysis showed no obvious clustering by pre-defined groups, suggesting that the overall community structure of the saliva microbiome was not stricken by short-term

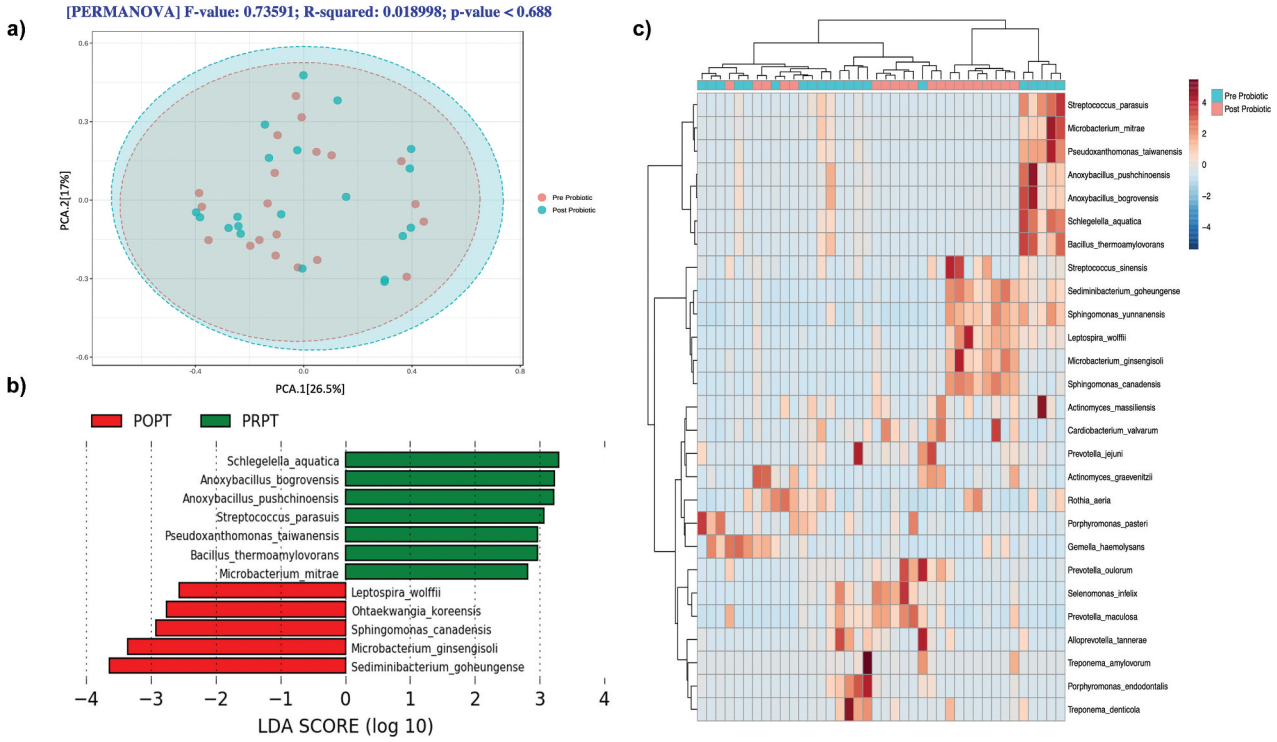

Figure 3. Structural composition and diversity comparison of the salivary microbiome before and after probiotic intervention. The overall structure of the saliva microbiome is not affected by short-term probiotic intake. (**A**) α-diversity boxplot (Shannon, Chao1 and Simpson-reciprocal indices) of the salivary microbiome between pre- and post-probiotic cohorts. Boxes represented the 25th to 75th percentile of the distribution; the median was shown as a thick dot in the middle of the box; whiskers extend between the 25th and 75th percentiles. (**B**) No apparent clustering trend can be noticed (P > 0.05) in the PCoA space when the non- quantitative unweighted UniFrac measure is used as beta-diversity, suggesting that a change in the relative abundances of the main components of the salivary microbiome is not the driving feature characterizing between probiotic and respective baseline samples. Species with a P value <0.1 on the univariate analysis with probiotic intervention were selected to define the top most abundantly differentiated across the study cohort. (**C**) Heatmap of the top 27 abundant species differentiates between probiotic and baseline samples. The color intensity in each box indicates the relative percentage of species in each sample. Relative abundance data were z-scored normalized by row. To show the distribution of abundant OTUs, the relative abundances of OTU data were normalized to have a mean of 0 and a standard deviation of 1 (z-score normalization). (**D**) Random Forest algorithm with 5 times 10- fold-cross validation used to identify a minimal set of bacterial species that maximally differentiated between pre- and post-probiotic intervention. *Schlegelella aquatica, Microbacterium mitrae, Microbacterium ginsengisoli*, and *Streptococcus parasuis* ranked the predominant difference between two cohorts under probiotic intervention. (**E**) P < 0.01 to detect species with the most statistically significant differences filtered using a q-value of 0.05 and effective size of 0.05 threshold in STAMP illustrating significant bacterial species in variations of different proportions using Welch's t-test. See also Supplementary Figure 2 for LEfSe results.

probiotic intake (PREMANOVA P value <0.688) (Supplementary Table 1) (Figure 3B).

Increased complexity of the salivary microbiome following short-term probiotic consumption

We performed a linear discriminant analysis effect size (LEfSe) to determine which microbial clades, if any, could explain the slight increase in the diversity of the salivary microbiome following short-term probiotic intervention. The LEfSe analyses identified 12 species in the salivary microbiome whose over-abundance discriminated between pre- and post-probiotic samples (Figure 3 B and C) (full details are given in Supplementary Table 2 and Supplementary Figures 2 and 3). Pre-probiotic samples had a significant enrichment of species (LDA log score at a threshold of 3.0): *Schlegelella aquatica* (*Proteobacteria), Streptococcus parasuis* (*Firmicutes), Anoxybacillus pushchinoensis* (*Firmicutes), Anoxybacillus bogrovensis* (*Firmicutes), Pseudoxanthomonas taiwanensis* (*Proteobacteria*). On the other hand, *Sediminibacterium goheungense* (*Bateriodetes*) and *Microbacterium ginsengisoli* (*Actinobacteria*) OTUs gave significance at the same effect size threshold for discriminating samples with probiotic consumption. Further analysis using 10-cross validation RF modeling (Figure 3D) and STAMP methods (Figure 3E) showed that few of these identified species, *S. aquatica, Microbacterium mitrae, M. ginsengisoli* and *S.* parasuis, reflected the predominant difference between pre- and post-probiotic cohorts under the short-time probiotic intervention.

We used the Boruta feature selection algorithm coupled with the RF classifier to examine which differential microbiome markers (predictors) at the species level accounted for 50% reduction of observed clinical indexes *i.e.* OHIS and PBI scores (response variables) with probiotic intervention. As evidenced by a decreased predictive mean square error compared to a random guess, a predictive model utilized 18 species with significant predictive power substantiated by LEFfSe analysis (Figure 4 C and 4D) (Supplementary Table 3). Among the identified microbiome markers, there was a reduction in disease-associated pathogens, such as *P. pasteri, Treponema* sp. and *Prevotella* sp., which correlated with the 50% reduction of the OHIS index score group (OHIS50-Y/Yes) (Figure 4G). Based on feature selection by Boruta, over-abundance of *M. ginsengisoli, S. canadensis* and *A. suimastitidis*, and reduced relative abundance of *F. fastidiosum* were highly discriminative for the 50% reduction of the OHIS index score with probiotic intervention (Supplementary Figure and Table 3) (Figure 4C). On the other hand, by both LEfSe and Boruta feature selection; overabundance of *C. leadbetteri* and decreased abundance of *K. oralis, P. acnes, S. aquatica*, and *A. bogrovensis* were identified in the 50% reduction of the PBI index score group by probiotic intake (PBI50-Y/Yes) (Supplementary Figure and Table 3) (Figure 4D). These species may independently be useful for predicting the 50% reduction of the PBI index score with probiotic intervention. The key species affected by probiotic intervention according to the clinical parameters are presented in heatmaps (Figure 4F and 4G). Moreover, the chord diagram was used to delineate species influenced by different clinical states before and after probiotic intake (Figure 4E). The chord diagram was divided into five sections representing the two clinical states of PBI and OHI-S before and after probiotic intake. Key species identified in two or more clinical states are indicated by arcs (chords) connecting the different clinical states.

Re-analysis of the overall salivary microbiome profiles using only the foregoing species with significant predictive power yielded some interesting results. PERMANOVA analysis significantly differentiated the salivary microbiome composition following probiotic intervention [between OHIS50-N/No and OHIS50-Y/Yes groups (P = 0.008) and PBI50-N/No and PBI50-Y/Yes (P = 0.001)] (Figure 4 A and B).

Persistence of the *Lactobacillus* genus in the salivary microbiome after probiotic intake

The *Lactobacillus* strain present in the probiotic product (*L. reuteri*) was not detected in the probiotic-intake samples. However, we observed the presence of a significantly higher-abundance OTU attributable to the *Lactobacillus* genus in the post-probiotic samples (Figure 2C).

The foregoing results suggested that the short course of the probiotic *L. reuteri* lozenges could potentially modulate the salivary microbiome, albeit the effect was limited in both the breadth of the change and in its temporal persistence.

Discussion

There has been some evidence from clinical and fundamental studies suggesting that fixed orthodontic appliances affect the oral health of the patients [27,28]. The effect of probiotics on various microbiological and clinical parameters in fixed orthodontic patients has produced conflicting evidence [1,29–35]. The present study evaluated the effect of oral probiotics on the salivary microbiome of periodontally healthy subjects under orthodontic treatment with fixed appliances using a prospective interventional study. The oral health of the subjects was significantly improved following 14 days' intake of the probiotic *L. reuteri* Prodentis lozenges. Although the overall diversity or evenness of the salivary microbiome did

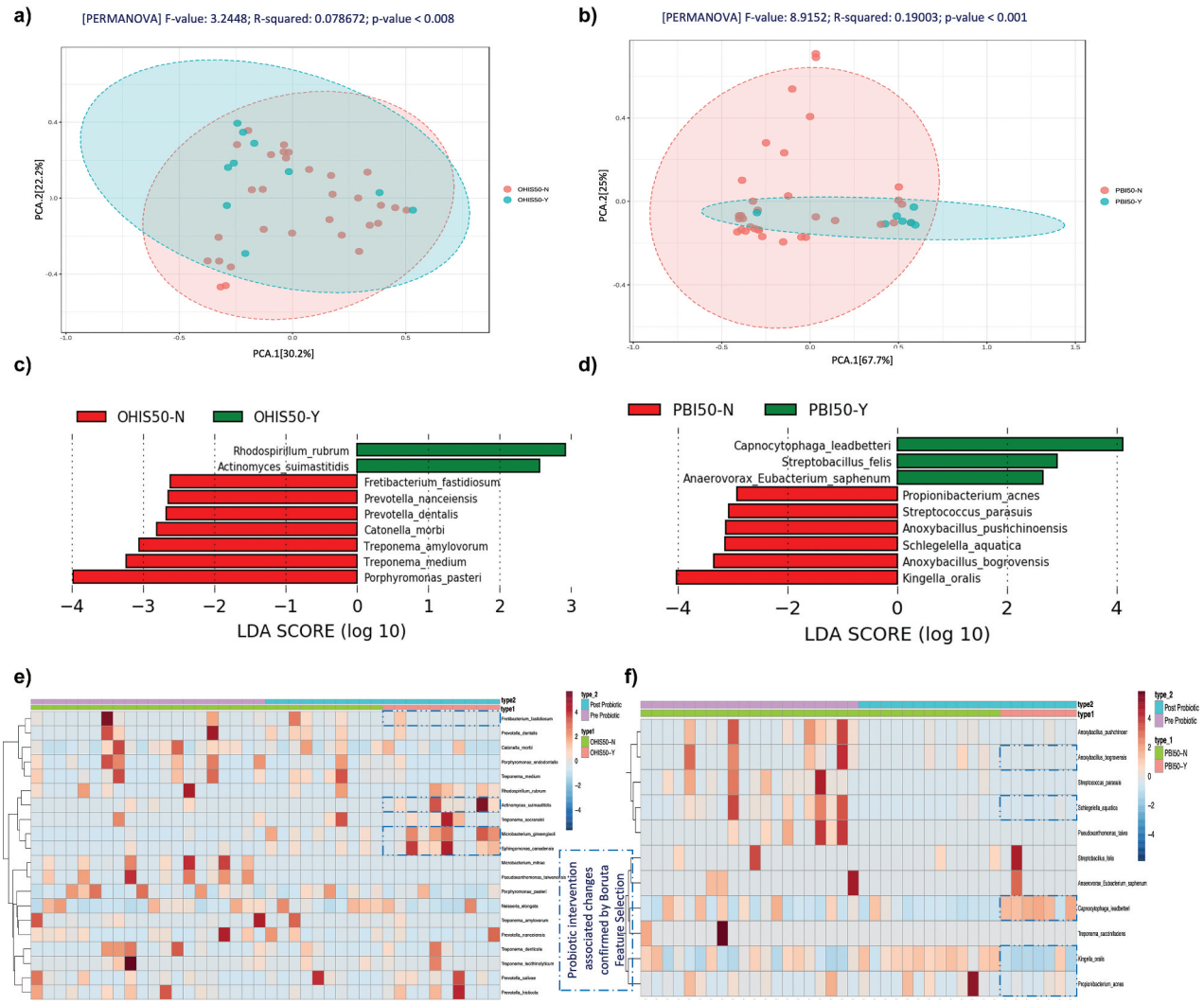

Figure 4. Increased complexity of the salivary microbiome in conjunction with 50% reduction of the oral hygiene index scores in subjects with fixed orthodontic appliances. (**A**) and (**B**) Apparent clustering in structural salivary composition observed when coupled with 50% reduction of the oral hygiene index scores (OHIS and PBI) after probiotic intervention. (**C**) and (**D**) Significant differentially abundant taxonomic biomarkers contribute to the change in the microbiome composition identified by LEfSe analysis. (**E**) The chord diagrams show the key taxa identified by the five different strategies (Baseline, post-probiotic; OHIS50-Y, OHIS50-N,PBI50-Y and PBI50-N). Key species identified in two or more clinical states are indicated by arcs (chords) connecting the different clinical states. (**F**) and (**G**) Heatmap of most abundant species among the OHIS50 groups differentiates between probiotic and baseline samples. Species highlighted in dotted blue squares are distinguished to have a strong predictive power for 50% reduction of both the OHIS and PBI index scores with probiotic intervention identified by the Machine learning Boruta algorithm coupled with the Random Forest classifier. See also Supplementary Figure 4. Abbreviations: PRPT- Pre-Probiotic group, POPT- Post-Probiotic group, OHIS50-N/No and PBI50-N/No – baseline and no change of OHIS and PBI index score groups, OHIS50-Y/Yes and PBI50-Y/Yes – 50% reduction of OHIS and PBI index score groups in probiotic intake.

not change significantly, there were minor changes in the abundance of disease-associated species following probiotic intervention.

Fixed orthodontic treatment may pose a significant challenge to maintain oral health of the patients as the appliance traps dental plaque biofilm leading to increased level of potential oral pathogens [36,37]. The use of probiotics as an adjunct therapy has been suggested for various diseases including periodontitis. Overall, the administration of probiotics, either as an adjunct to mechanical plaque control or as a single intervention, has yielded conflicting results [38]. Some studies have proven the advantages of probiotic consumption in reducing the extent of gingival inflammation and the amount of plaque [39,40]. In contrast, other studies have failed to exhibit additional clinical benefits [41–43]. Moreover, several investigations documented the varying impact of long-term probiotic intervention (maximum up to 1 month) on maintaining gingival health in orthodontic patients, some with beneficial improvements [44,45] while some without improvement [30,31]. There are ample evidence in the literature supporting the beneficial effects of oral *L. reuteri* such as its capability: (i) to inhibit plaque accumulation [46], (ii) to reduce cariogenic *mutans* streptococci [47,48], (iii) to improve clinical parameters when used as an adjunct therapy for periodontitis [7,8,46] and (iv) to exert beneficial

immunomodulatory and anti-inflammation effects [46,49]. *L. reuteri* has been considered as a potential probiotic adjunct together with non-surgical periodontal therapy for periodontitis patients [7,8,50]. In our study, we observed reduced gingival bleeding with improved PBI scores and overall oral hygiene improvement by the OHIS index in periodontally healthy individuals undergoing fixed appliances for orthodontic treatment after intake of the probiotic *L. reuteri* Prodentis lozenges. The observed difference in the improved oral health may be attributed to the short-term probiotic intervention. However, according to a recently published review, no consensus can be reached on the significant effect of oral probiotics on gingival inflammation and enamel decalcification in patients undergoing treatment with fixed orthodontic appliances [29]. Therefore, in the presented study, we investigated whether the impact of probiotic would be more pronounced by defining our study cohort with respect to 50% reduction of the oral clinical state from their respective baseline score.

In this context, there were some interesting findings with regard to the change in key species associated with the salivary microbiome. Two weeks' probiotic intake reduced the disease-associated pathogens, such as *Porphyromonas* sp., *Treponema* sp., *Prevotella* sp. and *F. fastidiosum,* which correlated with the 50% reduction of the OHIS index score group (OHIS50-Y), suggesting improvement of oral health. Moreover, probiotic interventions decreased the abundance of the periodontal pathogens *K. oralis* and *P. acnes*, which correlated with the 50% reduction of the PBI index score group (PBI50-Y). Hence, probiotic intervention considerably eliminated the dysbiotic oral microbiome by reducing disease-associated pathogens and enriching health-associated species. *K. oralis* is normally found in small numbers in the oral cavity in dental plaque biofilm [51]. It has been reported that the species increased significantly in periodontitis *i.e.* more than 5% of the total microbiota in periodontitis sites compared to the meagre number (0.4%) in periodontally healthy individuals. Additionally, *P. acnes* and *F. fastidiosum* have been shown to be associated with gingivitis [52] and periodontitis [53], respectively. Significant reduction of these species indicated the potential modulatory activity of probiotic intake. Moreover, *Capnocytophaga* species, known to be associated with healthy periodontium, increased following probiotic intervention [54].

Fixed orthodontic appliance could be considered as a condition that may significantly change the local microenvironment in dental plaque biofilm, consequently resulting in an altered salivary microbiome [55]. Previous studies have shown significant dysbiosis in both the saliva microbiome and supragingival plaque microbiome with substantial replacement of anaerobic bacteria in patients wearing fixed orthodontic appliances [36,37]. In the present study, we observed the presence of certain facultative anaerobes like *S. parasuis, Anoxybacillus* sp., *Bacillus thermoamylovorans* and mesophilic anaerobes like *M. mitrae* in the salivary microbiome of patients under fixed orthodontic treatment. Probiotic intervention reduced the proportion of these taxa. However, the absence of a placebo control group in the present study is a major limitation for a comprehensive comparison. Moreover, we did not detect the presence of the administrated probiotic strain *L. reuteri* in the post-intervention salivary microbiome. However, this does not exclude the probability that this probiotic strain colonizes in dental plaque biofilms. Therefore, future studies should aim at analyzing both saliva and dental plaque samples from the subjects. On the other hand, according to the key-stone plaque hypothesis, species with low-abundance may be able to modulate the niche environment [56,57]. Therefore, further research at a molecular level is warranted to investigate the molecular mechanism behind the modulation of the oral microbiome by the probiotic *L. reuteri* strain. It is also noteworthy that our results do not imply an explicit causative relationship between the microbiome changes with the reduction of clinical parameters, considering the potential confounders and a smaller sample size. Nevertheless, the changes of certain key microbes associated with health and disease are restructured during the transition from the baseline to the 50% reduction of the clinical state. This change could be attributed to the probiotic intervention.

> Future studies should be designed to examine the effect of long-term probiotic intervention

with an appropriate placebo control group in a large clinical cohort for more convincing evidence. Changes of the oral microbiota by the end of probiotic consumption after removal of fixed appliance as well as comparative evaluation of these changes prior to the bonding of orthodontic brackets should also be considered. Despite the foregoing limitation, the present study has provided valuable clinical and microbiological evidence on the health benefits of short-time administration of the probiotic *L. reuteri* lozenges on oral health. Hence, probiotic consumption may provide better oral health benefits by preventing accumulation of pathogenic microbiota while enriching symbiotic organisms during orthodontic treatment. These changes in the oral microbiome may have played a role in the observed improvement of the clinical parameters in the present study.

Conclusion

Within the limitations of the present study, it can be concluded that a short-term probiotic intervention

helped maintaining good oral health in patients undergoing fixed orthodontic therapy. Although there was no overall change to the microbiome structure, significant modulation in the abundance of the health and disease-associated species highlighted the health benefits of probiotic intervention on the oral microbiome and oral health of patients undergoing orthodontic treatment.

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Authorship contribution statement

ASW, CJS and JK designed the study. ASW, MIH and MOR collected the samples. MR performed the laboratory experiments. NS and CJS analyzed the data. ASW, NS and CJS wrote the paper. All authors discussed the results and commented on the manuscript.

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