

Alterations of the oral microbiome in patients treated with the Invisalign system or with fixed appliances

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Introduction: Although the Invisalign system has been used widely in recent years, the influences of this treatment on the oral microbiome and whether or not this influence is different from that of fixed appliances is still unknown. In this study, we investigated the changes in the oral microbiome in patients treated with the Invisalign system or with fixed appliances. **Methods:** Fifteen subjects were enrolled, comprising 5 fixed appliance patients, 5 Invisalign patient, and 5 healthy controls. Saliva samples were collected, and high-throughput pyrosequencing was performed based on the 16S rRNA gene. **Results:** Both fixed and Invisalign orthodontic treatments resulted in dysbiosis of the oral microbiome. *Firmicutes* and TM7 at the phyla level and *Neisseria* at the genus level displayed statistically significant differences between the 2 orthodontic groups. The effect of these changes with microbiome on oral health was inconsistent. The inferred microbial function of the Invisalign group suggested this group was more predisposed to periodontal diseases. **Conclusion:** The influence of the Invisalign system on the oral microbiome was no better for oral health compared with fixed appliances. The convenience of maintaining oral hygiene rather than changes in the oral microbiome may be the underlying reason for the performance of the Invisalign system on oral health. (Am J Orthod Dentofacial Orthop 2019;156:633-40)

Fixed orthodontic treatment is a common method for correcting malocclusions. The aesthetic and health effects after this therapy are apparent, but there are some side effects during the procedure. The most common side effects are periodontal complications¹⁻³ and root resorption,^{4,5} which is termed orthodontically-induced inflammatory root resorption.⁶ Orthodontic appliances also influence the oral

microbiota. For instance, they cause periodontitis- and caries-associated bacteria to become abundant in plaque⁷⁻¹⁰ and saliva.¹¹⁻¹³ In contrast, 1 study reported that some pathogenic bacteria decreased during orthodontic procedures.¹⁴

Invisalign (Align Technology, Santa Clara, CA) is a removable appliance with a series of transparent aligners previously designed using proprietary software.¹⁵ This Invisalign system offers many advantages compared with fixed appliances, such as better appearance, comfort level,¹⁶ periodontal health,¹⁷ and oral hygiene.¹⁸ Given these advantages, this orthodontic method has been adopted widely in recent years.

Although many reports have focused on differences in clinical parameters between traditional orthodontic appliances and the Invisalign system,^{5,16-20} only a few of them compared the microbiological aspect. Even in these few studies,^{17,18} they only compared 4 selected pathogenic species (*Prevotella intermedia*, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*) using real-time PCR. This means that the response of the entire microbiome to the Invisalign system is unknown, and whether it still performs better than fixed appliances in changing the oral flora aspect is unclear.

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Because an understanding of oral microbial changes associated with orthodontic therapy is beneficial in achieving a better and more comprehensive evaluation of this treatment, a high-throughput sequencing technique was used to determine the difference in changes to oral flora between Invisalign and fixed appliance treatment. To our knowledge, there has been no such report to date.

MATERIALS AND METHODS

This study was approved by the ethics committee of the First Hospital affiliated with Dalian Medical University, Dalian, China, and informed consent was obtained from all participating patients and volunteers. Twenty-six subjects who met the following criteria were enrolled in this study: aged 20–25 years, nonsmokers, without any diagnosed systemic diseases, and having received no antibiotics in the 3 months before sampling. Of them, 7 subjects received Invisalign treatment, 12 subjects received fixed orthodontic treatment, and 7 subjects were provided with no orthodontic treatment (control group). Orthodontic treatments had been carried out for 6 months at the First Hospital affiliated with Dalian Medical University, Dalian, China. All of the participants were instructed regarding oral hygiene procedures.

Salivary samples were taken at least 2 hours after meals or oral hygiene procedures. For each subject, 2 mL of saliva was collected and added to 2 mL of pre-cooled phosphate buffered saline. All samples were stored at -80°C .

Before DNA extraction, all saliva samples were centrifuged at 500 rpm for 10 minutes, then suspensions were centrifuged again at 13,000 rpm for 5 minutes, and the precipitates were collected with a swab. Total genomic DNA was obtained from the swab using the TIANamp Swab DNA Kit (Tiagen Biotech, Beijing, China) according to the manufacturer's instructions. All DNA was stored at -20°C before further analysis.

The 16S rRNA hypervariable V3–V4 region was amplified using the primer GC-341F (5'-CGCCCGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGCTACGGGAGGCAGCAG-3') and 518R (5'-ATTACCGCGGCTGCTGG-3'), the amplified product was analyzed by denaturing gradient gel electrophoresis (DGGE) on 8% polyacrylamide gel. The denaturant concentration gradient is 35%–60% (100% denaturant concentration is equivalent to 7M urea and 40% deionized formamide). Electrophoresis was performed at 200 V for 5 minutes and then at 65 V for 7 hours at 60°C . At the end of electrophoresis, ethidium bromide staining was performed for 1 hour. The DGGE map

was analyzed by Quantity One (version 4.6.2, Bio-Rad, Hercules, CA).

Five subjects were selected from each group for high-throughput pyrosequencing analysis. The 16S rRNA hypervariable V3–V4 region was amplified using the primer 319F (5'-ACTCCTACGGGAGGCAGCAG-3') and primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'), and sequencing was performed on the Illumina MiSeq platform (San Diego, CA). Quantitative Insights Into Microbial Ecology (version 1.8.0) was used to analyze the sequence data.²¹ The operational taxonomic units (OTUs) were clustered at a sequence similarity of 97%, and the GreenGenes database (version gg_13_8) was used for taxonomic analysis. Sequences were used for microbial community metagenome prediction with PICRUSt (version 1.1.0; <http://picrust.github.com/picrust/>), and functional inferences were identified using the Kyoto Encyclopedia of Gene and Genomes (KEGG) pathway.²¹ STAMP (version 2.1.3) was used to test for differentially abundant KEGG pathways in the 3 groups.

Statistical analysis

Differences in the relative abundance of phyla and genera among groups were determined using the Kruskal–Wallis test (R Development Core Team, Vienna, Austria). The posthoc multiple comparisons were analyzed by Student–Newman–Keuls test or Nemenyi test (SAS, version 8.02; SAS Institute Inc, Cary, NC) to examine whether there was a statistically significant difference between each pair of groups at phyla and genera level and for the Shannon index. Statistically significant differences between groups of the KEGG pathway were determined by the Welch's *t* test using STAMP.

RESULTS

As shown in the DGGE map (Supplementary Fig 1, available at www.ajodo.org), the species and number of bands increased in orthodontic treatment samples (F1–12, I1–7) compared with control samples (C1–7).

We performed the unweighted pair-group method with arithmetic means similarity clustering analysis by Quantity One (Supplementary Fig 2, available at www.ajodo.org). Samples were gathered into a cluster for each group, which indicated that there was no significant difference among individuals in each group. Therefore we selected 5 samples in each group randomly for high-throughput pyrosequencing based on the 16S rRNA gene.

A total of 572,000 sequences were obtained, and approximately 78% passed quality control, meaning that 444,957 sequences remained for analysis. The average number of sequences for each sample was

Table. Sequence data and Shannon index

Group	n	Mean no. of sequences	Mean OTU	Mean Shannon index
I	5	28,750	255.4	4.9100
F	5	28,109	224.8	4.5006
C	5	32,133	300.8	5.7277

29,664 (range, 21881–43680). These sequences were clustered into 3,905 OTUs, and the average number was 260.3 OTUs (range, 135–340). The average sequences and OTUs are shown in Table.

The Shannon indexes were calculated to investigate the diversity of oral microbial composition in the Invisalign (group I), fixed appliances (group F), and control (group C) group. The mean indexes were 4.9100, 4.5006, and 5.7277 for groups I, F, and C, respectively (Table). There were significant differences between groups I and C ($P = 0.0031$), and F and C ($P = 0.0001$); however, there was no significant difference between the I and F groups ($P = 0.0893$; Fig 1).

Rank–abundance curves were created to describe the richness and evenness of samples. The width of the curve represents the richness of the samples. If a curve shows a smoothly declining trend, it indicates that the species are evenly distributed; otherwise, it means that the proportion of dominant flora is high. As shown in Figure 2, there were more species and greater evenness in group C than groups I or F. There was no apparent difference between groups I and F.

After taxonomic analysis, sequences were classified into 21 phyla, and the top 10 are shown in Figure 3. The predominant phyla were *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, candidate division TM7, and Spirochaetes, which constituted approximately 98.7% of total sequences. Three of these phyla (*Firmicutes*, *Bacteroidetes*, and candidate division TM7) differed significantly in abundance among groups (Fig 4). For *Firmicutes*, the highest relative abundance was detected in group F ($P < 0.05$). Although group I had a greater abundance of *Firmicutes* than group C, the difference was not statistically significant. For *Bacteroidetes*, both groups I and F had a lower proportion than group C, but only group F was significantly different from group C ($P < 0.05$). The abundance of candidate division TM7 was significantly higher in group I than F. Although group C showed a median value between group I and F, there was no significant difference.

At the genus level, sequences were classified into 124 different genera. The top 10 genera were *Veillonella*, *Streptococcus*, *Prevotella*, *Haemophilus*, *Neisseria*, *Porphyromonas*, *Prevotella* (a member of the Paraprevotellaceae family), *Selenomonas*, *Rothia*, and

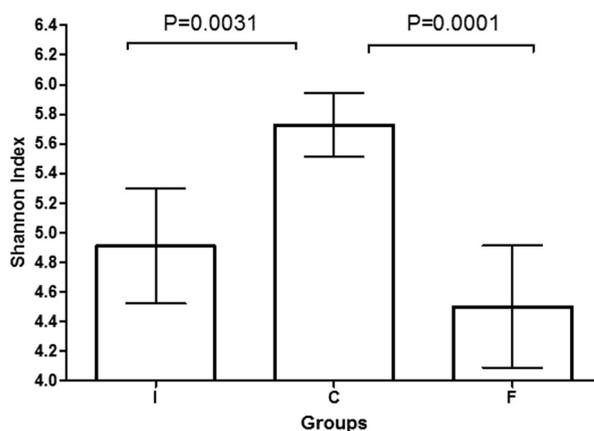


Fig 1. Shannon index of the Invisalign group and the fixed appliance group. * $P < 0.05$ represents significant differences among groups.

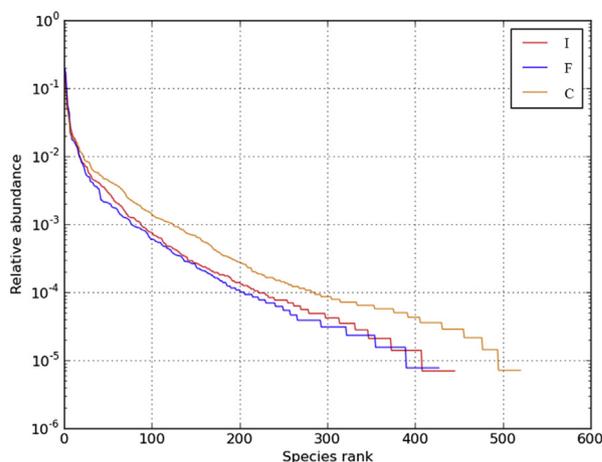


Fig 2. Rank–abundance curve.

Fusobacterium. The top 10 taxa comprised roughly 81.5% of the total sequences, and the distribution of these taxa in the samples is shown in Figure 5. To determine the variation of these genera, we then compared their relative abundances. Four genera (*Neisseria*, *Prevotella*, *Rothia*, and *Fusobacterium*) had significant differences among groups (Fig 6). For *Neisseria*, the proportion of this genus was higher in group I than group F ($P < 0.05$). Although the abundance of *Neisseria* was higher in group I than C, it was not statistically significant. Group C had a higher level of *Prevotella* and *Fusobacterium* compared with all groups, but was only significantly different than group F ($P < 0.05$). The abundance and proportion of *Rothia* in group C were lower than group I and F, with the latter group

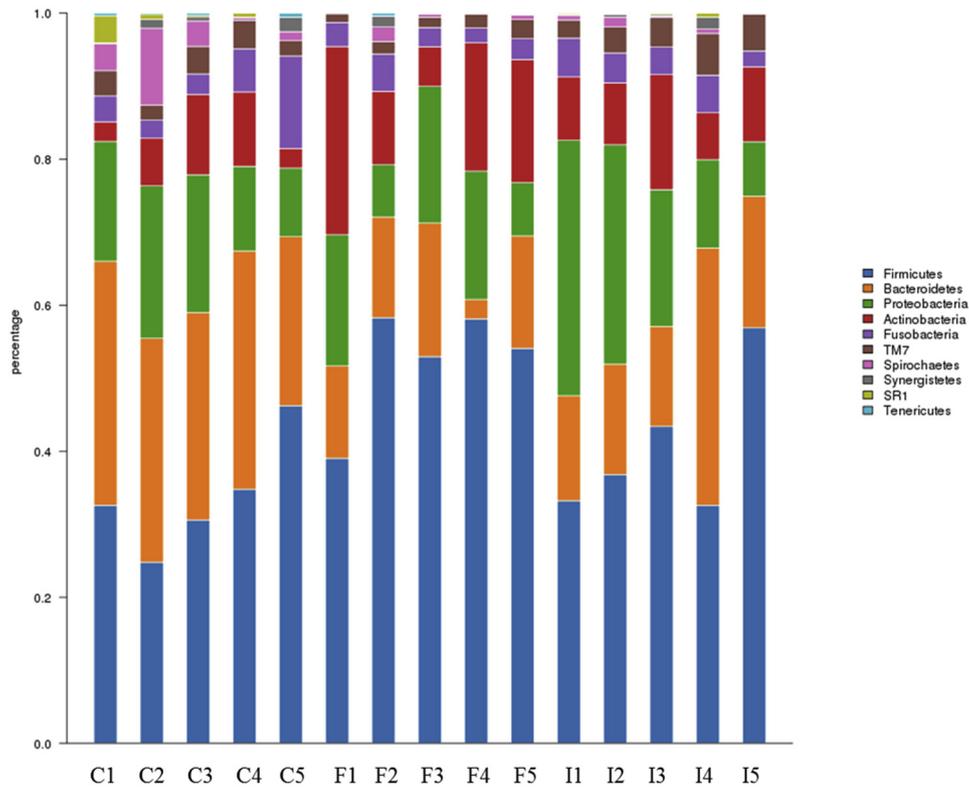


Fig 3. The relative abundance of the top 10 phyla in samples.

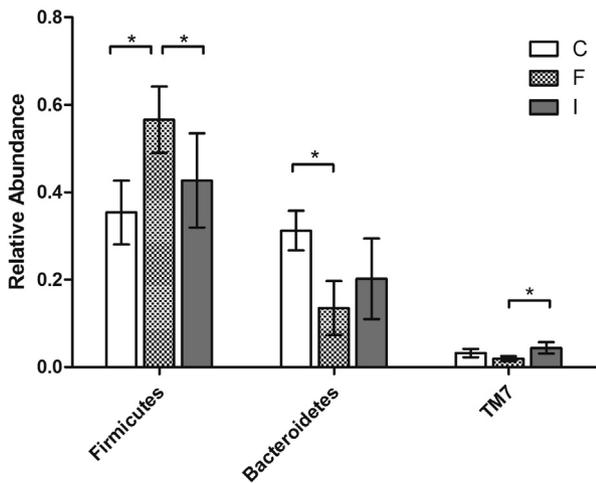


Fig 4. Histograms of phyla among groups. * $P < 0.05$ represents significant differences between groups.

exhibiting the highest relative abundance. There was also a significant difference between the abundance of group C and F ($P < 0.05$).

To investigate differences in microbial function, we assessed the KEGG pathways by inferred metagenomics using PICRUSt. We identified 6 enriched functional

orthologues in group I ($P < 0.01$; Fig 7). These enriched functions were related to energy metabolism (carbon fixation pathways in prokaryotes, oxidative phosphorylation, methane metabolism), amino acid metabolism (alanine, aspartate, and glutamate metabolism), carbohydrate metabolism (citric acid cycle) and metabolism of terpenoids and polyketides (geraniol degradation). Group F exhibited functions enriched in membrane transport (transporters, phosphotransferase system) and nucleotide metabolism (nucleotide metabolism_unclassified).

DISCUSSION

To identify and compare oral microbial changes that related to the 2 different orthodontic treatments, we analyzed microbial and functional aspects of Invisalign system patients, fixed appliance patients, and controls. This study demonstrated that both the Invisalign system and fixed orthodontic appliances cause microbial dysbiosis compared with normal oral flora; whereas, the Invisalign group was not significantly different from the fixed appliance group (I vs F). However, the abundance of some types of bacteria differed between the 2 orthodontic treatment groups, as well as between the treatment and control groups at

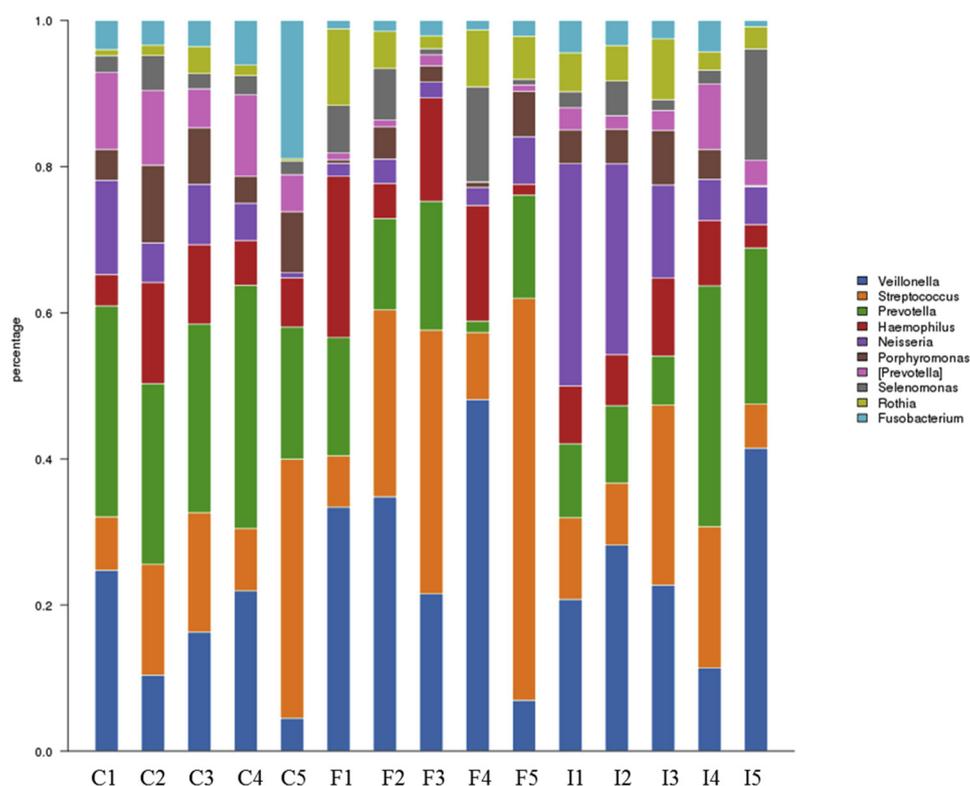


Fig 5. The relative abundance of the top 10 genera in samples.

both phylum and genus level. We also observed functional differences between the Invisalign and fixed appliance microbiomes.

It is well known that the ecosystem of the oral cavity is complex and delicate, maintaining the balance between the environment and the oral microbiome.²² However, when the balance is disturbed for some reason, composition and quantity changes of the microorganisms can induce pathological conditions and disease.²³ A systematic review study reported sufficient evidence that fixed orthodontic appliances affected the oral microbiota, but that more sensitive and specific microbiological analysis methods were needed to confirm these findings.²⁴

Recent advances in DNA sequencing technology, such as high-throughput sequencing, has provided a more comprehensive and sensitive method to analyze the oral microbiome. As such, we used this technique to analyze the microbiome of orthodontic patients and controls and observed that the diversity of the oral ecosystem decreased as a result of orthodontic treatments, regardless of the type (Fig 1). The richness and evenness of the oral microbiota were also disturbed by both orthodontic treatments (Fig 2). Collectively, these findings demonstrate that microbial dysbiosis occurred

in both Invisalign and fixed appliance groups. This ecosystem dysbiosis could be a reason for the increased caries, white spot lesions, and periodontal problems which have been observed with these orthodontic treatments.

To further identify compositional changes, we compared the relative abundance of the oral microbiome at phylum and genus level. Consistent with previous studies,²⁵⁻²⁷ our results indicated that the oral microbiome was dominated by the phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, candidate division TM7, and Spirochetes. Among these phyla, 3 showed differences among groups (Fig 4). In particular, *Firmicutes* and TM7 were significantly different between the orthodontic treatment groups (I and F). A previous study demonstrated that *Firmicutes* increased in the initial week of fixed appliance use, but then showed no difference at 6 or 12 weeks.²⁸ In this study, the fixed appliance group had an increased relative abundance of *Firmicutes* compared with the control group (F vs C). Because our samples were collected at least 6 months after orthodontic treatment, this result represented a long-term change of this phylum. For the Invisalign group, the abundance of *Firmicutes* was less than the fixed

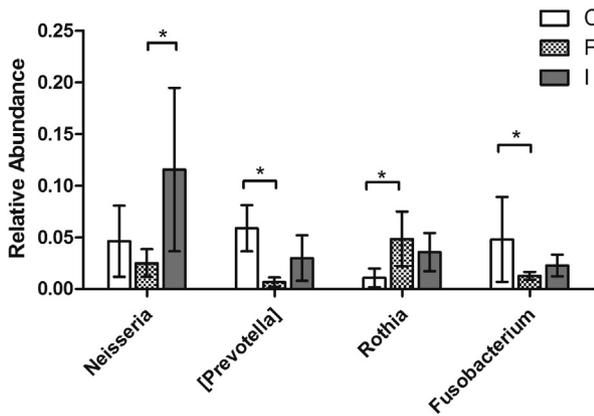


Fig 6. Histograms of genera among groups. * $P < 0.05$ represents significant differences between groups.

appliance group (I vs F) with no difference than the control group (I vs C), which indicated that the Invisalign group was more similar to controls.

Candidate division TM7 have been detected at various sites in the human body,²⁹⁻³¹ and are also widespread in the oral cavity.^{32,33} In our study, the relative abundance of TM7 was significantly higher in the Invisalign vs fixed appliance group (I vs F). Combining our results with data from previous studies which have reported that TM7 is associated with gingivitis^{28,33} and periodontitis,³⁴ the Invisalign group seemed to be more susceptible to periodontal complications associated with TM7. Although there was no significant difference between orthodontic treatment and control group, there was a tendency for decreased TM7 abundance in the fixed appliance group. Considering that treatment with fixed orthodontic appliances has the side effects of gingivitis and periodontitis TM7 can be assumed to be increased in group F, which was opposite to our result. This contradiction might be caused by differences in the sampling sites of plaque and saliva.

At the genus level (Fig 6), our results showed that *Neisseria* was more abundant the Invisalign than fixed appliance group (I vs F). *Neisseria* is an early colonizer of tooth surfaces²⁶ and some studies have indicated that *Neisseria* is associated with improved oral health³⁵ or reduced gingivitis.³⁶ From this viewpoint, our results might suggest that Invisalign was beneficial to periodontal health. This finding was similar to the condition of TM7, although there were no significant differences between orthodontic treatment and the control group, there was a tendency for the decreased abundance of *Neisseria* in the fixed appliance group (F vs C). This tendency was consistent with a previous study which

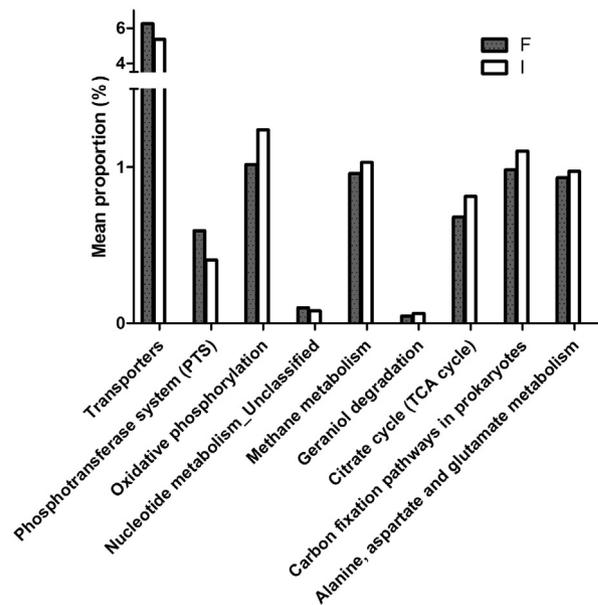


Fig 7. Functional comparison of the oral microbiome between the fixed appliance and Invisalign groups. $P < 0.01$ represents significant differences between groups.

reported that *Neisseria* became lower in abundance over time following treatment with fixed appliances.²⁸

We also observed that the abundance of *Prevotella* genus (a member of the Paraprevotellaceae family), which most studies investigating the oral microbiome during orthodontic treatment did not report, was decreased in the fixed appliance compared with the control group (F vs C). A previous study reported that Paraprevotellaceae were increased in the saliva of a cohort of urban Indian patients,³⁷ and this family demonstrated a relationship with intestinal diseases.³⁸⁻⁴⁰ Thus, these results suggested that the oral microbiome might have a relationship with the gut microbiome.

In the present study, we observed that changes in the microbiome associated with Invisalign treatment showed different effects on oral health, with some beneficial and some harmful. Thus, from the viewpoint of microbial composition and diversity, the Invisalign system did not show improved performance compared with fixed appliance treatment.

The results of inferred metagenomic function analysis (Fig 7) showed that the Invisalign group microbiome was enriched in energy metabolism, amino acid metabolism, carbohydrate metabolism, and metabolism of terpenoids and polyketides; whereas, the microbiome of the fixed appliance group was enriched in membrane transport and nucleotide metabolism. This result was consistent with a previous study,⁴¹ which reported that metaproteomic analysis of human salivary supernatant

was most represented by carbohydrate metabolism, amino acid metabolism, energy metabolism, translation, membrane transport, and signal transduction. Of these, carbohydrate, amino acid, and energy metabolism were associated with the early stages of biofilm formation⁴² and were reported to have potential importance in periodontal pathogenesis.^{43,44} Combined with data from these previous studies, our results might suggest an unexpected tendency for the microbial function of the Invisalign group to be more predisposed to periodontal diseases.

CONCLUSIONS

In general, both fixed and Invisalign orthodontic treatments resulted in dysbiosis of the oral microbiome. From the microbiome composition and functional aspects, the Invisalign system did not show improved performance compared with fixed appliance treatment. As such, the previously reported advantages of Invisalign on periodontal health were not supported by our current results. This contradiction suggests that the improved periodontal health performance associated with the Invisalign method may not be because of oral microbial conditions but may be the result of the convenience of implementing oral hygiene measures.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.ajodo.2018.11.017>.

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