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# Oral probiotics reduce halitosis in patients wearing orthodontic braces: A randomized, triple-blind, placebo-controlled trial

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**Oral probiotics reduce halitosis in patients wearing orthodontic braces: A  
randomized, triple-blind, placebo-controlled trial**

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Compounds

## Oral probiotics reduce halitosis in patients wearing orthodontic braces: A randomized, triple-blind, placebo-controlled trial

### Abstract

Orthodontic braces can impede oral hygiene and promote halitosis. The aim of the study was to investigate the effect of the oral probiotic *Streptococcus salivarius* M18 on oral hygiene indices and halitosis in patients wearing orthodontic braces. The study was a prospective, randomized, triple-blind, placebo-controlled trial. Patients undergoing fixed orthodontic treatment were randomly allocated to a probiotic group (n=32) and a placebo group (n=32). Patients consumed two lozenges per day for one month. Assessments were taken at baseline, at the end of the intervention, and at a 3-month follow-up. The outcome measures were plaque index (PI), gingival index (GI) and halitosis-causing volatile sulfur compound (VSC) levels. The dental biofilms before and after the intervention were analysed utilising next-generation sequencing of bacterial 16S rRNA genes. PI and GI scores were not significantly influenced by the probiotic intervention (intervention  $\times$  time:  $p > 0.05$ ). The level of VSCs decreased significantly in both the probiotic group (VSC reduction = -8.5%, 95%CI = -7.4% to -9.1%,  $p = 0.015$ ) and the placebo group (-6.5%, 95%CI = -6.0% to -7.4%,  $p = 0.039$ ) after 1-month intervention. However, at the 3-month follow-up, the VSC levels in the placebo group returned to baseline levels whereas those in the probiotic group decreased further (-10.8%, 95%CI = -10.5% to -12.9%,  $p = 0.005$ ). Time, but not treatment, was associated with the decrease in microbial community alpha diversity and a modest effect on beta diversity. Oral probiotic *S. salivarius* M18 reduced the level of halitosis in patients with orthodontic braces, but had minimal effects on plaque index, gingival index and dental biofilm microflora.

**Registry number:** ANZ ACTRN12615000341527

**Oral probiotics reduce halitosis in patients wearing orthodontic braces: A  
randomized, triple-blind, placebo-controlled trial**

**Introduction**

Orthodontic fixed appliances, also known as “braces”, interfere with tooth brushing and facilitate accumulation of dental plaque (biofilm). Failure to maintain good oral hygiene during orthodontic treatment can result in adverse effects such as dental enamel demineralization, gingival inflammation and halitosis [1]. A positive association between halitosis and orthodontic braces has been confirmed, and halitosis has been considered an important indicator of oral health during orthodontic treatment [2].

Products to prevent biofilm build-up, such as interdental brushes, specialized orthodontic toothbrushes, antimicrobial mouth rinses and toothpastes, are commercially available, but their efficacy appears to be very limited [3,4].

Probiotics have been shown to be effective in reducing dental biofilm formation, preventing caries development and decreasing halitosis in children, adolescents, adults and the elderly [5-10]. However, to date, there are few randomised controlled trials (RCTs) examining the efficacy of oral probiotics in enhancing oral health. Even fewer studies have investigated the role probiotics can play in orthodontic patients [5]. Of the RCTs that have examined the effect of probiotics on orthodontic patients [11-15], none have tested the effect of probiotics on halitosis or used the probiotic *S. salivarius* M18, a strain unique in its isolation from the mouth, not the gastrointestinal tract.

The aim of this trial was to determine the effect of oral probiotic *S. salivarius* M18 on oral hygiene indices (*i.e.* plaque index and gingival index) and halitosis in patients wearing orthodontic braces.

## Materials and Methods

### *Study design*

The study was a prospective, randomized, triple-blind, two-arm parallel group, placebo-controlled trial with a 1-month intervention and a 3-month follow-up. Study participants were recruited from patients undergoing orthodontic treatment at the Faculty of Dentistry, University of Otago. Data were collected between July 2015 and December 2015. An oral examination was taken at baseline, then after one and four months (Fig. 1). The investigators (GB, LM, MF) and participants were all blinded to the intervention given (probiotic or placebo). The study was approved by the University of Otago Ethics Committee (H14/103) and registered (ANZ ACTRN12615000341527).

### *Participants*

Sixty-four orthodontic patients (mean age  $14.9 \pm 3.2$  years, range 10-30 years; Table 1) wearing fixed appliances were recruited. The sample size determination was based on previous estimates of plaque index (PI) variability in adolescents requiring orthodontic treatment (SD = 0.4) [1,16]. To detect a decrease in the  $PI \geq 30\%$  with 80% power, a total of 58 patients (29 per group) were needed. To allow for possible dropout during the study, we recruited 64 patients.

Inclusion criteria were: having  $\geq 20$  natural teeth, wearing traditional braces (*i.e.* stainless steel brackets, Victory Series, 3M Unitek, Monrovia, California, USA) in both arches, and willingness to participate in the trial. Exclusion criteria were: living in a non-fluoridated area; periodontal disease; antibiotic therapy; wearing lingual braces; dental fluorosis; smoking; and allergy to dairy products. Eligible participants were informed of the study design and gave their written consent.

### *Randomization and allocation concealment*

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Balanced block randomization, with a block size of four and stratified by gender, was used to ensure equal patient allocation to each intervention group. The randomization sequence was created using an online random generator. Allocation concealment was achieved using sequentially numbered opaque sealed envelopes. The different lozenges were labeled 1 or 2 by the manufacturer. The label code was broken only when the intention-to-treat (ITT) analysis and statistical analyses were completed.

***Probiotic intervention and placebo control***

*Oral probiotic group:* participants took two lozenges of *S. salivarius* BLIS M18 bacteria (BLIS Technologies Ltd, Dunedin, New Zealand) each day during the 1-month intervention. The dose was  $3.6 \times 10^9$  *S. salivarius* colony forming units (CFUs)/lozenge. The remainder of the lozenge was isomalt (sugar substitute), tableting aids and peppermint flavoring.

*Placebo control group:* participants took two placebo lozenges (BLIS Technologies Ltd) each day during the 1-month intervention. The packaging, appearance and taste of lozenges in both intervention and control groups were identical. An additional 20 mg isomalt was used in place of the probiotic in the placebo lozenges.

To enhance self-dosing compliance, each participant was contacted each morning and night by text message and email to remind them to take the lozenges. Additionally, each participant was asked to fill in a reminder chart at home and return the blister packs at the end of the intervention period. The number of empty cavities on the blister packs was compared with the reminder chart completed by the participant to assess their compliance. All participants were informed not to use any other probiotic-containing products during the study.

***Study protocol and follow up***

The study comprised the following timepoints: baseline, 1-month intervention, 3-month intervention-free follow-up. Plaque Index (PI), Gingival Index (GI), Volatile Sulfur

Compound (VSC) levels, and plaque samples were collected at each timepoint. After baseline assessments, participants chewed a plaque-disclosing tablet (Disclotabs, Colgate-Palmolive, Sydney, NSW, Australia) until dissolved and removed the disclosed dental biofilm by brushing with a new toothbrush (Colgate® Ortho) and toothpaste (Colgate® Cavity Protection Toothpaste) under supervision (LM). The participants then rinsed their mouths with 30 ml chlorhexidine mouthwash (Savacol®, Colgate-Palmolive) and water before sucking the appropriate lozenge.

The participants were required to suck two lozenges each day, one after brushing their teeth in the morning and one after brushing their teeth at night.

### ***Study assessments***

The modified PI [17] was used to assess the amount of plaque on teeth and scored according to Löe [18]. In this PI scoring system, the teeth were divided into mesial, distal, gingival, and incisal (occlusal) regions in relation to the bracket. Plaque was then scored for each of the four areas based on four plaque index codes as used by Löe [18]. Four PI values were averaged for each tooth to give a mean PI tooth score. An overall mean PI for all teeth in the mouth was then calculated for each participant.

The modified GI was measured using a periodontal probe. Four areas around each tooth were scored according to Löe [18]. A tooth GI mean and a participant GI mean was calculated. The modified PI and GI indices used in this study accounted for the presence of brackets, making them more appropriate for use in an orthodontic sample [17].

VSCs in patients' breath samples were measured using a Halimeter (Interscan Corp., Chatsworth, USA). The validity of this equipment for the assessment of halitosis has been previously shown [19]. Following the manufacturer's instructions, the participants were asked to open their mouths slightly, and a straw connected to the Halimeter was placed inside the mouth without touching the teeth, tongue or other tissues. The patients were instructed to

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2  
3 breathe from the nose, not to blow or suck nor close lips. The VSC levels in breath were  
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5 recorded as parts per billion (ppb) of sulfide equivalents. Three measurements were taken for  
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7 each participant and the mean value calculated.  
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10 Supragingival plaque samples were collected with sterile dental probes from  
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12 buccal/labial/palatal and buccal/labial/lingual surfaces of all teeth mesial to, and including,  
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14 the first molars in both upper and lower arches and pooled. The samples were transferred to a  
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16 microfuge tube containing 400  $\mu$ L of buffer (25 mM Tris-HCl, pH 8.0, 2.5 mM EDTA and  
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18 1% [vol/vol] Triton X-100) and stored at -80°C.  
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### 21 ***Bacterial DNA extraction and purification***

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23 DNA was extracted and purified from the plaque samples using PureLink Genomic DNA  
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25 Mini Kits (Thermo Fisher Scientific, Auckland, New Zealand) in two batches. Each batch  
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27 contained 65 samples prepared for DNA sequencing (64 samples and 1 buffer control). The  
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29 first batch comprised baseline samples and the second batch samples taken at the end of the  
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31 1-month intervention. Each sample, which contained at least 20 ng of purified genomic DNA  
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33 at a concentration of at least 5 ng/ $\mu$ L was subjected to PCR amplification (11 cycles) and  
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35 Illumina Miseq sequencing (New Zealand Genomics Limited, Palmerston North, New  
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37 Zealand).  
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### 42 ***16S library preparation and Illumina MiSeq DNA sequencing***

43  
44 Barcoded amplicon libraries of the 16S rRNA V3-V4 regions were prepared for each sample  
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46 batch. The libraries were pooled by equal molarity before being subjected to Illumina MiSeq  
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48 sequencing. Amplicon sequences (2 x 250 bp) were processed using the DADA2 package  
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50 (version 1.6.0) in R [20]. The taxonomy was annotated using the naïve Bayesian classifier  
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52 method with the Silva reference database version 128 [21]. Downstream analyses were  
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54 performed using R (version 3.4.3), packages vegan (version 2.4.6) and phyloseq (version  
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56 1.22.3) [22]. For analysis of alpha diversity and beta diversity with Unifrac metrics, samples  
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were rarefied to a depth of 15,000 reads per sample. The rarefied data were also used to investigate the presence and abundance of *S. salivarius* M18, using the 16S rRNA gene of GCF\_000225385.1\_ASM22538v2 as a reference.

### ***Statistical Analysis***

An intention to treat analysis was carried out using mixed-models by SPSS (20.0, IBM, Chicago, Illinois, USA). The response variables were “PI”, “GI” and “VSC”. The variables “gender”, “intervention group” and “time” were entered as fixed factors, while study participants were entered as a random term. Where appropriate, Bonferroni-corrected post-hoc multiple comparisons were run. The efficacy of the probiotic versus the placebo intervention was tested by the interaction term treatment  $\times$  time.

The metagenomic analysis tested the null hypotheses that the treatment had no significant effect on alpha diversity (the number and distribution of taxa within samples) or beta diversity (differences in taxa between samples). The observed, Shannon, and Inverse Simpson alpha diversity metrics were calculated. The DESeq2 package (version 1.18.1) [23]; and Benjamini & Hochberg-corrected pairwise Kruskal-Wallis tests were used to test the null hypotheses that the abundance of genera was not different between timepoints nor treatment groups.

## **RESULTS**

The baseline characteristics of the participants are summarized in Table 1. The participant’s baseline values, including age, sex, PI, GI and VSC, were all similar.

The compliance of participants was generally good. Each participant was asked to take 60 lozenges in total (*i.e.* two lozenges/day for 1-month) and, on average,  $57.2 \pm 8.9$  lozenges were taken by each participant during the study. No significant discrepancy was found between participants’ reporting on the lozenge reminder chart and the number of lozenges

taken, as assessed by counting the empty cavities on the returned blister packs (paired Student's t-test,  $P > 0.05$ ; Pearson's correlation coefficient = 0.77,  $P < 0.001$ ). No adverse events were recorded during the trial.

***Plaque Index (PI) and Gingival Index (GI)***

PI scores were not significantly influenced by the intervention ( $F = 0.3$ ;  $P = 0.569$ ), or gender ( $F = 0.2$ ;  $P = 0.685$ ), or time ( $F = 1.6$ ;  $P = 0.201$ ). There was no significant interaction between intervention group and time ( $F = 1.0$ ;  $P = 0.353$ ), indicating that PI scores did not differ between the probiotic and placebo group over time (Fig. 2A). GI scores were not influenced significantly by the intervention ( $F = 0.1$ ;  $P = 0.867$ ), or gender ( $F = 0.6$ ;  $P = 0.455$ ). The interaction between intervention group and time was not significant either ( $F = 0.2$ ;  $P = 0.844$ ) (Fig. 2B).

***VSC levels***

The VSC levels were not significantly influenced by gender ( $F = 0.3$ ;  $P = 0.561$ ) but changed significantly over time ( $F = 7.9$ ;  $P = 0.001$ ). There was a significant interaction between intervention group and time ( $F = 6.4$ ;  $P = 0.01$ ). This interaction term represents the parallelism between VSC trajectories and its statistical significance indicates that the changes of VSC measurements differed between the probiotic and the placebo groups over time. Indeed, the VSC scores decreased significantly in the probiotic group (by 17 ppb,  $P < 0.015$ ) and placebo-control group (by 13 ppb,  $P = 0.039$ ) after the 1-month intervention. However, at the 3-month intervention-free follow-up, the VSC levels in the placebo group returned to a value ( $203 \pm 59$  ppb) similar to the baseline ( $204 \pm 69$  ppb) ( $P = 0.619$ ), whereas the VSC levels in the probiotic group decreased further ( $180 \pm 47$  ppb) from baseline ( $201 \pm 71$  ppb) ( $P = 0.005$ ) (Fig. 2C and Table 1).

The proportion of participants with high VSC levels (>150 ppb) at the baseline, 1-month intervention, and 3-month intervention-free follow-up was 81.3%, 78.1%, and 75.0% for the oral probiotic group, and 75.0%, 68.8%, and 84.4% for the placebo-control group.

### **Metagenomic analysis**

Treatment had no effect on any measure of alpha diversity ( $P > 0.05$ , Kruskal-Wallis test), but observed alpha diversity was significantly reduced for both treatment groups between time points ( $P < 0.001$ , Kruskal-Wallis test) (Fig. 3).

The contributions of time and treatment to total beta diversity was analysed by redundancy analysis, as assessed by Bray-Curtis, weighted Unifrac, and unweighted Unifrac distances. Total contribution ranged from 3.7% (Bray-Curtis) to 6.4% (Unweighted Unifrac). The null hypothesis of homogenous beta dispersion between groups was rejected for Bray-Curtis distance for time ( $P < 0.005$ ), and for weighted Unifrac distance for both time and treatment ( $P < 0.05$ ). Using the unweighted Unifrac distance, a significant effect of time ( $P < 0.001$ ), but not treatment, was found on community beta diversity using permutation-based anova. The stress of ordination (0.22) by unweighted Unifrac distance indicated poor representation of data in reduced dimensions (Fig. 4).

Abundance of genera was significantly associated with time (Table 2, Fig. 3). Nine genera were significantly associated with time by DESeq2 analysis and five of these by both DESeq2 analysis and the Kruskal-Wallis test. *Rothia* was the only genus significantly associated with treatment (DESeq2 analysis; Fig. 3).

One operational taxonomic unit (OTU) in the dataset was a 100% BLAST match for the *S. salivarius* BLIS M18 16S rRNA sequence and also a perfect match to only *S. salivarius* ATCC7073 within the NCBI Bacterial & Archaeal 16S rRNA sequence database. There was no significant difference between the abundance and presence/absence of this OTU between

treatment groups, time points, nor treatment groups stratified by time (Fig. 5; all  $P > 0.05$ , Kruskal-Wallis test and Chi square test).

## DISCUSSION

This RCT assessed the efficacy of oral probiotic *S. salivarius* M18 to manage oral hygiene and halitosis in patients with orthodontic braces by investigating the changes in plaque index (PI), gingival index (GI), volatile sulfur compound (VSC) and microbiota. It was found that the regular use of oral probiotics did not influence PI and GI scores, but resulted in a prolonged reduction in VSC levels.

Orthodontic treatment has become increasingly popular due to its benefits in improving smiles, self-esteem and jaw function. However, braces make it more difficult to brush teeth effectively, and can promote the level of halitosis [2,4]. Many dental products are commercially available to prevent plaque buildup, with little effect. These include interdental brushes, specialized orthodontic toothbrushes, antimicrobial mouth rinses and toothpastes[3,4]. The World Health Organization defines probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” [24]. Regular consumption of oral probiotics has been reported as an effective way for managing plaque in preschool children, primary-school children, adolescents, adults and the elderly [5-10]. However, few studies have assessed the effects of probiotics on the biofilm microbiota in patients with orthodontic braces. Previous research has mainly focused on the effect of probiotics on levels of mutans streptococci and lactobacilli [11-15]. Also, only one long-term trial (17 months) has investigated clinical outcomes [12]. The present study is the first to assess the effect of oral probiotics on halitosis in patients with orthodontic braces. It is also the first time probiotic *S. salivarius* M18 has been tested in an orthodontic population. *S. salivarius* M18 was the probiotic of choice as it was isolated from the oral cavity, and so is

likely to be well adapted to colonising the mouth where it may have beneficial effects. In addition, *S. salivarius* M18 has strong antimicrobial activity against dental pathogens, in particular *S. mutans*, due to the production of salivaricins M, 9 and A [25].

Some of our findings are consistent with the results of a recent systematic review and meta-analysis of the effect of probiotics on the oral health of non-orthodontic patients [26] which determined that there was no significant reduction in PI, but it did find a small, yet significant, improvement in GI. When compared with general dental patients, orthodontic patients have a higher risk of caries and biofilm formation due to the impact of fixed orthodontic appliances on oral hygiene [27,28]. The only other study of probiotics on clinical parameters of white spot lesions in adolescent orthodontic patients found no difference in white spot lesion incidence over time [12]. The probiotic did not perform better in one or other gender and there was no difference between genders in their compliance with consuming the lozenges. A recent trial has found that daily consumption of Kefir and probiotic toothpaste usage decreased the *S. mutans* and *Lactobacillus* levels in the saliva of orthodontic patients [29].

The mean level of VSCs in patients with orthodontic braces has been found to be significantly higher than in the normal population, possibly due to the poorer oral hygiene caused by braces [27,28]. In a general dental population, *S. salivarius* K12 has been shown to reduce VSC levels in 85% of participants when delivered by lozenges [30]. The present study is the first to examine the effect of *S. salivarius* M18 on VSC levels in orthodontic patients. The VSC levels decreased after one month in both participant groups (Fig 2C). The drop in VSC levels in the control group may have been due to the improved oral hygiene achieved by following the study protocol (*i.e.* Hawthorne effect) [31].

After the 3-month treatment-free follow-up, the VSC levels in the control group returned to baseline levels, which may indicate that the microflora had returned to its original state. In

contrast, the VSC levels in the oral probiotic group reduced further. The main contributor to VSCs is considered to be the anaerobic biofilm located on the dorsum of the tongue [32]. *S. salivarius* preferentially colonises the tongue and can seed saliva from this niche. This could explain the detection of *S. salivarius* M18 in the saliva of participants in the probiotic study of Burton *et al.* [8]. The biofilm samples analysed in the present study, however, were collected from the teeth. Therefore, *S. salivarius* M18 may have colonised the tongue and affected the VSC-generating biofilm on the tongue with no detectable effect on the dental biofilm microbiota found in this study. A recent trial has found that oral administration of *Lactobacillus* in patients with severe periodontitis improved periodontal health and halitosis [33]. Recent systematic reviews also suggest that probiotics are beneficial in treating intra-oral halitosis [34,35]. Short-term (average of two weeks) administration of probiotics, mainly *Lactobacillus* strains, had a moderate effect on halitosis, as assessed by organoleptic scores [36].

Considering orthodontic treatment duration is usually about two years, oral probiotics may have additional oral health benefits if used throughout treatment. However, the clinical relevance of the VSC reduction found in the current study (from  $201 \pm 71$  ppb to  $180 \pm 47$  ppb) is unclear since 150-160 ppb is considered as a threshold for the clinical diagnosis of halitosis [19,37].

We analysed the metagenomes of 130 samples (64 from participants plus one negative control) before and after the probiotic intervention. Alpha diversity analysis revealed a significant decrease in community richness in both groups between time points, perhaps due to the oral hygiene regime. Time but not probiotic treatment had a significant effect on community beta diversity. Nine genera were significantly associated with time. The abundance of *Actinomyces*, *Rothia*, *Streptococcus*, *Selenomonas*, *Laurotropa*, and *Olsenella* decreased, while the abundance of *Capnocytophaga*, *Escherichia*, and *Fusobacterium*

increased. Only *Rothia* had a significant difference between probiotic treatment groups after accounting for the effect of time.

A previous culture-based study reported that *S. salivarius* strain M18 was detectable in the mouths of 62% of subjects after 28 days of treatment [8]. In addition, this study showed that *S. salivarius* strain M18 inhibited a variety of bacterial strains *in vitro*, including *S. mutans*, *Actinomyces naeslundii*, and *Rothia mucilagenosa*. The inhibition of *Rothia* is supported by our metagenomic analysis. However, the 16S rRNA sequence of *S. salivarius* strain M18 was detectable in only 21% of samples in this study. This may indicate that strain M18 was not persistent in this study. Alternatively, strain M18 may persist at levels in which it is culturable but below the sequencing detection limit of this study. The 16S rRNA V3/V4 hypervariable region of *S. salivarius* strain M18 is 100% identical to the 16S rRNA sequence of other *S. salivarius* strains such as ATCC 7073. Therefore, our analysis cannot discriminate between strain M18 and other commensal *S. salivarius*.

The use of probiotics has advantages over chemical agents because of a reduced risk of inducing antibiotic resistance and causing other side effects. However, current evidence for the efficacy of probiotics in improving oral health is inconclusive due to the use of different probiotic strains and administration strategies across studies, but also due to methodological issues, such as small sample size, and short-term follow-up [34]. Future studies should address these limitations.

## CONCLUSION

Oral probiotic *S. salivarius* M18 reduced the level of halitosis in patients wearing orthodontic braces, but did not decrease their plaque or gingival indices. Time, but not treatment, was associated with both an overall decrease in alpha diversity (richness), and with a modest effect on overall beta diversity (compositional similarity between samples) as measured by

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unweighted Unifrac distance. The abundance of the genera *Streptococcus*, *Rothia*, *Selenomonas*, *Actinomyces*, and *Lautropia* decreased between time points, but only *Rothia* also changed in abundance between treatment groups after the intervention. A longer intervention and follow-up period should be investigated.

**Conflict of interest**

The authors all declare no conflict of interest.

**Acknowledgments**

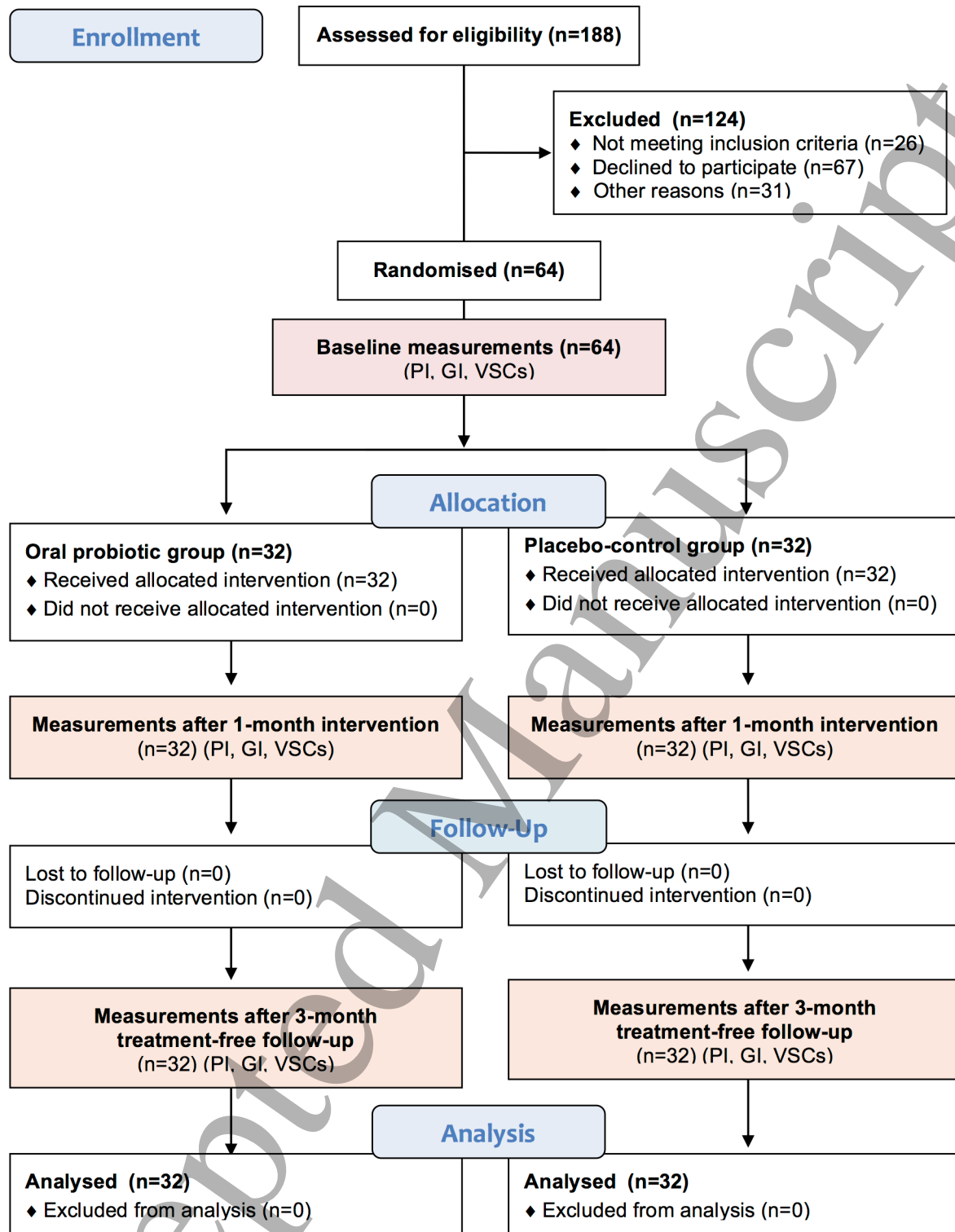
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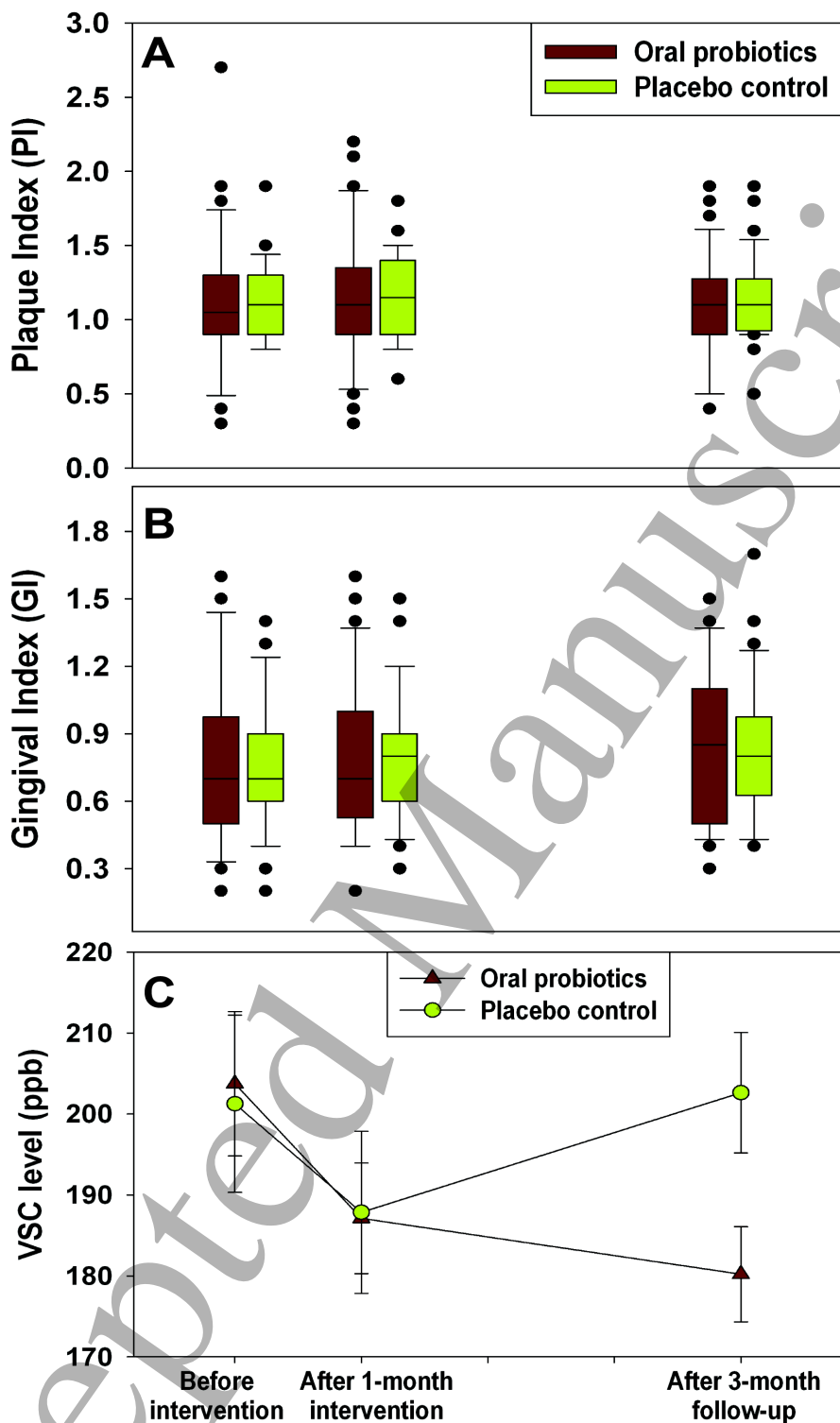
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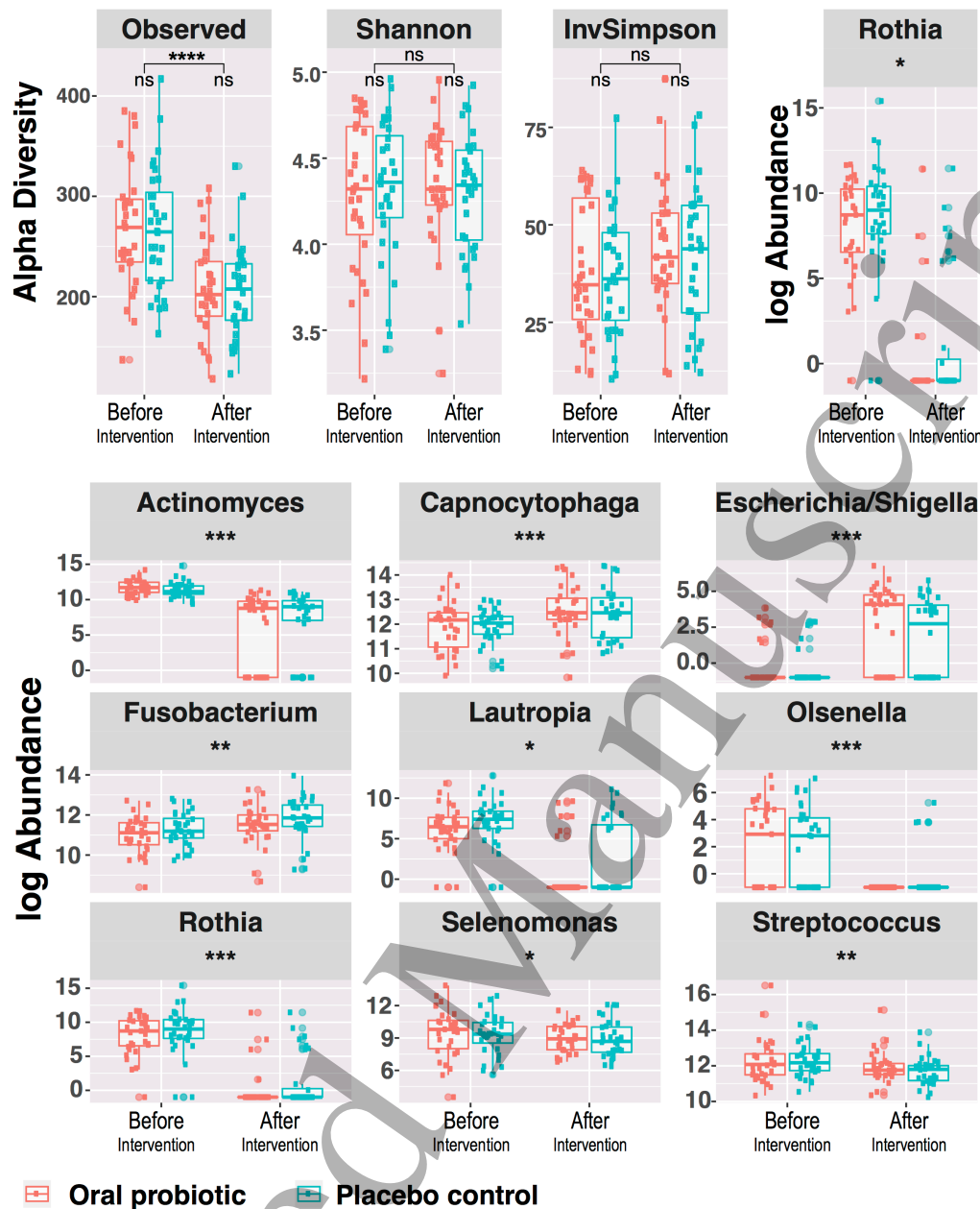
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**Figure 1.** CONSORT study flow chart showing patient flow during the trial. A total of 188 patients were assessed for eligibility to be included in the study and 64 were randomized to either the oral probiotic group (n = 32) or placebo control group (n = 32). No participants were lost during the study and all participant data were included for analysis.

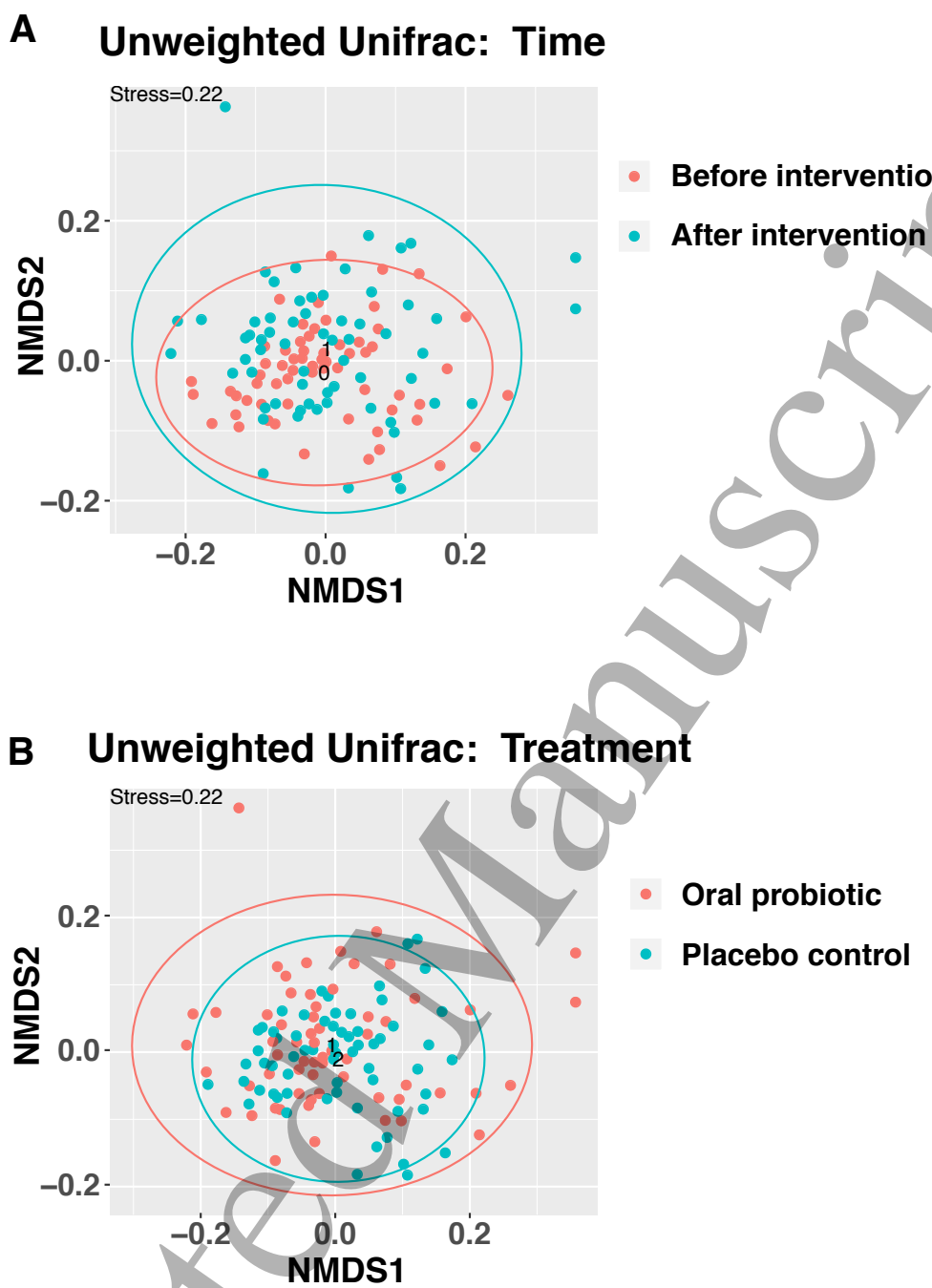


**Figure 2.** Effect of probiotic treatment on plaque index (PI), gingival index (GI) and volatile sulfur compound (VSC) levels. Box-and-whisker plots of the PI (A) and GI (B) scores of the two groups at baseline, after 1-month intervention and 3-month intervention-free follow-up. (C) Mean VSC levels of the two groups at baseline, after 1-month intervention and 3-month intervention-free follow-up (data represent means  $\pm$  standard errors).

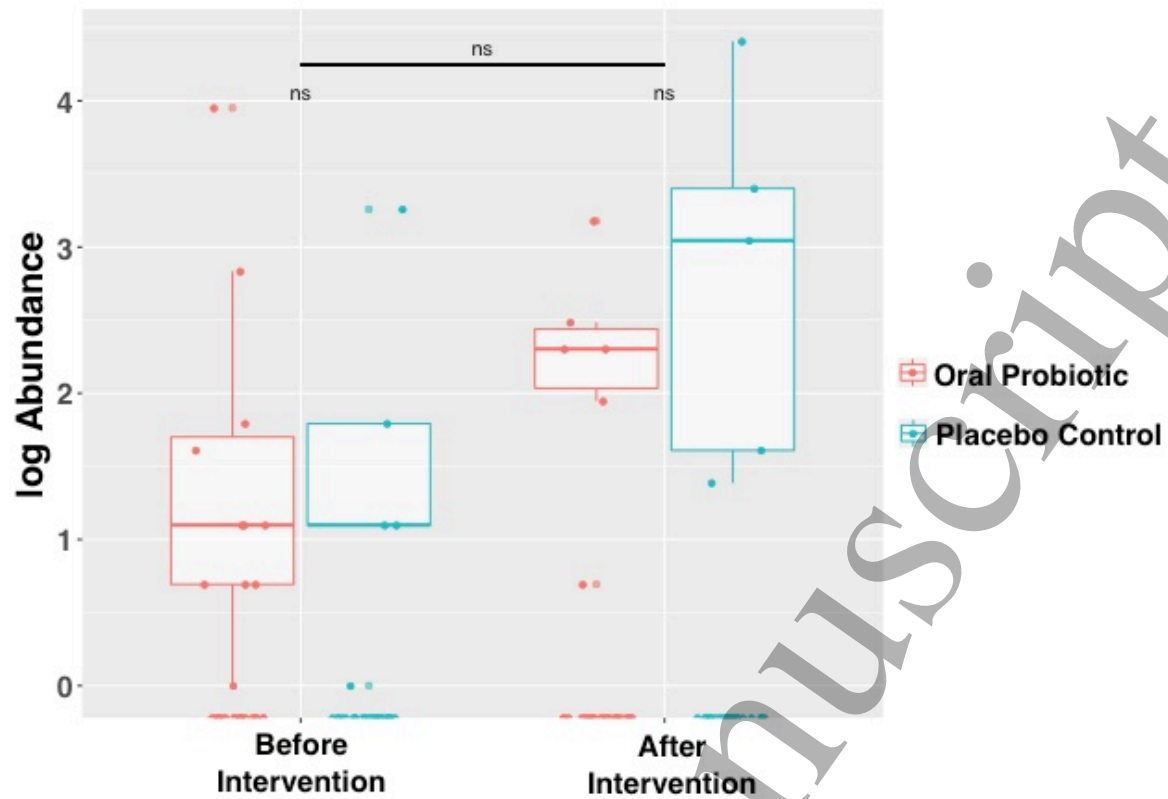


**Figure 3.** Relationships between time, treatment, and microbiota. (A) Alpha diversity of samples, as measured by observed amplicon sequence variants, Shannon diversity, and inverse Simpson diversity. Color indicates treatment group. Lines indicate significant differences between groups as measured by Kruskal-Wallis test. (B) The genus (*Rothia*) associated with treatment after correcting for the effect of time. (C) Genera significantly associated with time. Nine genera (*Actinomyces*, *Capnocytophaga*, *Escherichia/Shigella*, *Fusobacterium*, *Lautropia*, *Olsenella*, *Rothia*, *Selenomonas*, *Streptococcus*) were significantly associated with time by DESeq2 analysis (C), while six genera (*Actinomyces*, *Lautropia*, *Rothia*, *Selenomonas*, *Streptococcus*, *Veillonellas*) were associated with time by the Kruskal-Wallis test. Five genera (*Actinomyces*, *Lautropia*, *Rothia*, *Selenomonas*, *Streptococcus*) were associated with time by both methods. Details of significance values are given in the Table 2.

For all figures, \* =  $P < 0.05$ , \*\* =  $P < 0.01$ , \*\*\* =  $P < 0.001$ , NS = not significant. For panels B and C, asterisks on top bars indicate p-values determined by BH-corrected Wald test.



**Figure 4.** Non-metric multidimensional scaling (NMDS) ordinations of data by unweighted Unifrac distance. Samples are colored according to their respective time point (A) or treatment (B). Ellipses indicate 95% confidence interval for the standard deviations of points in each group.



**Figure 5.** The abundance of *S. salivarius* stratified by time and treatment group. This abundance reflects the presence of either the *S. salivarius* BLIS M18 or the commensal *S. salivarius* (OTU). This OTU did not differ between treatment groups, time points, nor treatment groups stratified by time (all  $P > 0.05$ , Chi square test and Kruskal-Wallis test).

**Table 1.** Baseline characteristics of participants in the two treatment groups.

	Oral probiotics (N=32)	Placebo control (N=32)
<b>Age (N, %)</b>		
<14 years	14 (22%)	22 (34%)
≥14 years	18 (28%)	10 (16%)
<b>Sex (N, %)</b>		
Male	12 (19%)	11 (17%)
Female	20 (31%)	21 (33%)
<b>Plaque Index (mean ± SD)</b>	1.1 ± 0.4	1.1 ± 0.2
<b>Gingival Index (mean ± SD)</b>	0.8 ± 0.4	0.8 ± 0.3
<b>VSCs (ppm) (mean ± SD)</b>	204 ± 69	201 ± 85



**Table 2.** Details of significance values for the relationships between time, treatment and microbiota. Nine genera (*Actinomyces*, *Captocytophaga*, *Escherichia/Shigella*, *Fusobacterium*, *Lautropia*, *Olsenella*, *Rothia*, *Selenomonas*, *Streptococcus*) were significantly associated with time by DESeq2 analysis, while six genera (*Actinomyces*, *Lautropia*, *Rothia*, *Selenomonas*, *Streptococcus*, *Veillonellas*) were associated with time by the Kruskal-Wallis test. Five genera (*Actinomyces*, *Lautropia*, *Rothia*, *Selenomonas*, *Streptococcus*) were associated with time by both methods. One genus (*Rothia*) was associated with treatment by DESeq2 analysis.

Time-associated: DESeq2	Genus	Log2-fold change	p-value	padj
	<i>Streptococcus</i>	0.70	0.00026	0.0030
	<i>Capnocytophaga</i>	-0.69	0.000023	0.00036
	<i>Escherichia/Shigella</i>	-3.7	0.00	0.00
	<i>Rothia</i>	5.1	0.00	0.00
	<i>Actinomyces</i>	2.7	0.00	0.00
	<i>Fusobacterium</i>	-0.57	0.00026	0.0030
	<i>Selenomonas</i>	0.90	0.0014	0.014
	<i>Olsenella</i>	4.8	0.00	1.0 e-7
	<i>Lautropia</i>	1.9	0.0037	0.032
Time-associated: Kruskal-Wallis	Genus	p-value		BH
	<i>Veillonella</i>	0.0035		0.034
	<i>Streptococcus</i>	5.2 e-06		7.2 e-05
	<i>Rothia</i>	1.1 e-16		0.00
	<i>Selenomonas</i>	0.0057		0.046
	<i>Actinomyces</i>	7.7 e-19		0.00
Treatment-associated: DESeq2	Genus	Log2-fold change	p-value	padj
	<i>Rothia</i>	-2.5	0.00022	0.020