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Los Angeles

Characterization of the
Oral Microbiome in Orthodontic Patients

A thesis submitted in partial satisfaction of the
requirements for the degree Master of Science
in Oral Biology

by

Edward Lorilla Vilorio

2019

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ABSTRACT OF THE THESIS

Characterization of the Oral Microbiome in Orthodontic Patients

by

Edward Lorilla Vilorio

Master of Science in Oral Biology

University of California, Los Angeles, 2019

Professor Renate Lux, Chair

There are approximately 700 bacterial species present in the oral cavity that exist in a complex, delicately balanced ecosystem. Orthodontic treatment with fixed appliances has been shown to disrupt this balance leading to an increased risk of white spot lesions, caries and periodontal disease. With the advent of clear aligners, orthodontic patients are now able to remove their appliances and perform oral hygiene more efficiently. Previous studies have examined the effects of fixed and removable orthodontic appliances on periodontal health with varying results.

We examined patients at the UCLA Orthodontics Clinic with fixed appliances (n=12) and clear aligners (n=12) through the first six months of treatment. Periodontal status was evaluated using the Turesky et al. Modified Quigley-Hein Plaque Index (PI) and the Löe and Silness Gingival Index (GI). Plaque was collected from anterior teeth, posterior teeth and from the inner

surface of the clear aligner trays. DNA from the plaque samples was extracted and subjected to next generation sequencing of the 16S rRNA gene.

There was a significant increase in PI and GI in the fixed appliance group over the first six months of treatment. In the clear aligner group, there was no significant increase in PI, but there was a significant, yet transient increase in GI that eventually returned to baseline levels. Distinct, patient-specific shifts in the microbial communities were observed in both groups upon starting treatment. The microbial community inside the clear aligners is unique and less diverse than those found on teeth, but the overall composition of tooth-associated biofilm was similar between both groups.

Patients treated with fixed appliances experienced significantly more plaque accumulation on the surfaces of the teeth compared to patients treated with clear aligners. This increase in plaque accumulation in the fixed appliance group was associated with a significant increase in gingival inflammation, which was localized to the posterior teeth. The inner surfaces of the clear aligner trays harbor a unique microbial community that is less diverse than those found in tooth-associated biofilms.

The thesis of Edward Lorilla Vilorio is approved.

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2019

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INTRODUCTION

Orthodontic tooth movement has traditionally been achieved using fixed appliances. Until the 1980s, the primary method of placing fixed attachments was to cement a preformed band on every tooth [1]. With the advent of bonding techniques, the only teeth that are now routinely banded are maxillary and mandibular first molars [2]. Although treatment with fixed appliances is the tried-and-true method of treating malocclusion, it also has several disadvantages for patients, including poor esthetics and a decrease in the patient's ability to effectively remove oral biofilm [3]. In the late 1990s, Align Technology established a computerized system of making casts with incremental changes that could be used to fabricate removable aligners [1]. With the introduction of this new treatment modality, orthodontic patients were now able to remove their appliances to eat, brush and floss, thus allowing for excellent maintenance of oral hygiene [4-8].

The oral cavity has one of the most diverse microbiomes in the human body [9]. Approximately 280 bacterial species have been isolated in culture from the oral cavity, but it has been estimated that less than half of the bacterial species in the oral cavity can be grown in culture [10, 11]. Cultivation-independent molecular techniques, specifically 16S rRNA gene-based cloning studies, have shown that there are 700 microbial species that live in the human oral cavity as part of a complex, delicately balanced ecosystem [9-12]. Orthodontic treatment with fixed appliances results in an imbalance in this ecosystem and leads to an increased risk of white spot lesions, caries and periodontal disease [1, 11, 13] (Figure 1). The metabolic activities of oral biofilm on the surface of teeth can demineralize the enamel, forming white spot lesions (WSLs) that are precursors to the development of dental caries. Biofilm at the gingival margin can also lead to gingival inflammation, a precursor to periodontitis. These complications are

common during orthodontic treatment with fixed appliances and represent the two most common bacterial diseases in humans: caries and periodontal disease [9-11].

Orthodontic brackets themselves do not directly damage the teeth; however, they lead to plaque accumulation and make plaque removal more difficult [1]. The development of WSLs and gingival inflammation are caused by a shift from a benign, commensal microbial community toward an acidogenic community (in caries) or an inflammatory community (in periodontal disease) [11]. White spot lesions are areas of enamel decalcification that can be carious or non-carious [1, 14]. Previous studies have shown that 23% of orthodontic patients developed WSLs during treatment, being 2.5 times more frequent in the maxillary arch [14]. Five significant risk factors have been implicated in the development of these lesions: patients with poor oral hygiene, patients whose oral hygiene declined during treatment, treatment time in excess of 36 months, teeth without fluorosis and preexisting white spot lesions [14, 15]. Gingivitis is an inflammatory disease that causes erythema, swelling, bleeding and pain. Although gingivitis after orthodontic treatment is transient, white spot lesions may require dental restorations to improve facial esthetics in up to 15% of orthodontic patients, requiring an estimated \$500 million per year for restorative dentistry [16].

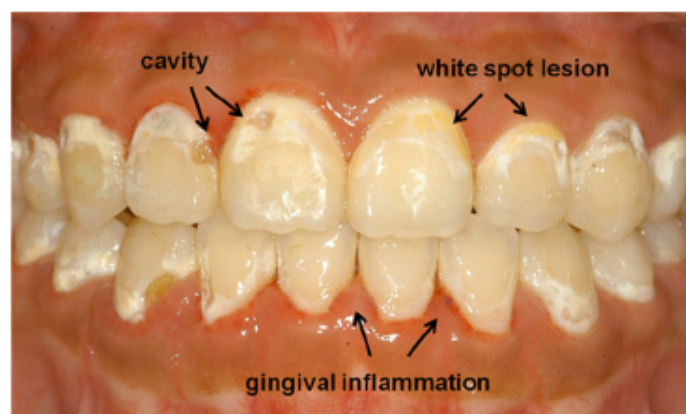


Figure 1: Biofilm formation in orthodontic patients may result in gingival inflammation, white spot lesions and caries [16].

Despite the increasing popularity of clear aligners, relatively few studies have compared the effects of this new treatment modality on periodontal health, and the studies that are available present conflicting results. For instance, Miethke and Vogt (2005) reported that plaque index was significantly lower in the clear aligner group, but the periodontal condition in both groups was nearly identical [5]. Other studies have reported better periodontal health in patients treated with clear aligners compared to fixed appliances [7, 17-19]. In contrast, Chhibber et al. (2018) concluded that there was no significant difference in plaque index, gingival index or papillary bleeding index between patients with clear aligners and fixed appliances [20].

In addition to examining the periodontal health of patients with fixed and removable appliances, some studies have made attempts to characterize the oral microbiome in these patients. The current literature has reported conflicting findings and many of them have reported only short-term data. For instance, Levrini et al. (2015) used real-time PCR to detect periodontal pathogens and biofilm mass. They discovered that *Aggregibacter actinomycetecomitans*, which is a gram negative anaerobe involved in chronic periodontitis, was present in 1 of 35 patients with fixed appliances and not present in the clear aligner group [6]. Karkhanechi et al. (2013) used the hydrolysis of BANA (N-benzoyl-DL-arginine-naphthylamide) by plaque samples as a semi-quantitative marker for the presence of gram-negative anaerobic bacteria strongly associated with chronic periodontitis: *Treponema denticola*, *Porphyromonas gingivalis* and *Tannerella forsythia* [8]. They found that BANA scores were significantly greater for the fixed appliance group, which supports their findings of decreased plaque and gingival inflammation as a result of improved oral hygiene in the clear aligner group [8]. Sifakakis et al. (2018) used qPCR to evaluate the change in select bacterial species in the saliva, but found no changes in the salivary counts of *Streptococcus mutans* or *Lactobacillus acidophilus* resulting from either fixed

or aligner treatment [21]. Guo et al. (2018) performed comprehensive 16S rRNA sequencing and found that clear aligners induced a microbial shift, but these changes were nonpathogenic over the first three months of treatment [22]. A systematic review by Guo et al. (2017) concluded that even though there was an initial increase in pathogenic bacteria shortly after beginning treatment, this transient increase tended to decrease to pre-treatment levels when followed for a longer period [23].

The limitations of the current literature include (1) short 3-month follow-up periods and (2) lack of biofilm analysis present inside the aligners themselves. Similar to plaque build-up on the surface of teeth, biofilm also forms on the surfaces of the aligner trays. Patients are instructed to wear their aligners for at least 22 hours per day, which means that the biofilm present within the trays is in contact with the surfaces of teeth for a majority of the time during treatment. Levirini et al. (2015) and Lombardo et al. (2017) evaluated different methods to remove biofilm from clear aligners but did not analyze the microbial composition, and there are no studies that investigate the microbial community within the aligner trays themselves [24, 25].

Clear aligners have become a popular orthodontic treatment option that provides an esthetic and hygienic alternative to fixed appliances. However, relatively little research has been done to investigate the effects of clear aligners on plaque accumulation, gingival inflammation and the oral microbiome. In this study, we provide for the first time a detailed, longitudinal analysis of microbial communities present on the teeth and aligner trays, as well as an assessment of relevant clinical parameters.

OBJECTIVES AND SPECIFIC AIMS

Orthodontic treatment may lead to caries and periodontal disease, but the underlying microbial causes have not been well studied. While there are a few studies that have investigated changes in the microbial ecosystem during orthodontic treatment, most have focused on fixed appliances. We hypothesize that although clear aligners can be removed allowing for better oral hygiene, they may cause significant changes in the balance of the microbial species similar to fixed appliances. In order to test our hypothesis, we proposed the following specific aims:

Aim 1: To analyze plaque and gingival indices from all teeth in both fixed appliance and clear aligner groups

Aim 2: To analyze the relative abundance of each microbial species and how the microbial community changes over time in both groups by:

- a) Plaque collection from anterior teeth, posterior teeth and the inside of the aligner trays
- b) Microbial community analysis using 16S rRNA sequencing

MATERIALS AND METHODS

Study participants were recruited at the UCLA Orthodontics Clinic under IRB #16-001258. Twenty-four patients who were preparing to start orthodontic treatment with either fixed appliances or clear aligners were included. Thus, twelve patients in each group were recruited and the groups had similar gender and age distributions. Oral consent was obtained from each patient, or from the parent or guardian if the patient was a minor. For the fixed appliance group, we did not specify whether the patients were treated with conventional twin brackets or self-ligating brackets because Cardoso et al. (2015) found no significant difference in plaque accumulation or gingival inflammation between patients treated with either appliance [26]. We excluded patients with active caries, advanced periodontal disease, patients with chronic systemic diseases, patients who currently use or have used antibiotics in the last 30 days, patients with significantly reduced saliva production and patients who have had radiation therapy to the head and neck region.

Clinical data and plaque samples were collected at the pre-treatment baseline (T0), 1 month (T1), 3 months (T2) and 6 months (T3). Plaque index (PI) was measured using the Turesky et al. Modified Quigley-Hein Plaque Index (TQHPI), which requires the use of disclosing solution and scores supragingival plaque formation on a numerical scale from 0 to 5 (Figure 2, Table 1) [27-29]. The level of gingival disease was measured using the Löe and Silness Gingival Index (GI), which is based on two of the characteristic signs of inflammation: swelling and bleeding (Table 2) [30, 31]. The GI does not consider quantitative changes of the periodontal tissues, such as pocket depths, but rather focuses on the qualitative changes [32].

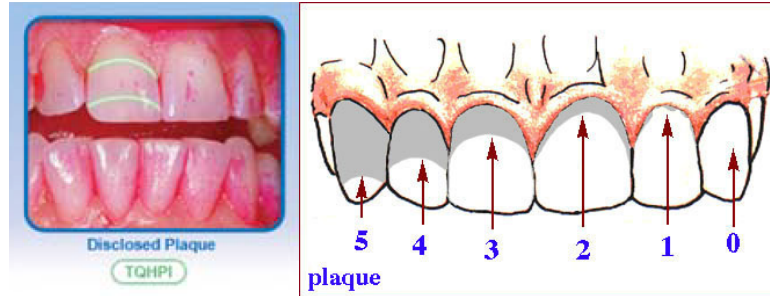


Figure 2: The image on the left shows plaque colored with disclosing solution [29]. The image on the right indicates the different criteria of the TQHP index described in the table below [29].

PI Scores	Criteria
0	No plaque
1	Separate flecks of plaque at the cervical margin of the tooth
2	A thin continuous band of plaque (up to one mm) at the cervical margin of the tooth
3	A band of plaque wider than one mm but covering less than one-third of the crown
4	Plaque covering at least one-third but less than two-thirds of the crown of the tooth
5	Plaque covering two-thirds or more of the crown of the tooth

Table 1: Criteria for the Turesky et al. Modified Quigley-Hein Plaque Index (TQHP)

GI Scores	Gingival Status	Criteria
0	Normal Gingiva	Natural coral pink gingiva with no evidence of inflammation
1	Mild Inflammation	Slight changes in color, slight edema. No bleeding on probing.
2	Moderate Inflammation	Redness, edema and glazing. Bleeding upon probing
3	Severe Inflammation	Marked redness and edema/ulceration/tendency to bleed spontaneously

Table 2: The Löe and Silness Gingival Index scoring system [30-32]

Supragingival plaque was collected using sterilized periodontal scalers from anterior and posterior teeth, specifically the gingival third of the buccal and lingual surfaces of the central incisors and first or second premolars depending on whether the patient was treated with extractions. Plaque was also collected from the patient's most recent aligner trays using

interproximal brushes [33]. All plaque samples were deposited into separate sterile collection tubes containing 15% glycerol in phosphate buffered saline (PBS) [34].

DNA from the plaque samples was extracted and microbial community analysis was performed via next generation sequencing of the 16S rRNA gene using the MiSeq platform (Illumina) available at the UCLA Microbiome Core [35]. Bioinformatic data analysis was performed using the following procedures: after demultiplexing and trimming of barcodes, low quality sequences containing bases with Phred quality values <20 as well as sequences with >3% uncertain basepairs were removed. The 16S rRNA sequences were clustered into operational taxonomic units at a 98% similarity level using QIIME [36] and taxonomically assigned by comparison to the Human Oral Microbiome Database (HOMD) [37].

Alpha-diversity (Shannon Index), Beta-diversity (Weighted UniFrac) and principal coordinate analyses were calculated in QIIME. Alpha-diversity is defined as “within-sample diversity,” “biodiversity” or richness [38], and can be measured using phylogenetic diversity, which is a calculation of diversity that considers phylogenetic distance or relatedness of all sequences found in a given sample [39]. Beta-diversity is defined as the difference between each sample [40]. Principal coordinates analysis (PCoA) was performed to calculate “coordinates” for each sample to give a visual representation of each sample relative to every other sample. The closer each sample appears to another, the more similar the samples [41].

The power of this study was calculated using the G*Power statistical analysis program [42, 43]. Normality of the data was then determined using the Shapiro-Wilk analysis [44]. Statistical significance was calculated using t-test for the PI data and the Mann Whitney U-test for the GI data at a level of $p \leq 0.05$.

RESULTS

The average age of the clear aligner group (29.83 ± 11.98 years) was greater than the average age of the fixed appliance group (20.83 ± 13.35 years), but this difference was not statistically significant ($p=0.096$). Each group had twelve subjects with four males and eight females in the fixed appliance group, while the clear aligner group had three males and nine females (Table 3, Supplemental Table 1). Due to the variation in patient recall intervals and missed appointments, each patient was collected at T0 and at least one of the three remaining time points (T1, T2, T3).

	Fixed Appliance	Clear Aligner
Subjects	12	12
Male	4	3
Female	8	9
Mean Age	20.83	29.83
SD	13.35	11.98

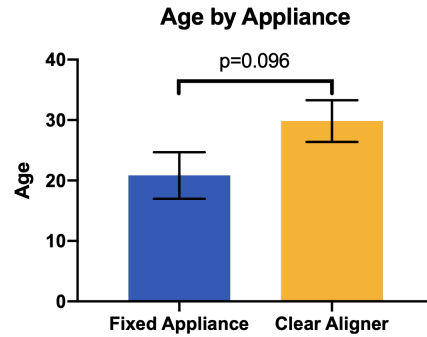


Table 3: Gender and age distribution for fixed appliance and clear aligner groups

Shapiro-Wilk Statistic analysis was performed to test for normality of the data [44]. For the threshold $p=0.05$, the calculated W value should be greater than 0.916 to indicate that the data has a normal distribution. Given the W values shown in Table 4, the PI data seems to follow a normal distribution, while the GI data does not follow a normal distribution.

	Plaque Index	Gingival Index
n	73	73
Mean	1.80	0.42
SD	0.81	0.44
W	0.9697	0.8451
Distribution	Normal	Not Normal

Table 4: Summary of Shapiro-Wilk Statistic Analysis. Given a threshold $p=0.05$, a W value greater than 0.916 implies the data follows a normal distribution

The implied α and power of the study was then calculated using the G*Power statistical analysis program [42, 43]. The effect size for the PI data was 0.4274, and the calculated power was 0.7495. The effect size for the GI data was 0.6139, and the calculated power was 0.8583.

The clinical data (PI and GI) for each time point is outlined in Table 5. Given the normality of the PI data, a two-tailed t-test was used to calculate p-values. PI scores showed an overall increase in plaque levels from baseline (T0) to treatment (T1-T3) in both groups, with a significant increase observed in the fixed appliance group ($p < 0.01$, Figure 3A). When PI scores were separated by time point, there was a significant increase in the fixed appliance group from T0 to T2 ($p < 0.01$) and T0 to T3 ($p < 0.01$); however, the clear aligner group did not have any significant differences in PI from T0 to T3 (Figure 3B). When PI scores were further divided into Anterior and Posterior PI scores, a similar result was observed. For Anterior PI, there was a significant increase in the fixed appliance group from T0 to T2 ($p < 0.01$) and T0 to T3 ($p < 0.01$), but no significant differences in the clear aligner group (Figure 4). Additionally, for Posterior PI, there was a significant increase in the fixed group from T0 to T2 ($p < 0.05$) and T0 to T3 ($p < 0.01$), but no significant differences in the aligner group (Figure 4).

Appliance	Time Point	n	PI (Mean \pm SD)	GI (Mean \pm SD)
Fixed Appliance	T0	12	1.38 \pm 0.66	0.28 \pm 0.37
	T1	6	1.91 \pm 0.79	0.39 \pm 0.52
	T2	11	2.22 \pm 0.73	0.58 \pm 0.48
	T3	7	2.65 \pm 0.40	1.07 \pm 0.29
Clear Aligner	T0	12	1.49 \pm 0.93	0.18 \pm 0.22
	T1	6	1.25 \pm 0.85	0.09 \pm 0.14
	T2	10	1.90 \pm 0.70	0.48 \pm 0.36
	T3	9	1.78 \pm 0.63	0.35 \pm 0.44

Table 5: Clinical parameters at each time point for fixed appliance and clear aligner groups

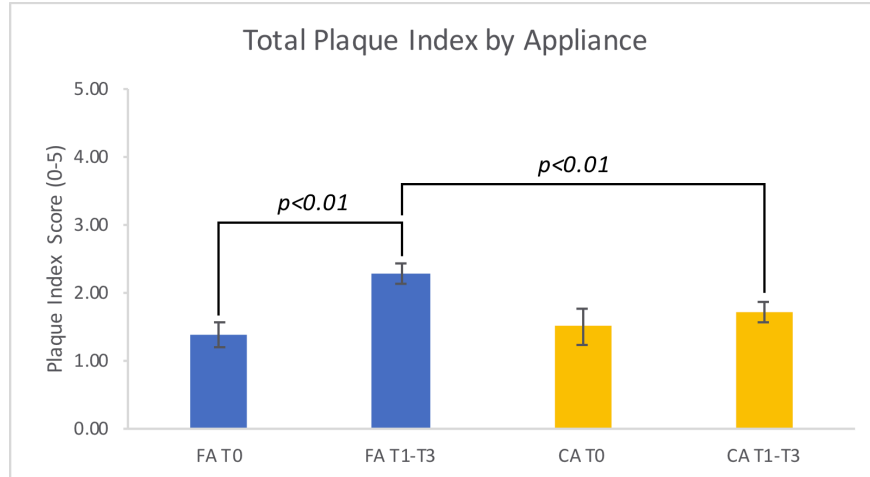


Figure 3A: Overall PI scores for fixed appliances (FA) and clear aligners (CA) at baseline (T0) compared to treatment (T1-T3)

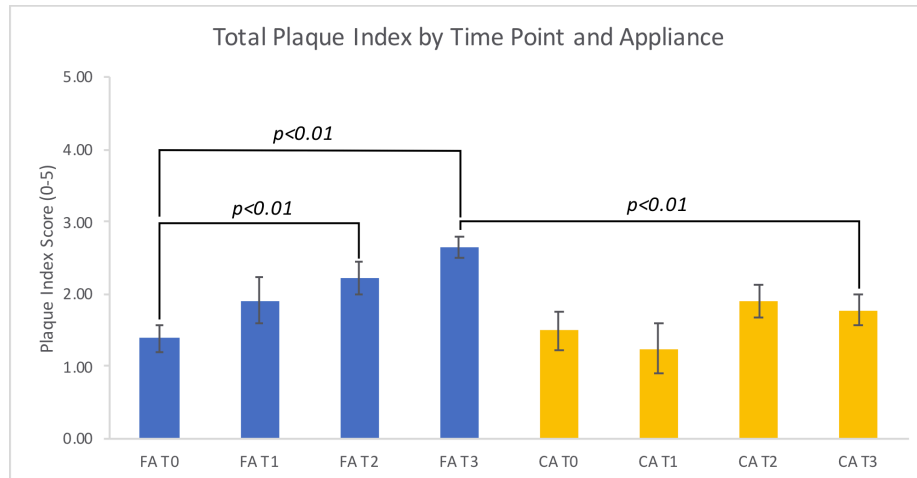


Figure 3B: Overall PI scores separated by time point

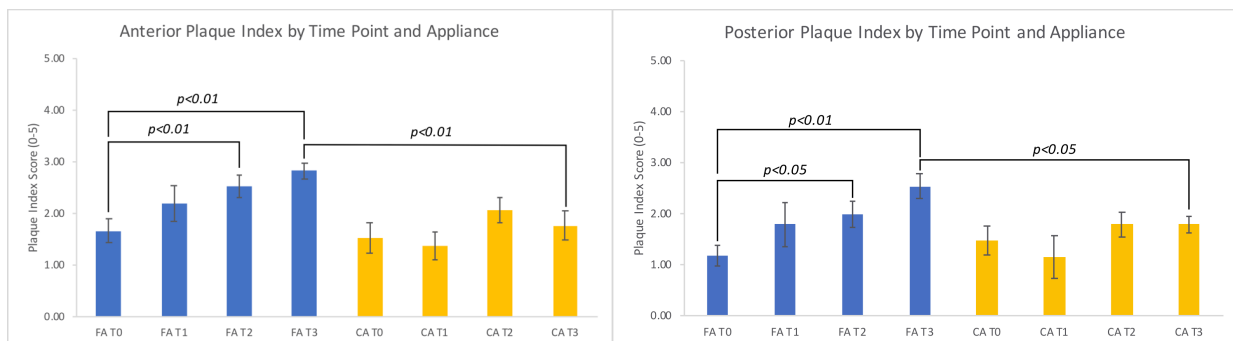


Figure 4: Anterior PI (left) and Posterior PI (right) scores separated by time point

Since the GI data did not follow a normal distribution, significance was calculated using the Mann-Whitney U-test. Similar to PI scores, the GI scores followed an upward trend from baseline (T0) to treatment (T1-T3) in both groups, with a significant increase in the fixed appliance group (Figure 5A, $p<0.05$). When GI scores were separated by time point, there was a significant increase in the fixed appliance group from T0 to T3 ($p<0.01$) and in the clear aligner group from T0 to T2 ($p<0.05$, Figure 5B). When further divided into Anterior and Posterior GI scores, there was a significant increase in Posterior GI from baseline to treatment in the fixed appliance group ($p<0.05$, Figure 6). However, there were no significant differences in Anterior or Posterior GI in the clear aligner group, nor was there a significant difference in Anterior GI in the fixed appliance group ($p=0.0633$, Figure 6). Next, we tested if there was a correlation between PI and GI. We found a strong correlation in the fixed appliance group ($r=0.8347$), but there was no correlation between PI and GI in the clear aligner group ($r=0.3172$, Figure 7).

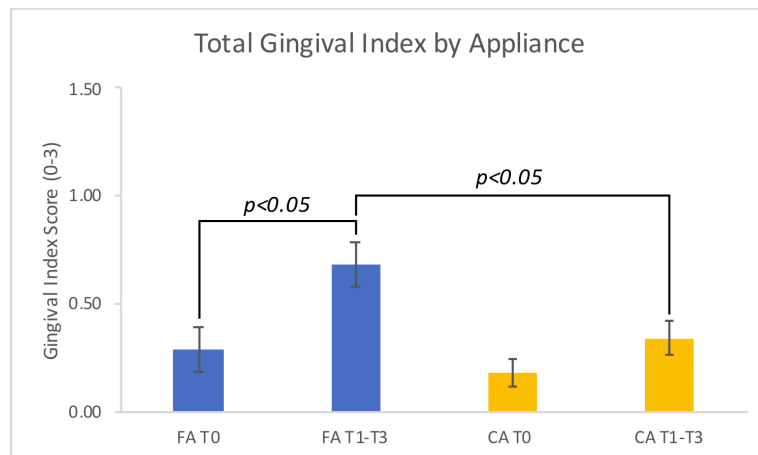


Figure 5A: Overall GI scores for fixed appliances (FA) and clear aligners (CA) at baseline (T0) compared to treatment (T1-T3)

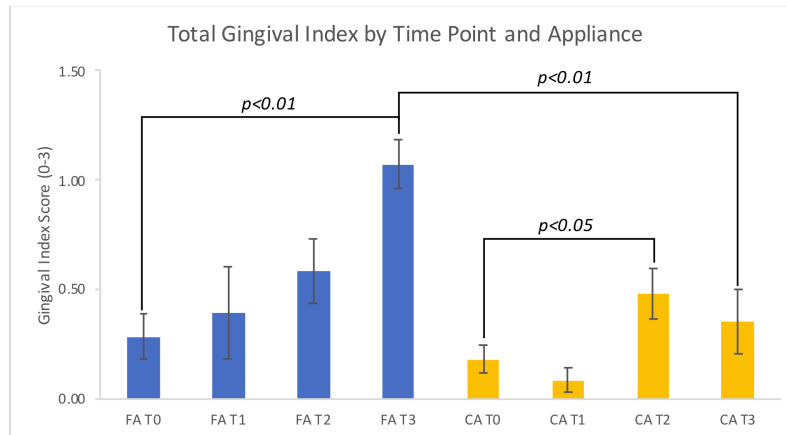


Figure 5B: Overall GI scores separated by time point

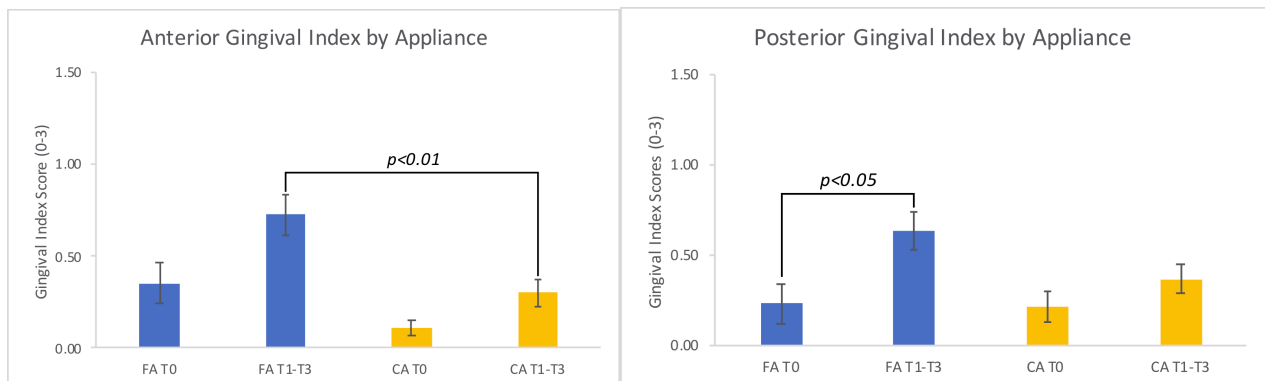


Figure 6: Anterior GI (left) and Posterior GI (right) scores for FA and CA at baseline (T0) compared to treatment (T1-T3)

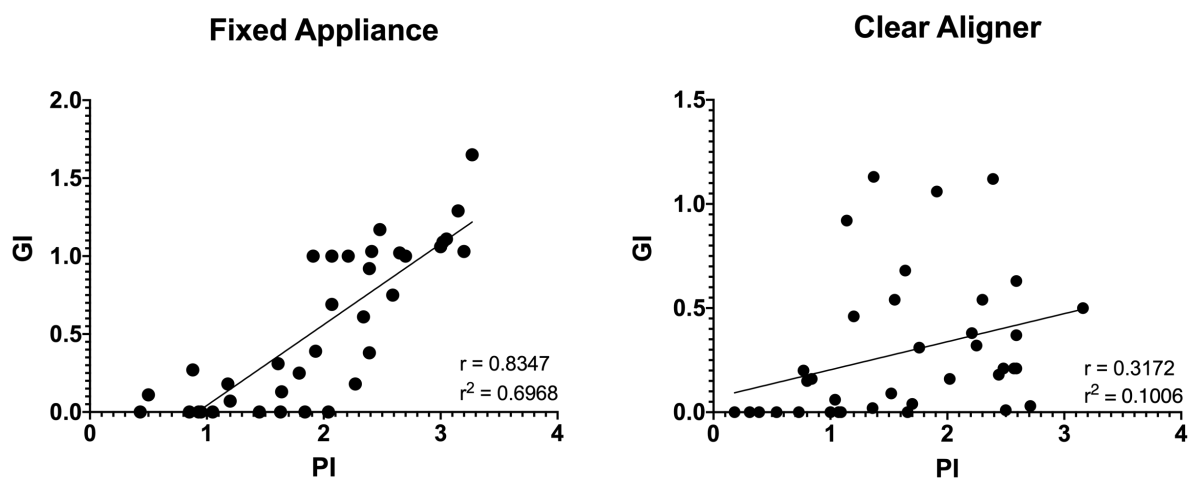


Figure 7: Correlation between PI and GI in FA (left) and CA (right) groups

Figure 8 shows taxa composition analysis from the 16S rRNA sequencing data. Each subject appears to have a unique bacterial community profile that shifts after beginning orthodontic treatment. Anterior and posterior plaque seem to have a similar microbial composition, while tray biofilm has a distinct composition and is less diverse than the composition of oral plaque samples. Figure 9 shows certain bacterial species that are significantly different in the tray biofilm compared to oral plaque samples. For instance, *Actinomyces*, *Corynebacterium* and *Campylobacter* species are greatly reduced in the clear aligner trays, while *Gemella* and *Neisseria* are significantly enriched in the trays (Figure 9). Alpha diversities were calculated for anterior, posterior and tray plaque samples at each time point. There was an apparent increase in alpha diversity in anterior and posterior plaque in the fixed appliance group at time points T2 and T3, but none of the differences were significant (Figure 10). In addition, tray samples are less diverse than anterior and posterior plaque in the clear aligner patients (Figure 10).

Beta-diversity analysis of baseline samples was performed to show that there is no significant difference between both groups at baseline. The comparison of the baseline samples explains more than 40% of the variation, which confirms that no significant difference exists (Figure 11A). When we compared the clear aligner patients only, anterior and posterior plaque generally overlap, while tray samples are close yet distinct (Figure 11B). Beta-diversity analysis of the clear aligner (Figure 11C) and fixed appliance groups (Figure 11D) by location shows that there is a tendency of samples to cluster by patient.



Figure 8: Taxa composition analysis showing bacterial profiles for patients in both groups

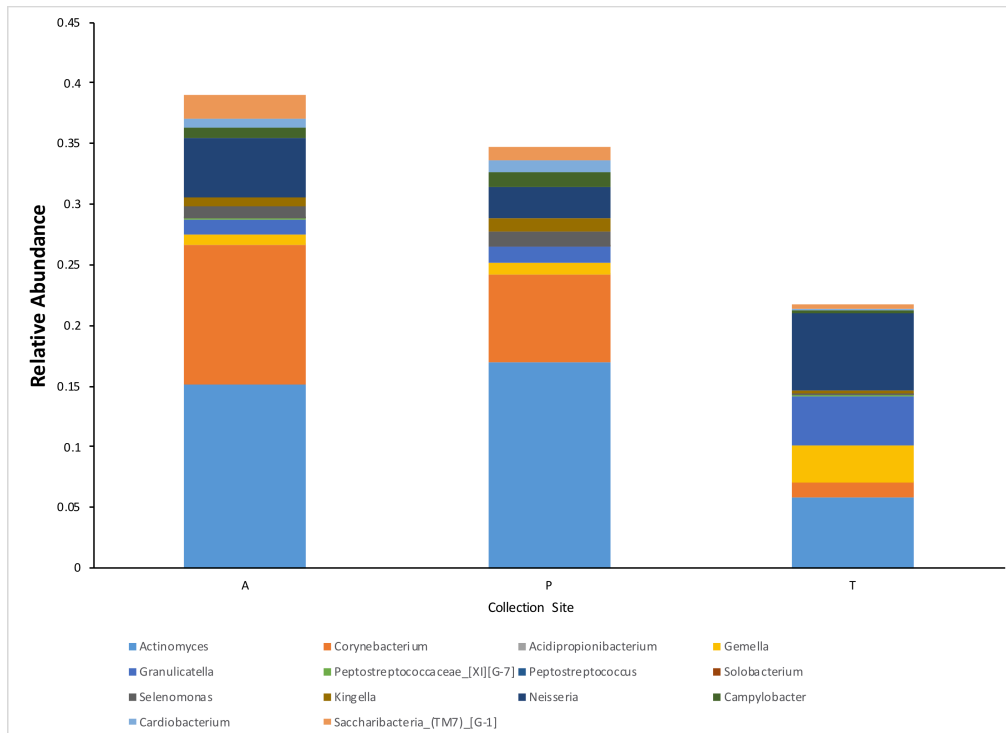


Figure 9: Taxa composition analysis highlighting significantly difference species present in anterior plaque (A), posterior plaque (P) and tray plaque (T).

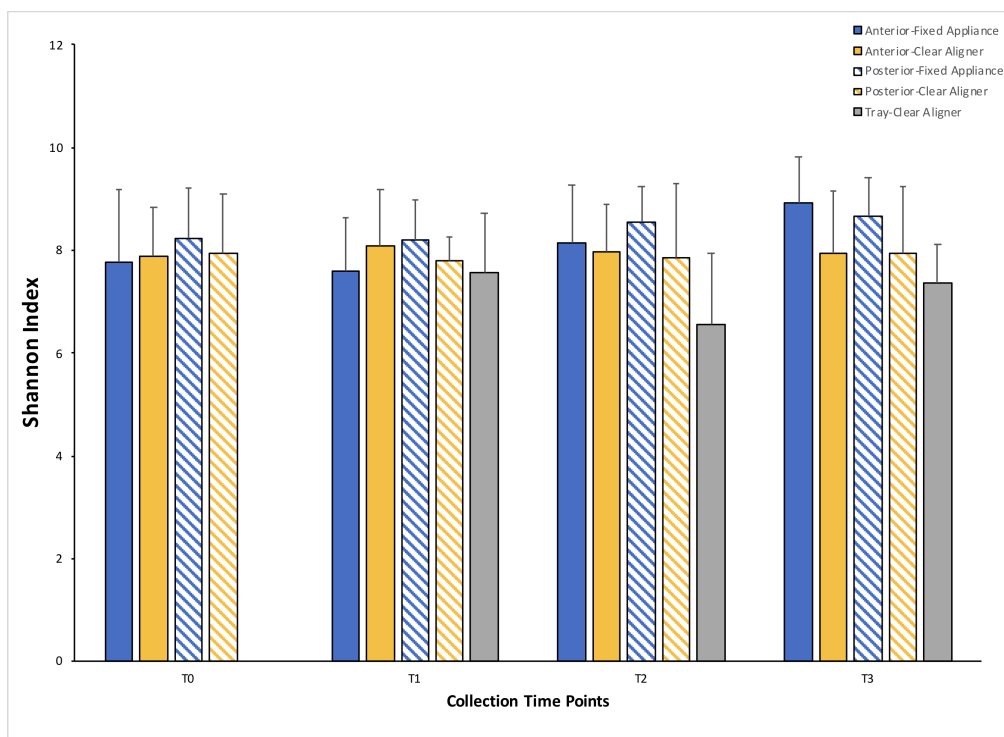


Figure 10: Alpha-diversity analysis showing the microbial richness of the biofilm samples at each time point for anterior, posterior and tray plaque

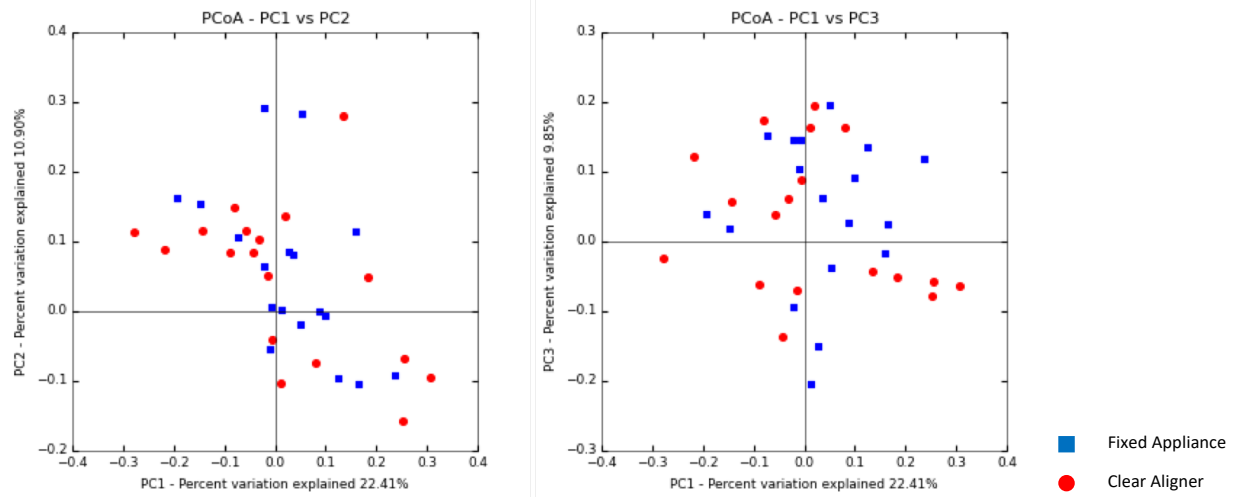


Figure 11A: Beta-diversity analysis showing comparison of Baseline (T0) samples

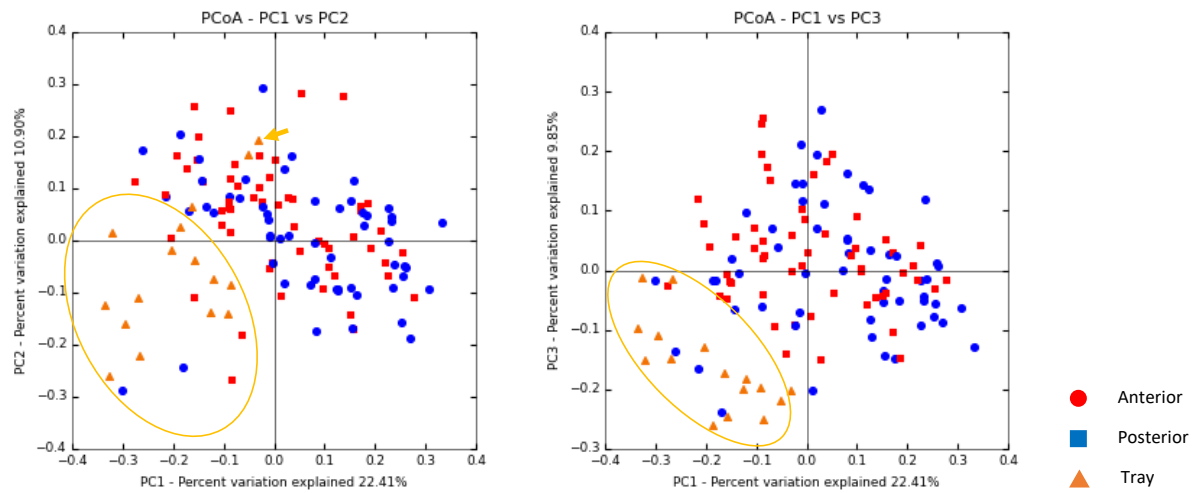


Figure 11B: Beta-diversity analysis of the clear aligner group by location

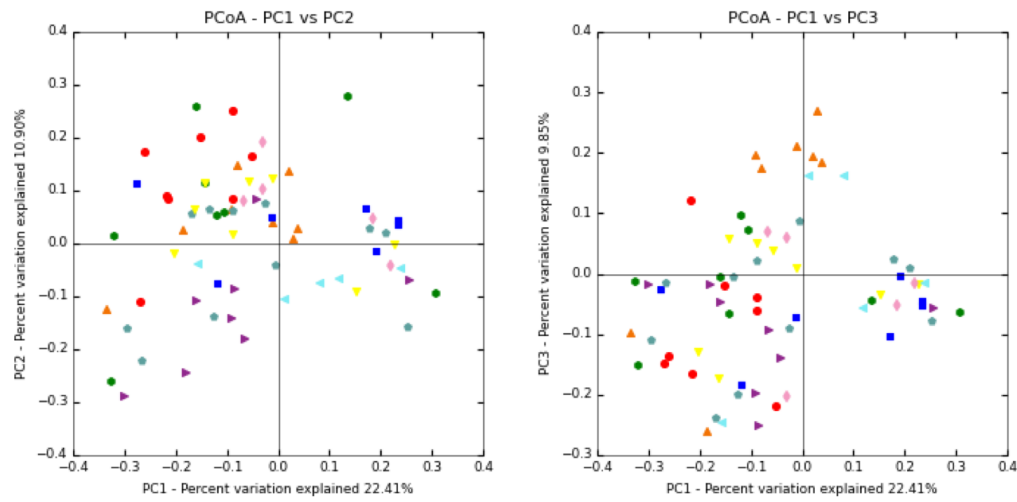


Figure 11C: Beta-diversity analysis of the clear aligner group by patient

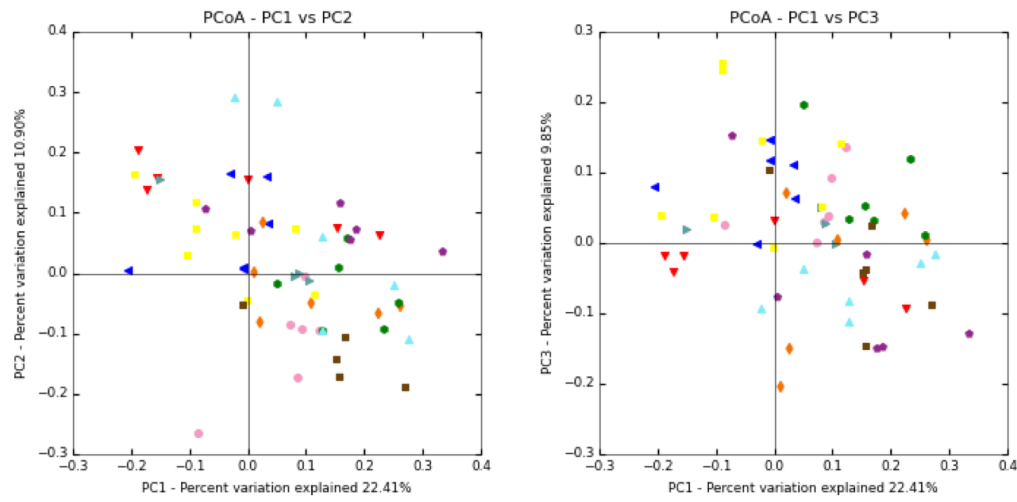


Figure 11D: Beta-diversity analysis of the fixed appliance group by patient

DISCUSSION

Orthodontic treatment with either fixed appliances or clear aligners may cause a hindrance to normal oral hygiene practices, which can result in increased plaque accumulation and gingival inflammation. Fixed appliances require the use of bands, brackets and wires, which make plaque removal and especially flossing more difficult. Clear aligner treatment introduces a foreign material covering the entire dentition for nearly 22 hours per day, and usually requires the use of bonded composite attachments to facilitate orthodontic tooth movement. Although clear aligners are removable and should result in better maintenance of oral hygiene, the current literature does not have a definitive conclusion. While several studies have shown better periodontal health status for patients treated with clear aligners compared to fixed appliances, other studies have concluded that there is no significant difference in periodontal health between the groups [5, 7, 17-20].

Relatively few studies have used next generation 16S rRNA sequencing to investigate changes in the oral microbiome during clear aligner therapy. Furthermore, the studies that are available did not compare microbial changes in patients treated with clear aligners to those with fixed appliances [22]. The nature of plaque accumulation in clear aligner therapy is different than fixed appliances because patients are able to remove their aligner trays for eating and performing oral hygiene. Due to the increasing popularity of clear aligners among orthodontic patients, a more comprehensive understanding of how this treatment modality affects the oral environment is necessary. Through this study, we were able to provide insights into how clear aligners and fixed appliances affect the oral microbiome and periodontal health, as well as provide, for the first time, microbial analysis of the community present within the clear aligners themselves.

The fixed appliance group experienced a significant mean increase in PI score of 0.89 ($p<0.01$) over the first 6 months of treatment, compared to the clear aligner mean increase of 0.21, which was not significant ($p=0.46$, Figure 3A). When separated by time point, there was a significant increase in PI for the fixed appliance group from baseline (T0) to 3 months (T2, $p<0.01$) and baseline to 6 months (T3, $p<0.01$), but there were no significant changes in the clear aligner group (Figure 3B). When PI was divided into Anterior and Posterior PI, a similar result was observed. Thus, this study concludes that clear aligner therapy results in less plaque buildup on teeth compared to fixed appliances. This observed lower level of plaque could be due to greater ease of plaque removal, decreased plaque accumulation or a combination of both.

The fixed appliance group also had a significant mean increase in GI score of 0.39 ($p<0.05$) over the first 6 months of treatment, compared to the clear aligner mean increase of 0.16, which was not significant ($p=0.26$, Figure 5A). When separated by time point, there was a significant increase in GI in the fixed appliance group from baseline to 6 months ($p<0.01$, Figure 5B). Additionally, there was a significant increase in GI in the clear aligner group from baseline to 3 months (T0 to T2, $p<0.05$), but this increase returned to baseline levels at 6 months (T0 to T3, $p=0.39$). When further divided into Anterior and Posterior GI scores, there was a significant increase in Posterior GI from baseline to treatment in the fixed appliance group ($p<0.05$, Figure 6B). However, there were no significant differences in Anterior or Posterior GI in the clear aligner group ($p=0.11$ and $p=0.18$, respectively), nor was there a significant difference in Anterior GI in the fixed appliance group ($p=0.063$, Figure 6A). Therefore, we can conclude that orthodontic treatment with fixed appliances results in increased gingival inflammation and that this increase is localized to the posterior teeth. Clear aligner therapy also resulted in an increase

in gingival inflammation, but this increase was transient and eventually returned to baseline levels.

Due to the strong correlation between PI and GI in the fixed appliance group ($r=0.8347$), we can conclude that the significant increase in PI in the fixed appliance group is associated with the significant increase in GI from baseline to 6 months. On the other hand, the clear aligner group had a very weak correlation between PI and GI ($r=0.3172$). This can help explain our finding that although there was no significant increase in PI in the clear aligner group, there was a transient increase in gingival inflammation at 3 months. In the fixed appliance group, there was an apparent increase in alpha diversity at time points T2 and T3 from baseline, whereas the clear aligner group remained relatively stable (Figure 10). Previous studies have shown that increased gingival inflammation is associated with a more diverse microbiota [45], which may help explain the significant increase in GI in the fixed appliance group.

Alpha-diversity analysis revealed that there were no significant differences between anterior and posterior plaque (Figure 10), and beta-diversity analysis showed that anterior and posterior plaque samples generally overlap (Figure 11B). These findings indicate that the microbial composition of tooth-associated plaque is relatively stable throughout the oral cavity. Therefore, future studies should choose a single, consistent location to sequence biofilm rather than collecting plaque from multiple teeth.

Microbial sequencing showed that each subject appears to have a unique bacterial community profile that shifts after beginning orthodontic treatment (Figure 8). Beta-diversity analysis of both clear aligner and fixed appliance groups indicated that there is a tendency of the samples to cluster by patient (Figures 11C, 11D). This suggests that there are patient-specific

shifts in the microbial communities from baseline, which is likely due to the host effect on the microbiome [46].

Alpha-diversity analysis also showed that tray samples were less diverse (lower Shannon Index) than anterior and posterior plaque. In addition, taxa composition analysis and beta-diversity revealed that the microbial composition of tray biofilm was significantly different from tooth-associated biofilm (Figures 9, 11B). Since patients are instructed to wear their aligner trays for at least 22 hours per day, the biofilm present in the aligners is in contact with the teeth for a majority of the time during orthodontic treatment. In general, the tray biofilm did not significantly alter the composition of the tooth-associated biofilm during treatment. However, in patients 17 and 37, there were a few anterior and posterior samples that co-localized with the tray samples. Future studies with a greater sample size are necessary to evaluate whether the tray biofilm may affect tooth-associated biofilm in a subset of clear aligner patients.

Orthodontic treatment with fixed appliances or clear aligners results in an imbalance in the microbial ecosystem and leads to an increased risk of white spot lesions, caries and periodontal disease. Improved understanding of the microbial species present during orthodontic treatment will lead to better prevention of these orthodontic-related complications. Future studies should include time points at 12 months after starting treatment, at the removal of appliances and 3 months after removal to further evaluate more long-term effects of orthodontic treatment with either treatment modality.

CONCLUSION

Patients treated with fixed appliances experienced significantly more plaque accumulation (measured by PI) on the surfaces of the teeth compared to patients treated with clear aligners over the first six months of orthodontic treatment. This increase in plaque accumulation was associated with a significant increase in gingival inflammation (measured by GI) in the fixed appliance group. There was no significant difference in the microbiome found in plaque collected from anterior teeth compared to posterior teeth. In addition, the inner surfaces of the clear aligner trays contain a unique microbial community that is less diverse than those found in tooth-associated biofilms. Future studies with a larger sample size and more long-term time points are needed to better understand the clinical and microbial changes that are induced by orthodontic treatment, and to evaluate whether these changes are permanent or return to pre-treatment levels once treatment is completed.

SUPPLEMENTAL TABLES/FIGURES

Patient	Appliance	Age	Gender
3	Fixed Appliance	21	Male
4	Fixed Appliance	40	Male
6	Fixed Appliance	12	Female
8	Fixed Appliance	17	Male
9	Fixed Appliance	14	Female
10	Fixed Appliance	14	Female
13	Fixed Appliance	13	Female
15	Fixed Appliance	50	Female
29	Fixed Appliance	11	Female
33	Fixed Appliance	15	Female
36	Fixed Appliance	8	Female
41	Fixed Appliance	35	Male
1	Clear Aligner	22	Male
11	Clear Aligner	26	Female
17	Clear Aligner	38	Female
18	Clear Aligner	24	Female
20	Clear Aligner	27	Female
23	Clear Aligner	56	Male
35	Clear Aligner	23	Female
37	Clear Aligner	25	Female
40	Clear Aligner	51	Female
60	Clear Aligner	23	Female
62	Clear Aligner	20	Male
63	Clear Aligner	23	Female

Supplemental Table 1: Patient age and gender distribution

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