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Initial Changes In The Oral Microbiome In Orthodontic Patients During Treatment

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Initial Changes in The Oral Microbiome in Orthodontic Patients During Treatment

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

by

Andrew R. Hopkins

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ABSTRACT OF THE THESIS

Initial Changes in The Oral Microbiome

in Orthodontic Patients During Treatment

by

Andrew R. Hopkins

Master of Science in Oral Biology

University of California, Los Angeles, 2023

Professor Renate Lux, Chair

Background: The oral microbiome is host to a diverse set of microbes and is extremely dynamic depending on the environment that it is placed in. Microbial profile changes are associated with a patient's ability to practice proper oral hygiene, the diet they maintain, adequate salivary flow and composition, and a myriad of other contributing factors. Orthodontic appliances pose as an obstacle as they act as plaque retentive surfaces on the teeth and hinder the patient's ability to perform oral hygiene successfully. The inability to remove plaque allows it to develop into a mature biofilm, capable of causing dental diseases such as caries and periodontal disease.

Objective: Our study aims to investigate how patients microbial profiles and gingival/plaque indices change over time comparing patients undergoing orthodontic therapy through fixed appliances with those receiving clear aligner therapy with a focus on early time points.

Methods: The study was performed on a total of nine patients, five of which received fixed appliances and four received clear aligner therapy. Both supragingival and subgingival bacterial samples were collected at six different timepoints: T0 (baseline directly before orthodontic treatment commences), T1 (one-week after orthodontic treatment commences), T2 (two-weeks), T3 (three-weeks), T4 (four-week) and T5 (three-months). Additionally, at each visit the gingival and plaque indices were recorded. The indices were compared within each group at successive time points as well as between both treatment groups. The composition of the plaque samples was elucidated by extracting the DNA and performing next generation sequencing of the 16S rRNA. Changes in plaque profiles, diversity, and overall composition were compared between timepoints and between both treatment groups.

Results: The results of the study showed that there was a general trend for higher gingival indices in patients undergoing FA when compared to CA therapy, which was statistically significant at T5 ($p < 0.01$). Similarly, patients undergoing treatment with FA showed statistically significantly higher plaque scores at timepoints T3-T5 than patients in the CA group ($p < 0.01$). The microbiological analysis revealed that the bacteria associated with the clear aligner tray had a unique microbial flora when compared to both the supragingival and subgingival flora. This flora exhibited significantly lower levels of *Actinomyces*, and higher levels of *Porphyromonas* and *Streptococcus*. The FA group showed significantly higher levels of various genera of bacteria known to be involved in the progression of periodontal disease such as, *Porphyromonas*, *Tannerella*, *Fusobacterium*, *Leptotrichia*, and Lachnospiraceae. Alpha – and beta-diversity showed no significant differences in composition of supragingival versus

subgingival microbial communities but did confirm the unique microbiota associated with the tray bacteria.

Conclusions: The results of the study correlate with previous studies that discussed a higher gingival and plaque index associated with patients undergoing orthodontic treatment with fixed appliances when compared to clear aligners. The microbiological analysis confirmed a unique biofilm community associated with the tray and not the supragingival nor the subgingival microbiota. More importantly than quantity of the bacterial load, the type of the bacteria is an important player in the progression of periodontal disease, specifically the red complex. Our analysis showed that the patients undergoing FA therapy showed significantly higher levels of periodontal pathogens compared to CA, which is consistent with the observation of significant differences in gingival indices between treatments.

The thesis of Andrew R. Hopkins is approved.

Bo Yu

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2023

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INTRODUCTION

The oral cavity hosts one of the most diverse and dynamic microbiomes, consisting of over 770 prokaryotic species.[1] The microorganisms play significant roles in aiding the host immune system, as well as in causing disease. The composition and relative abundance of different species can shift the oral microbiome from a state of health to a state of disease.[2] There are many contributing factors that alter the composition including feeding habits, oral hygiene, temperature, as well as alterations in the pH. When these factors lead to an unbalanced microflora, it has been shown to be associated with diseases such as dental caries, periodontal disease, diabetes, and circulatory problems.[3] The significance of maintaining a balanced oral microflora is crucial in maintaining a healthy state instead of a diseased one.

Dental malocclusions affect a great majority of the population and consist of problems in relation to skeletal asymmetries, or dental issues such as crowding and protrusive teeth.[4] Though often viewed as more of a cosmetic treatment, orthodontics plays a large role in maintaining the health and function in the oral cavity. Orthodontic problems such as severe crowding impedes adequate plaque removal and along with other factors can lead to periodontal disease.[5] Tondelli et al., showed that patients who have severe crowding associated with periodontal disease benefit from orthodontic treatment.[6] He showed that the resolution of crowding, combined with better ability to perform oral hygiene reduced plaque accumulation, which led to control of periodontal disease. In cases of severe crowding, orthodontic treatment might be viewed more as a therapeutic treatment than a cosmetic one alone.

It is commonly seen in patients undergoing orthodontic treatment to have increased levels of plaque surrounding the brackets on the teeth. Orthodontic appliances in the traditional form of braces act as a retentive surface for plaque accumulation.[7] In modern orthodontics, a differential effect of various orthodontic appliances on plaque retention is being debated. For instance, Chhibber et al., noted no differences in long-term plaque retention when comparing conventional brackets versus self-ligation brackets or clear aligner therapy.[8] It was previously demonstrated that through full coverage of dental crowns and a decrease in the ability for salivary flushing of the teeth, the treatment through clear aligner therapy is no better

than fixed orthodontics in limiting plaque accumulation.[9] On the other hand, studies have shown that there is a statistically significant increase in plaque accumulation over a six-month treatment time for fixed orthodontic therapy when compared to clear aligner therapy.[10] Similarly, Issa et al. noted a statistically significant improvement for parameters such as gingival index, plaque index, bleeding on probing, when patients were treated using clear aligners. They showed such improvement is due to the ease of access and better oral hygiene performed by the patients.[11] It can be elucidated from the aforementioned studies that our understanding of the true impact of different orthodontic therapies on plaque index is still incomplete and should be evaluated further.

Similarly, when comparing clear aligners versus fixed appliances it was noted that salivary samples demonstrated a statistically significant decrease in microbial diversity when comparing clear aligner therapy and fixed appliances to a control group receiving no orthodontic treatment.[12] Perhaps more importantly than if plaque index is increased between different treatment modalities, is if a shift in the microbial diversity and composition is seen. Shokeen et al. noted that there was a patient-specific shift in the microbial environment whereby there was no similarities across patients.[10]

Often seen in orthodontic treatment are the formation of white spot lesions. These white spot lesions are the earliest stages of the caries process, and we often identify *Streptococcus mutans* as the initiating bacterium in this process.[13] Evaluating how *S. mutans* levels are affected through orthodontic therapy can give insight into why these lesions are so common. Jing et al. noted no significant increase in total bacteria when patients underwent fixed orthodontic therapy, but noted an increase in *S. mutans*.[13] They demonstrated that when comparing *S. mutans* levels to total bacteria collected over four time points, there was a statistically significant increase in *S. mutans* in proportion to total bacterial level.[13] Interestingly, it has been shown that when comparing clear aligner therapy to fixed orthodontic appliances, clear aligner therapy had significantly lower plaque and gingival indices, but no difference in *S. mutans* levels.[14] It is demonstrated through these previous studies that the true shift in total bacterial levels, as well as alterations in specific bacteria is largely unknown.

Similar to the alterations in the microflora associated with supragingival bacteria, the subgingival plaque may be altered through orthodontic treatment which can affect periodontal structures. Guo et al. collected subgingival plaque from patients undergoing fixed orthodontic therapy and noted a transient increase in *Porphyromonas gingivalis, Fusobacterium nucleatum, Prevotella intermedia*, and *Tannerella forsythia*.[15] Each of these bacterial species have been determined to be key players in the development of periodontal disease and thus determining how they change during orthodontic therapy is of much importance.[16] Gujar et al. collected plaque samples from conventional brackets, lingual brackets as well as clear aligner therapy and compared their microbial compositions. The results of their study noted that *F. periodontium* and *P. intermedia* were increased in the conventional bracket group when compared to the other treaments.[17] The aforementioned studies show that there is an alteration in the environment of the oral cavity when undergoing orthodontic therapy, which causes a shift in the microbiome that may lead to an increase in pathogenic bacteria.

There is a great need to elucidate the dynamic changes that occur in the microbiota during orthodontic treatment because of the clinical implications that result from such a shift. Recent studies have shown that the prevalence of a new carious lesion for a patient undergoing orthodontic therapy is 45.7% and the prevalence for formation of a white spot lesion is 68.4%.[18] Another study noted that 57.9% of patients undergoing orthodontic therapy had white spot lesions, with an average of 4.8 teeth involved, and an even greater prevalence when treatment time exceeded 17 months.[19] In addition to the effects that orthodontics has on the demineralization process of dental crowns, it may also negatively impact the periodontium. It has been shown that during fixed orthodontic therapy there was a statistically significant increase in both pocket depths and bleeding on probing, which if remain uncontrolled could negatively impact the patient's periodontal health.[20] There is a need to explain the true impact that both fixed orthodontic therapy and clear aligner therapy have on both supragingival and subgingival biofilm composition, and how this may alter the patient's overall oral health.

The current study aims to answer the question about the changes in gingival index, plaque indices, and plaque composition between fixed appliances and clear aligner therapy after the initiation of orthodontic therapy. Unlike previous studies, our collections will include the

earliest stages of therapy in weekly intervals and thus allow to determine precisely at what point significant changes in the clinical parameters and microbiome occur. Additionally, we will be able to track the maturation of the dental biofilm and assess which, if any, of the known pathogenic bacteria in dental caries or periodontal disease become more prevalent when patients are undergoing orthodontic therapy.

Overall Objectives and Specific Aims:

Objective: The objective of this study is to evaluate the shift in oral microbial plaque and salivary compositions in patients undergoing orthodontic treatment through fixed and clear aligner therapies throughout the initial stages of orthodontic treatment. It is hypothesized that there will be a significant shift in the microbial composition and diversity in the plaque and salivary samples.

Specific Aims:

1) To analyze differences in gingival and plaque indices in patients undergoing fixed orthodontic appliances versus clear aligner therapy
    a) Analyze the change in clinical indices within each group at successive timepoints
    b) Compare the plaque and gingival indices between different treatment modalities
2) To analyze relative abundance, shift in diversity, and changes in the microbial composition over the first four-weeks and at three months of orthodontic treatment.
    a) Compare microbial composition of supragingival and subgingival plaque, as well as bacteria associated with the tray surface using alpha-diversity and beta-diversity measures.
    b) Using the same analyses, analyze these changes within each group at sequential visits as well as comparing fixed appliances versus clear aligner therapy.

Materials and Methods

Study participants were patients from the orthodontics clinic at UCLA under IRB #20-000316-AM-00001. Nine patients were recruited who were to begin treatment through either fixed orthodontic appliances or through clear aligner therapy. Patients who agreed to participate in the study signed consent forms. A legal guardian signed on behalf of a participant, who was a minor.

The inclusion criteria included patients of any age undergoing orthodontic treatment through either fixed appliances or clear aligner therapy. The exclusion criteria consisted of patients with active caries or periodontal disease, patients who had chronic systemic diseases, patients who had taken antibiotics within the previous 30 days, pregnant patients or those planning to be pregnant, and patients claiming to have any salivary flow anomalies. The exclusion criteria also consisted of patients who had an initial stage of orthodontic treatment that was not accompanied by fixed or clear aligner therapy., i.e. patients being treated with expanders for extended periods of time.

Study participants were seen at six different time points, T0 (baseline, before bonding), T1 (one-week following the start of orthodontic treatment), T2 (two-weeks), T3 (three-weeks), T4 (four-weeks), and T5 (three-months). At T0, each patient was given a baseline set of oral hygiene instructions including the proper brushing and flossing techniques, as well as frequency and duration. At each visit the gingival and plaque indices were taken. The gingival index was measured using the Löe and Silness Gingival Index (GI) (Table 1).[21] This index was performed by a single clinician consisting of a two-step process: (1)utilizing clinical appearance of the gingival tissue; and, (2) assessing inflammation based on the induction of bleeding with a probe. A periodontal probe was lightly brushed into the gingival sulcus around all surfaces of the teeth. Depending on the appearance of the tissue as well as its reaction to periodontal probing, the sites were be scored as shown in Table 1.

The plaque index was measured using the Turesky et al. Modified Quigley-Hein Plaque Index (PI) (Table 2, Figure 1).[22] The plaque index was performed through the utilization of a plaque disclosing solution. Five drops of plaque disclosing solution was be placed underneath the patient's tongue. The patient was advised to swish the solution around for 30 seconds.

After expectorating the solution, the patient rinsed his/her mouths out with water three separate times. Immediately following the plaque staining, a single clinician examined four different surfaces of each tooth (mesial, buccal, distal, and lingual). Based on the clinical appearance of the teeth after application of the solution, the supragingival plaque was scored based on the criteria listed in Table 2.

Table 1: The Loe and Silness Gingival Index scoring system [21]

Scoring:		
0	Normal gingiva	Natural coral pink gingival w/ no e/o inflammation
1	Mild inflammation	Slight changes in color, slight edema.
2	Moderate inflammation	Redness, edema, and glazing.
3	Severe inflammation	Marked redness and edema/ulceration/tendency to bleed

Table 2: Turesky et al. Modified Quigley-Hein Plaque Index [22]

PI Score	**Criteria**
0	No plaque
1	Separate flecks of plaque at the cervical margin of the tooth
2	≤1mm continues band of plaque at the cervical margin of the tooth
3	A band of plaque >1mm but <1/3 the crown of the tooth
4	Plaque covering 1/3 – 2/3 the crown of the tooth
5	Plaque covering >2/3 the crown of the tooth

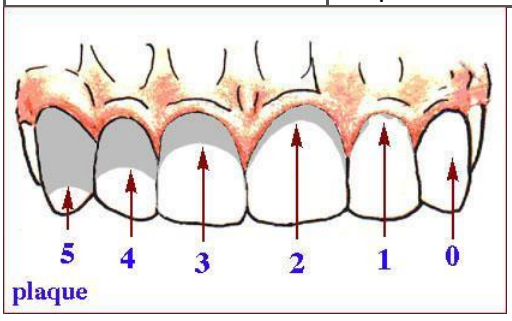


Figure 1: Turesky et al. Modified Quigley-Hein Plaque Index [22]

Following the evaluation of the GI and PI scores, various plaque samples were taken from the patient's tooth surface to assess the bacterial composition in their oral cavity. Using sterilized periodontal curettes, plaque was collected from the crown surface of the eight

premolars. Both supragingival plaque and subgingival plaque collections were taken and placed into 15% glycerol in phosphate buffered saline. In order to prevent the possibility of taking plaque collections of joined origin, supragingival collections consisted of areas mesial/distal/occlusal to the bracket or attachments. In patients undergoing clear aligner therapy, a dental microbrush was used to collect plaque from the inner portions of the tray.

Plaque samples were utilized to compare the changes in microbial profile between different timepoints as well as between treatment groups. DNA was extracted from the supragingival, subgingival as well as tray samples using the MasterPure DNA extraction and Purification Kit (Epicentre, Madison, Wis) according to the manufacturer's instructions.[23] After isolation, the 16S rRNA gene was amplified according to the HOMINGS protocol but using V4 primers and followed by cleaning with AMPure XP beads (A63881; Becman Coulter, Irving, Tex).[24] Next, barcodes were trimmed and base pairs removed in those with low-quality sequences, and finally the 16S rRNA sequences were clustered into operation taxonomic units using QIIME2 and assigned by comparing to the Human Oral Microbial Database.[23] Overall microbial composition, microbial profile changes, and microbial diversity (alpha- and beta-diversities) were assessed.

Results

The mean age of the patients in the fixed appliances group was 33.35 years (SD= 8.80 years), compared to 33.38 years (SD= 4.80 years) in the clear aligner group, this was not statistically significant (p= 0.99). The fixed appliances group was composed of five patients, all of them being male patients. The clear aligner group contained a total of four subjects, 1 of them being a male and 3 being female. The demographics for the patient in the study have been given in Table 3, and depicted in Figure 2.

Table 3: Patient number, gender distribution and mean ages compared between the fixed appliance and clear aligner groups.

	Fixed Appliances	Clear Aligners
Subjects	5	4
Male	5	1
Female	0	3
Mean Age	33.35	33.38
SD	8.80	4.80

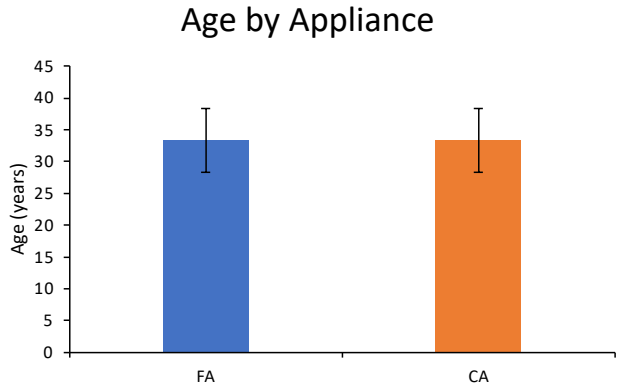


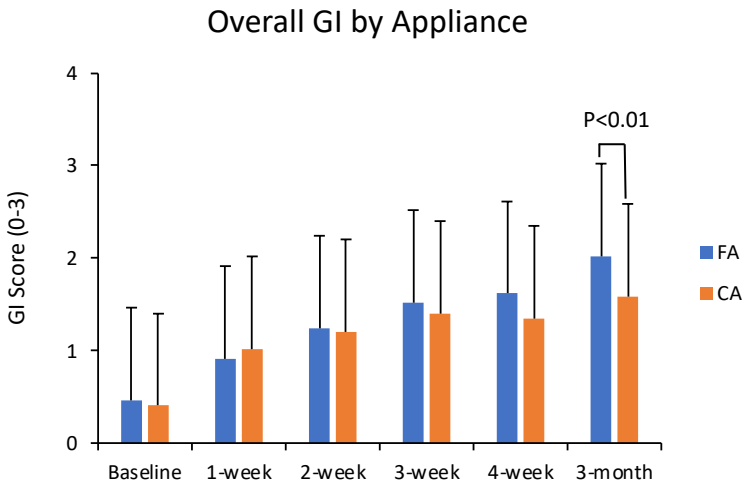
Figure 2: Mean ages compared between the fixed appliance and clear aligner groups. The fixed appliances group is shown in blue, and the clear aligner group shown in orange. There is no significant differences in age when comparing the two treatment groups.

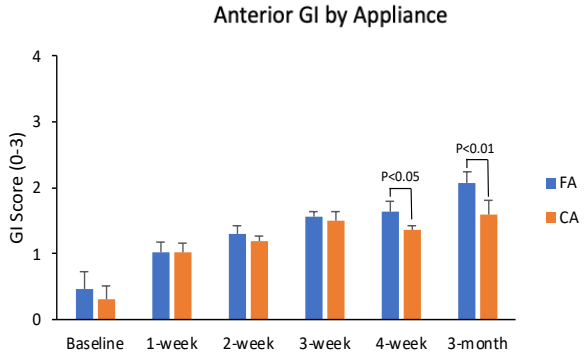
The clinical data comparing both the gingival index (GI) and plaque index (PI) between fixed appliances and clear aligners are summarized in Table S1. Both groups showed a general trend

of an increase in both GI and PI scores throughout treatment, which is depicted in Figures 3 and 5. As indicated in Figure 3, there were no statistically significant differences in GI scores between FA or CA at timepoints T0-T4, but there was a statistically significantly higher GI score in the FA group compared to CA group at T5 ($p< 0.05$). The data was stratified to test for any significant differences between the GI score in the anterior portion of the mouth (from the incisors and canines) versus the posterior portion of the mouth (premolars and molars). When evaluating the anterior portion of the mouth, there was a significant difference between the FA and CA groups at timepoints T4 ($p<0.05$) and T5 ($p<0.01$) (Figure 4). On the contrary, there as only a significant difference in GI scores in the posterior portion of the mouth at T5 (Figure 4, $p< 0.01$). When evaluating the overall differences in the PI between both groups, the FA had a significantly higher score at T3 ($p<0.05$), T4 ($p< 0.01$), and T5 ($p<0.05$) (Figure 5). When evaluating the anterior sector of the mouth there were significant differences at T4 ($p= 0.05$) and T5 ($p= 0.04$) (Figure 6). The posterior portion showed no statistically significant differences between the groups, though the FA consistently showed greater PI scores. The GI and PI scores were then evaluated comparing the baseline scores to the scores of the combined treatment T1-T5 (Figures 7 and 8). When comparing the GI from T0 to T1-5 for the CA and FA group there was a statistically significant increase in the GI score in both groups ($p<0.05$, $p< 0.01$, respectively). There was a statistically significant difference in the combined GI score during treatment for the FA group when compared to the CA group ($p= 0.02$) (Figure 7). Figure 8 depicts the change in PI scores in both groups from baseline (T0) to the combined treatment (T1-5). The CA group did not show a statistically significant increase in PI score between the timepoints ($p= 0.21$). On the contrary, the FA group showed a statistically significant increase

from baseline to T1-5 (p<0.01). There was a statistically significant difference in PI score when comparing the groups for the combined treatment (p= <.01).

Figure 3: Overall gingival index score comparing fixed appliances to clear aligners over each time point.





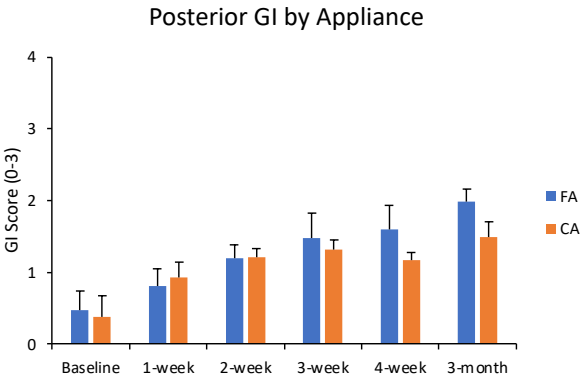


Figure 4: Anterior and posterior gingival indices comparing fixed appliances to clear aligners over each time point.

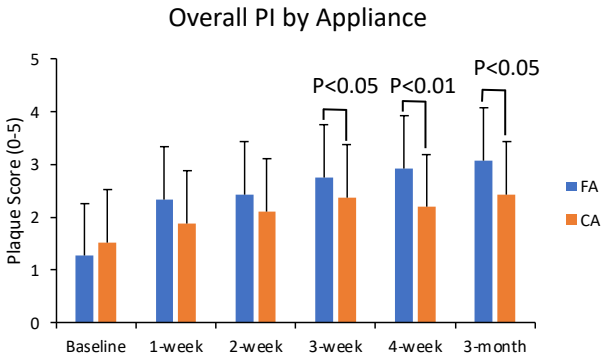


Figure 5: Overall plaque index score comparing fixed appliances to clear aligners over each time point.

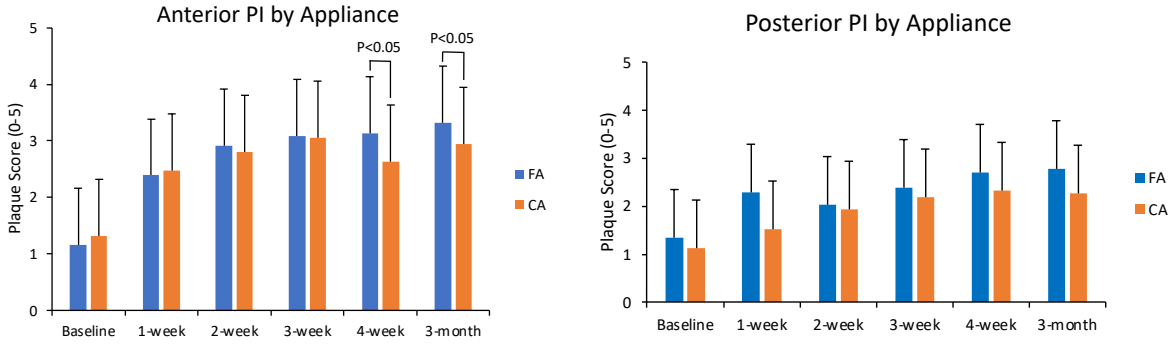


Figure 6: Anterior and posterior plaque indices comparing fixed appliances to clear aligners over each time point.

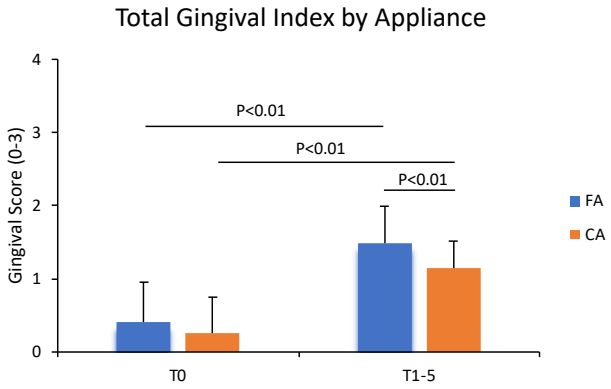


Figure 7: Overall gingival index score comparing fixed appliances and clear aligners from baseline (T0) compared to treatment (T1-5).

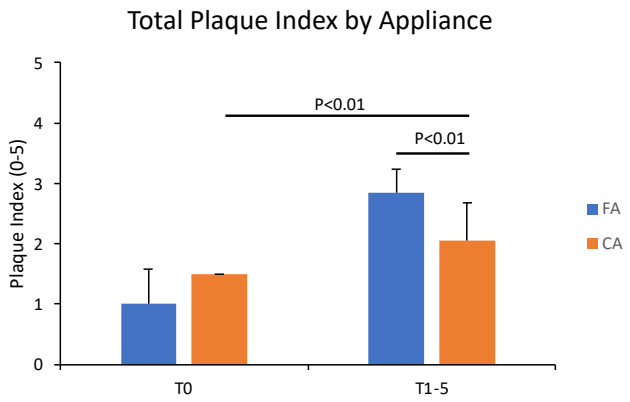


Figure 8: Overall gingival index score comparing fixed appliances and clear aligners from baseline (T0) compared to treatment (T1-5).

Figure 9 shows the 16S rRNA sequencing results comparing the bacterial composition in the clear aligner group. The figure elucidates that there was a significantly lower relative abundance of Actinomyces present in the tray compared to both the subgingival and supragingival plaque ($p<0.01$). The opposite pattern was observed for the level of *Porphyromonas* in the tray compared to supragingival plaque ($p<0.05$), but not the subgingival colony. Lastly, the figure illustrates the increased levels of *Streptococcus* in the tray biofilm when compared to both the subgingival and supragingival microbial communities ($p<0.01$). The microbiological analysis was also used to compare the relative abundance of various bacterial genera between the clear aligner and fixed appliances group (Figure 10). This figure illustrates a significantly higher level of *Leptotrichia* in the supragingival bacteria in the FA group when compared to the CA group ($p<0.05$). A similar trend of increased *Leptotrichia* in the FA group is seen in the subgingival biofilm, but it is not statistically significant. *Porphyromonas* and *Tannerella* both showed a significantly increased relative abundance in the FA group for both the supragingival and subgingival plaque communities.

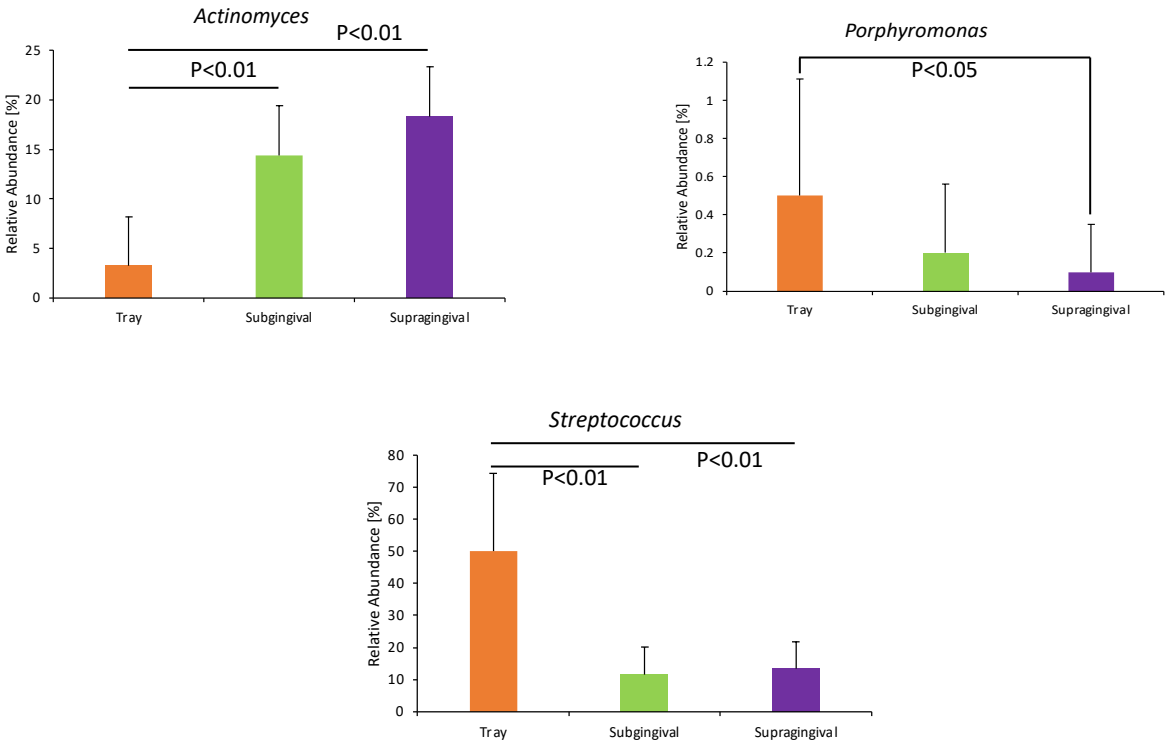


Figure 9: Relative abundance of bacterial genera isolated to only the CA group. There is a significantly higher level of Streptococcus in the tray group (orange) than subgingival (green) and (supragingival).

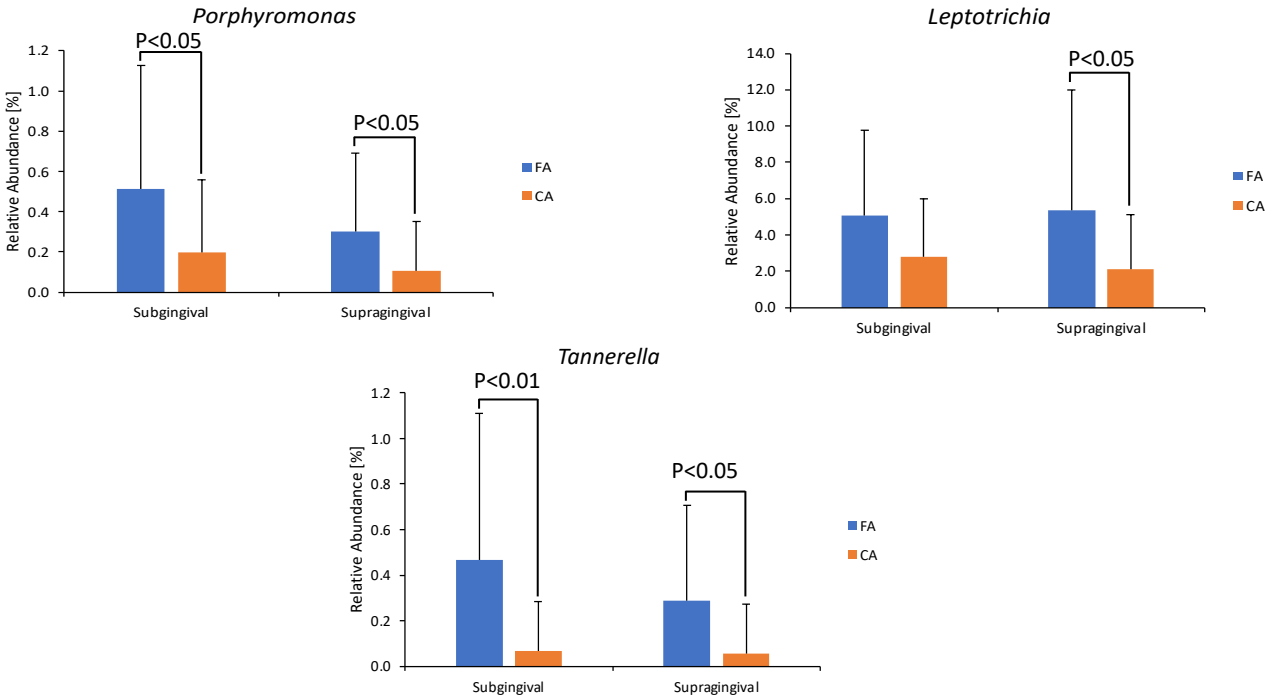


Figure 10: Comparing relative abundance of bacterial genera Porphyromonas, Leptotrichia, and Tannerella, between CA (orange) and FA (blue) groups in both the supragingival and subgingival bacteria.

The results of the microbiological analysis were then stratified to evaluate associations between different bacterial abundances and either GI or PI score. Though not statistically significant, patients who had higher GI score in the FA group showed increased levels of *Aggregatibacter*, whereas the CA group showed statistically significant decreased levels (Figure 11). In patients with an increased GI score of 2, there was significantly higher abundance of *Campylobacter*, *Tannerella* and *Porphyromonas* in the FA group when compared to the CA group ($p<0.05$). When evaluating the patients based on their PI score, patients in the FA who had higher PI score showed significant increases in *Campylobacter*, *Tannerella*, *Lachnospiraceae*, and Porphyromonas (Figure 12). Additionally, patients with the highest PI scores showed significantly

higher levels of *Tannerella*, *Leptotrichia*, *Lachnospiraceae*, *Fusobacterium*, and *Porphyromonas* in the FA group when compared to the CA group.

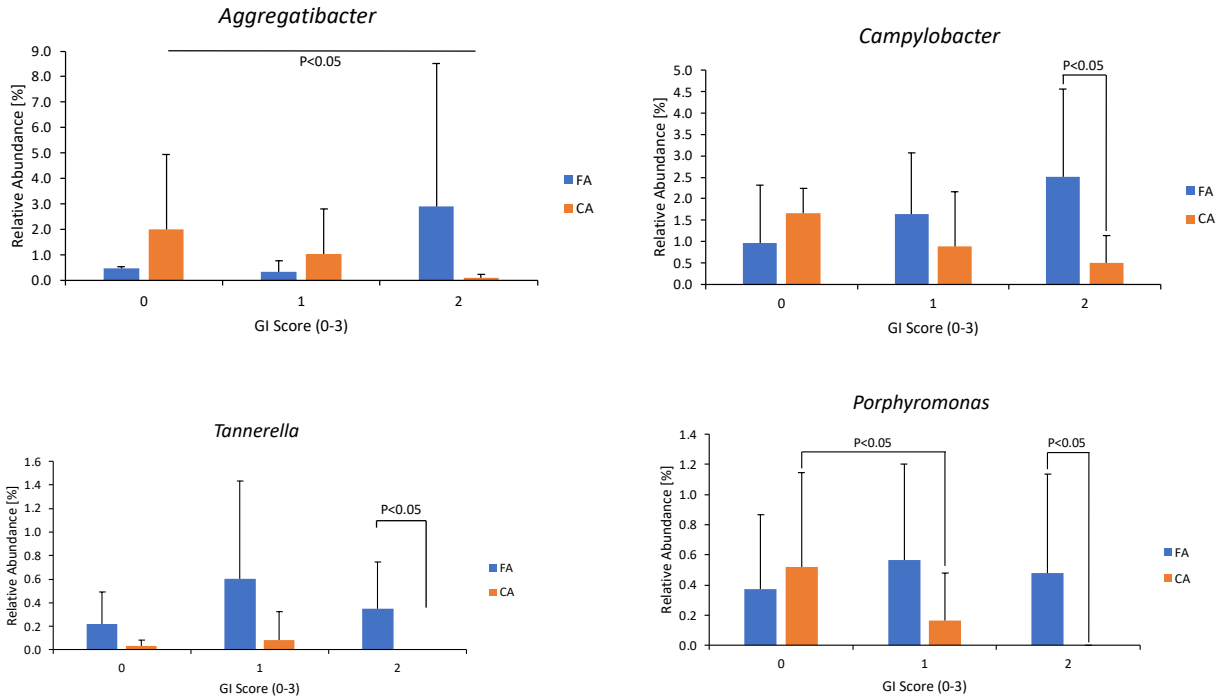
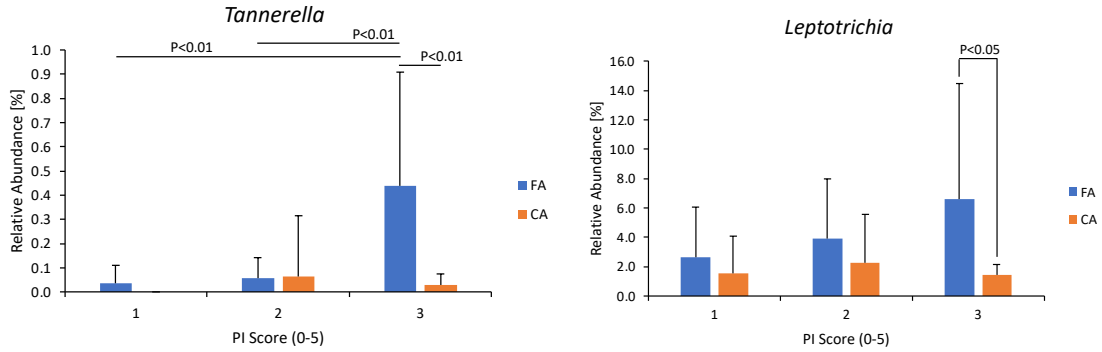


Figure 11: Comparing relative abundance of bacterial genera between FA and CA based on GI score.



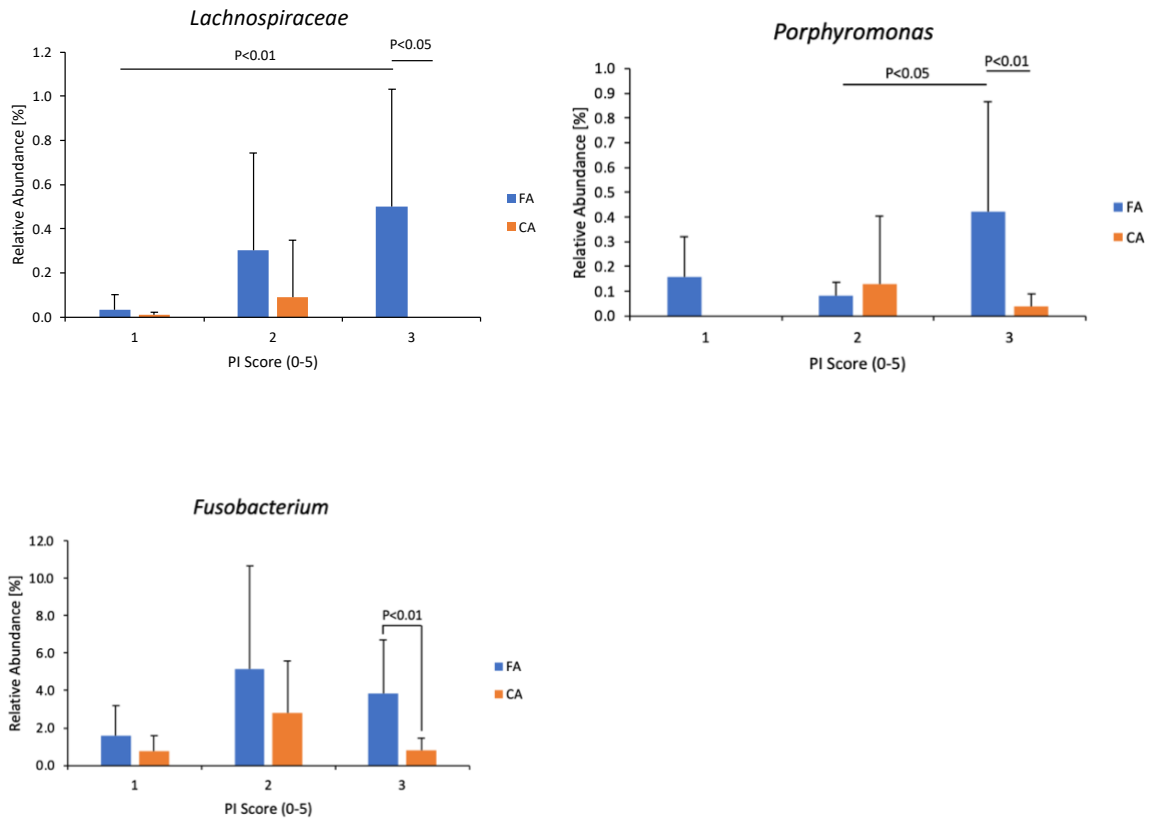


Figure 12: Comparing relative abundance of bacterial genera between FA and CA based on PI score.

Beta-diversity analyses via weighted UniFrac were performed on the samples and the features driving the first two dimensions taken together explain more than 35% of the variation between samples. Figure 13 displays a conglomeration of all samples and shows no clear distinction between the subgingival and supragingival plaque samples with the tray samples seemingly accumulating in a certain section of the plot. Separate analysis of FA group confirmed that there was again no clear delineation between the subgingival and supragingival samples in this treatment group. Examination of the CA alone, revealed an overlap between the supragingival and subgingival bacteria, but tray samples were isolated into one general location. Alpha-diversity analysis using Shannon Index was also performed, and it was found that the CA subgingival, FA subgingival, and FA supragingival biofilms are significantly different from that of the tray samples (Figure 14). Beta- and alpha-diversity analyses were performed comparing

subgingival plaque to gingival index and supragingival plaque to plaque index, but no significant results were noted (Figure S1 and S2, respectively).

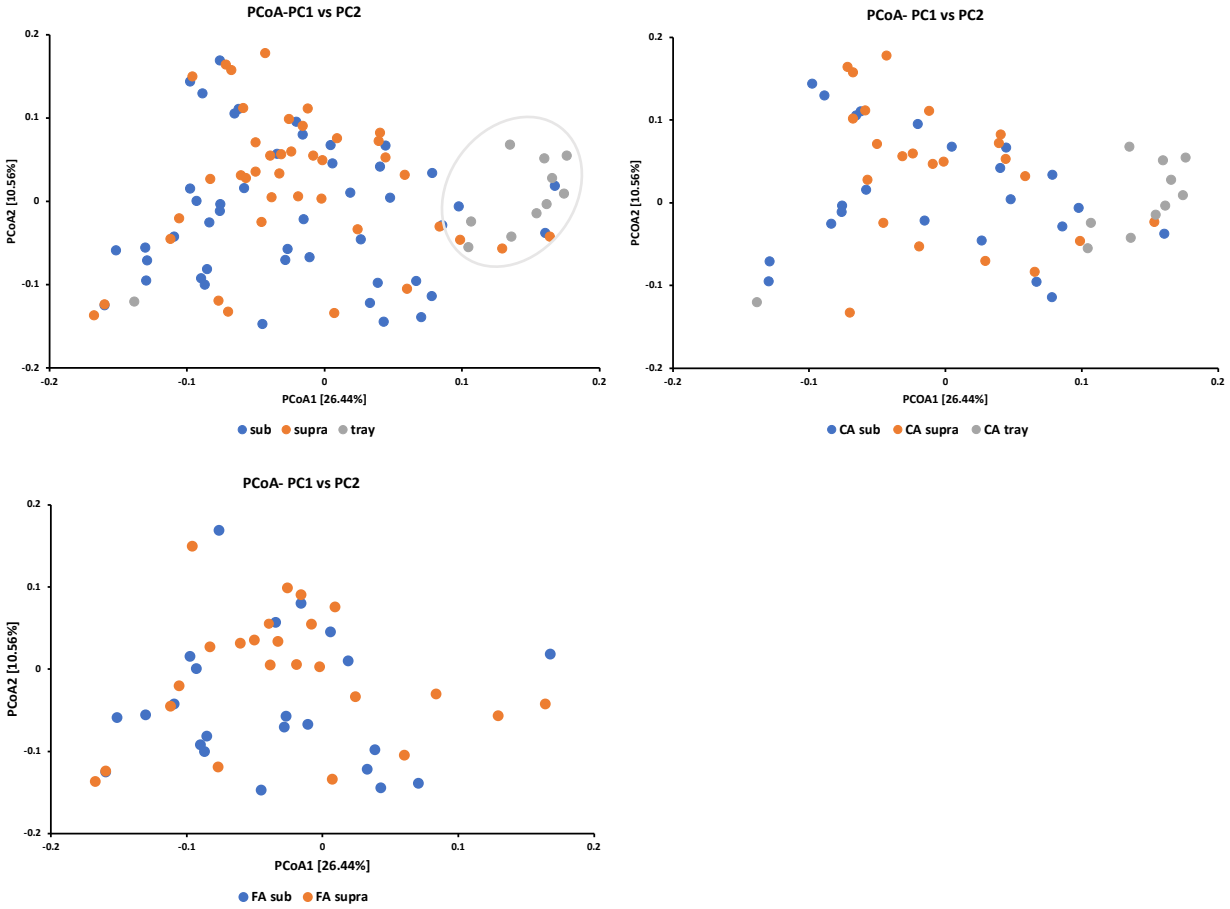


Figure 13: Beta-diversity analysis. The upper left panel combines the FA and CA groups and showed only a clear distinction in the tray bacteria (gray). The upper right panel isolates only the CA aligner group and shows the distinct group of tray bacteria. The bottom left panel is the FA group and shows no distinction between supragingival (orange) and subgingival (blue) bacteria.

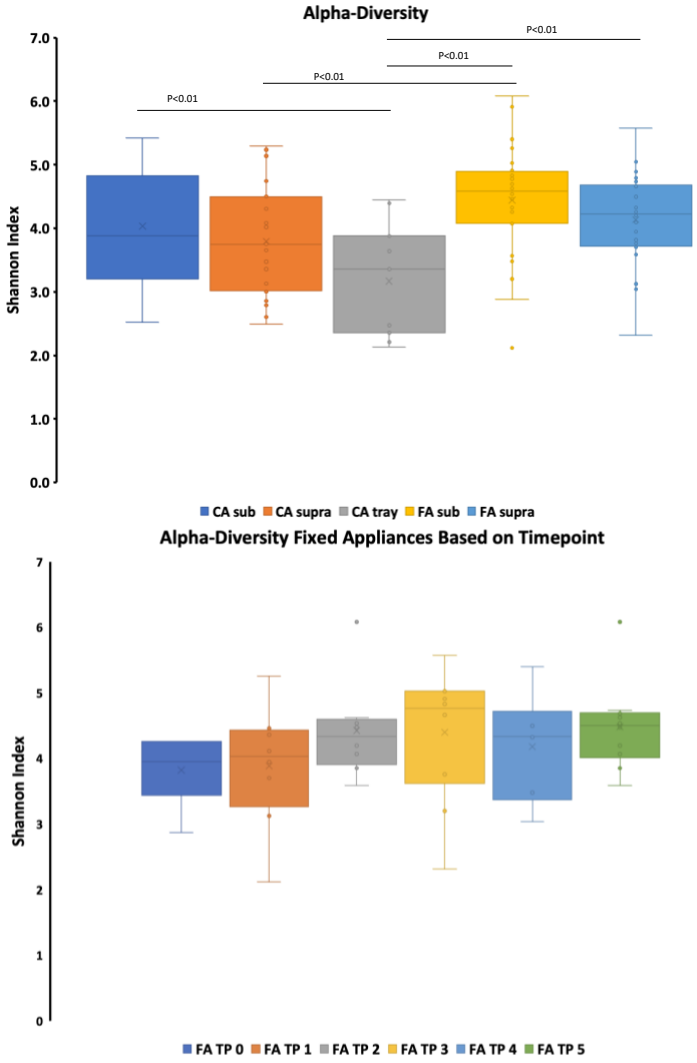


Figure 14: Alpha-diversity analysis. The top diagram shows both the CA and FA group and distinguishes based on site of the bacterial samples. It is shown that they tray bacteria shows significantly lower level of diversity than the other sites. The bottom diagram is separated based on time point in the FA and showed no significant differences, but did show a general increase in diversity over successive timepoints.

DISCUSSION

It is well documented that one of the largest drawbacks in orthodontic treatment is the physical barrier to proper oral hygiene with a concomitant increase in bacterial plaque accumulation, followed by typical side effects of erythematous/edematous tissue and a higher prevalence of bleeding on probing.[1-3] With the advances currently occurring in the orthodontic field with technological advances allowing for different treatment modalities, it is important to assess whether these advances have any impact on the patient's ability to perform proper oral hygiene practices and subsequent effects on plaque and gingival index scores.

It is currently thought that due to the design of traditional metal brackets there is increased mechanical retention of bacterial plaque and thus higher plaque indices in these patients. In accordance with Kitada et al., our study showed that when compared to baseline, the patients in the fixed appliance group showed increased plaque scores, which were significant at the third-week, one-month, and three-month timepoints.[7] Similarly, Shokeen et al., showed that over successive timepoints from baseline to six-months, the FA group showed significantly increased levels of PI scores, which were also significantly higher than the CA group.[10] The results of our study are contradictory to Chhibber at al., who noted that when comparing fixed appliances to clear aligners, there were no differences in long-term plaque retention.[8] The possible explanation for this was discussed previously as Addy et al., who believed that the full occlusal coverage of the teeth by plastic in patients with clear aligners decreased the salivary flushing. The decrease in salivary flushing prohibits the natural removal of the dental biofilm and thus increases plaque accumulation.[9] Though this may be true, which may be explained by the general increase in plaque index scores in the CA group in our study,

we found that when comparing the baseline PI to that during treatment (T1-5) there were no statistically significant differences. In a similar way, Issa et al., discussed that when patients were treated with CA they found decreased GI scores, PI scores, and bleeding on probing which can be attributed to the ease of access and better oral hygiene practices.[11] The ability of patients being treated with clear aligners to remove the trays for brushing and flossing may prove to be a differentiating factor in oral hygiene practices, allowing for a decrease in overall plaque accumulation and thus better gingival index scores and less bleeding on probing.

The results of the microbiological data reveal the changes in quantity of the microbiome is accompanied by a shift in individual microbial genera in patients undergoing orthodontic therapy in both FA and CA. Figure 9 as well as Figures 13 and 14 show an interesting phenomenon where the tray biofilm is significantly distinct from both the subgingival and supragingival ones. Though there are few significant differences between the supragingival and subgingival plaque communities, there are stark differences when they are compared with the ones colonizing the tray. Both the alpha- and beta-diversity analyses display a significant difference in the tray biofilm bacterial composition when compared to other bacterial samples collected. This indicates that there may be a unique microbial composition that specifically associates itself with the tray in clear aligner therapy. A possible rationale for this unique biofilm could be due to differential affinities of microorganisms to the tray material. It was previously shown that the tray bacterium was composed of a unique microflora at the six-month timepoint, but our study illustrates that this change in flora happens quite early after orthodontic treatment commences.[10]

The result of the analysis when comparing the relative abundance of various bacterial genera in the FA to the CA groups showed differences that could have an impact on oral health. For instance, *Leptotrichia* which has been shown to be associated with patients with periodontal disease, had significantly higher levels in the supragingival bacteria in the FA when compared to the CA group.[25] Interestingly, even though there was not a significant difference in relative abundance of *Leptotrichia* in the subgingival plaque, it was increased in the FA group when compared to the CA group. A similar observation was noted by Shokeen et al., who found significant differences in *Leptotrichia* abundance between CA and FA groups.[10] In a similar fashion, there were significantly increased levels of both *Porphyromonas* and *Tannerella* levels in the subgingival and supragingival bacteria of the FA when compared to the CA group. It is well documented that *Porphyromonas* and *Tannerella*, two bacterial species in the "red complex", are key bacteria in the progression of periodontal disease.[26-7] da Silva-Boghossian et al., collected subgingival samples from patients ranging from good health to chronic periodontitis and found patients with periodontitis had significantly higher levels of *Actinobacter* as well as *Porphyromonas* and *Tannerella*.[26]In a meta-analysis, Nath et al., showed that in patients with periodontal disease there is a high prevalence of the "red complex" and after periodontal therapy there is a large decrease in *Tannerella* and *Porphyromonas*.[27] The importance that the red complex plays in the progression of periodontal disease cannot be understated and our study found significantly higher levels of these bacteria in the FA group when compared to the CA group.

The data was then stratified to determine any associations of people with better or worse GI and PI scores with abundance of different genera. The data from Figure 11 illustrates

the relative abundance of certain bacterial genera as the GI score increases from a level of 0 to a level of 2. This data revealed that when patients have generally low GI scores (0 or 1) the levels of most of the genera are of equal levels between the FA and CA groups. On the other hand, as the GI score increased to a level of 2, we saw that there were significantly higher levels of *Campylobacter*, *Tannerella*, and *Porphyromonas* in the FA group when compared to the CA group. A possible rationale this phenomenon could be that the plaque is more frequently disturbed in the CA group than the FA. If this were the case, there is potential that this disturbance would inhibit the proliferation of strictly anerobic bacteria, such as the red complex bacteria. Thus, the induction of gingivitis in the CA group could be due to overall plaque load rather than the accumulation of pathogenic bacteria. In a similar fashion, relative abundance was evaluated based on different levels of PI scores. The results were similar as with the GI score, where in patients with lower PI scores, the levels are more similar between the two groups but as the PI scored increased to a level of 3, there are significant differences between the FA and CA groups. There is a steady but non-significant increase in *Campylobacter* in the CA group, whereas the abundance is significantly increased from PI score 1 to that of 2 and 3 in the FA group. Cai et al., discussed that Campylobacter plays a role in oral and bowel diseases due to the fact that it secretes pro-inflammatory cytokines which initiates the inflammation necessary for disease formation. They concluded that *Porphyromonas gingivalis*, *Fusobacterium*, and *Campylobacter* may play an important role in the progression of periodontal disease.[28] Interestingly, it was also found that patients who had a higher plaque scores showed significantly higher levels of *Fusobacterium*, a bacteria important in the progression of periodontal disease.[28] The aforementioned red complex bacterium, *Tannerella* and

Porphyromonas showed similar changes with significant increases in abundance in only the FA group, and a significantly higher level in patients with a plaque score of 3 in the FA group when compared to the CA group. Two additional bacteria genera that showed significant differences were *Lachnospiraceae* and *Leptotrichia*. Lu et al., took subgingival plaque samples from healthy patients, patients with gingivitis and patients with Stage I/II periodontitis. They found that patients with increased levels of *Lachnospiraceae* were associated with patients with Stage I/II periodontitis, and patients with increased levels of *Leptotrichia* were found in patients with gingivitis.[29] The results of the analysis compared the relative abundance between the groups as well as based on their GI and PI scores reveals interesting connections. Firstly, there appears to be a unique composition of bacteria that is associated with the tray when compared to subgingival and supragingival bacterial. When compared to the CA group, many of the bacterial genera associated with gingivitis and periodontal disease are found in higher quantities in the FA group. Lastly, as the level of gingival and plaque indices increases, we find these disease inducing genera to be significantly higher in the FA group.

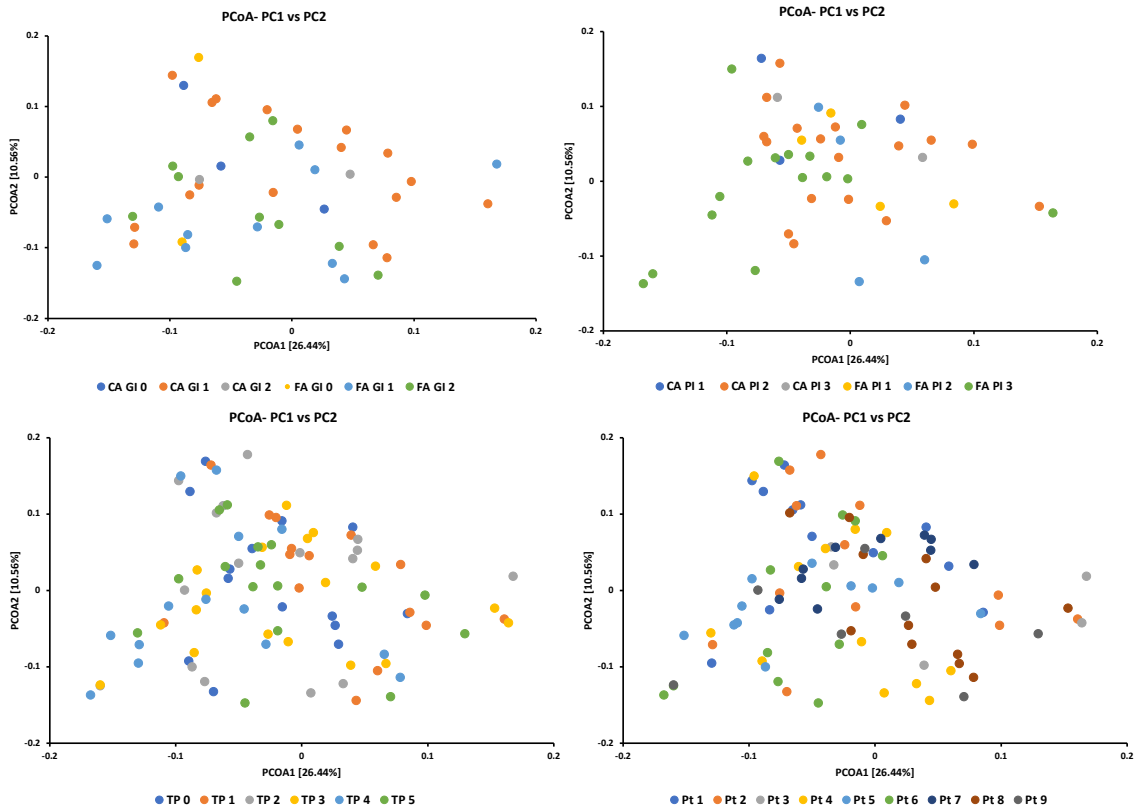
The limitations of the study mainly are associated with the small sample size that was collected. The difficulty was due to the frequent visits that the patients would need to attend in order for the plaque samples and GI/PI scores to be performed. Additionally, the distribution of males to females in the groups differed significantly and did not allow us to compare any differences based on sex. Future directions for the study would be to increase the sample size and distribution of the population. Additionally, an investigation into the properties of the tray material that hosts a unique biofilm could be performed. Lastly, a one-way and two-way

ANOVA analysis will be carried out to examine the association between orthodontic appliance and microbiome shift.

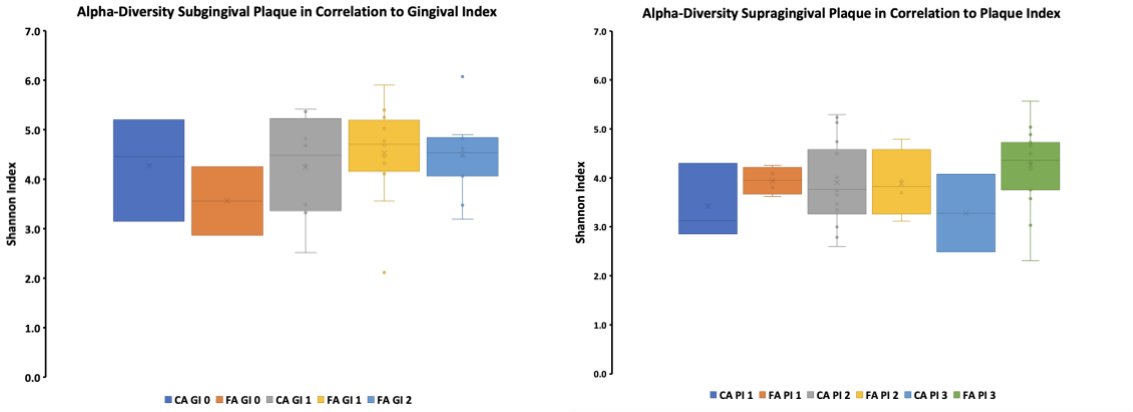
Supplemental Tables and Figures

Table S1: Mean Gingival and Plaque indices taken at each time point for the fixed appliance and clear aligner groups.

Appliance Type	Time Point	n	GI (mean ± SD)	PI (mean ± SD)
Fixed Appliances	T0	5	.47 ± .26	1.26 ± .30
	T1	5	.91 ± .19	2.33 ± .23
	T2	5	1.24 ± .13	2.43 ± .43
	T3	5	1.52 ± .22	2.74 ± .02
	T4	4	1.62 ± .22	2.92 ± .10
	T5	5	2.02 ± .15	3.07 ±.06
Clear Aligners	T0	4	.40 ± .25	1.52 ± .46
	T1	4	1.01 ± .09	1.89 ± .43
	T2	4	1.20 ± .08	2.10 ± .31
	T3	4	1.40 ± .10	2.37 ± .19
	T4	3	1.35 ± .11	2.19 ± .03
	T5	4	1.59 ± .26	2.43 ± .13



S1: Beta-diversity analysis comparing different timepoints, subgingival bacterial levels to GI index, and supragingival bacterial levels to PI score.



S2: Alpha-diversity analysis comparing subgingival bacterial levels to GI index, and supragingival bacterial levels to PI score.

Appendix A
Sample Data Collection Sheet

Sample Collection Data Sheet

Note: Do not include any patient identifying information

Subject ID#: _____

Date of visit: _____ Visit # *(1, 2, 3, 4, 5, 6)*: _____

Did subject refrain from brushing teeth or eating? *(circle)* Yes No

Appliance Type *(Braces or Invisalign)*: _____

Samples ID#s:
Sample Label = [subject ID][type of sample: S=saliva, A=Supragingival Plaque, B=Subgingival Plaque, T=Tray][Visit #] *ex:* 1A3 (subject #1, supragingival plaque, visit #3)
Make sure matches label on collection tubes

_____

_____

_____

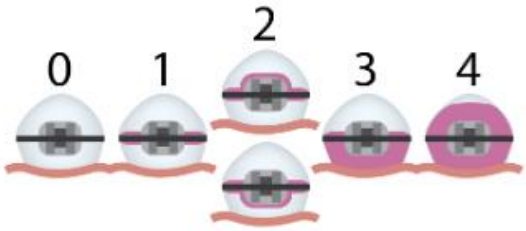
_____

Notes:

Modified Orthodontic Plaque Index

Score **ONLY BRACKETED/ ATTACHMENT** surfaces

Score	Criteria
0	No plaque
1	Interproximal plaque accumulation (mesial/distal of bracket)
2	Interproximal plaque accumulation (mesial/distal) AND incisal/cervical to bracket base
3	Continuous plaque from gum line to bracket base
4	Complete coverage by plaque



[blank] = no bracket/attachment present

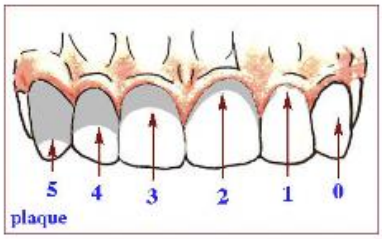
Tooth number	MOPI Score
2	
3	
4	
5	
6	
7	
8	
9	
10	
11	
12	
13	
14	
15	
Sum	

Tooth number	MOPI Score
18	
19	
20	
21	
22	
23	
24	
25	
26	
27	
28	
29	
30	
31	
Sum	

Plaque Index

Score **ALL** surfaces

Score	Criteria
0	No plaque
1	Separate flecks of plaque at the cervical margin of the tooth
2	≤1mm continues band of plaque at the cervical margin of the tooth
3	A band of plaque >1mm but <1/3 the crown of the tooth
4	Plaque covering 1/3 – 2/3 the crown of the tooth
5	Plaque covering >2/3 the crown of the tooth



Tooth number	Buccal Score	Lingual Score
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		
13		
14		
15		
Sum		

Tooth number	Buccal Score	Lingual Score
18		
19		
20		
21		
22		
23		
24		
25		
26		
27		
28		
29		
30		
31		
Sum		

Gingival Index

Score each tooth of their buccal, mesial, lingual and distal surfaces of the gingival tissues by using Loe and Silness gingival index.

Scoring:
0 = Normal gingiva: Natural coral pink gingival w/ no e/o inflammation
1= Mild inflammation: Slight changes in color, slight edema. No bleeding on probing.
2 = Moderate inflammation: Redness, edema, and glazing. Bleeding on probing.
3 = Severe inflammation: Marked redness and edema/ulceration/tendency to bleed spontaneously

Codes:
X = tooth absent
B = bracketed tooth
O = banded
N = no bracket/band
A = attachment

Upper tooth number	Code	Buccal surface score	Lingual surface score	Mesial surface score	Distal surface score
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					

Lower tooth number	Code	Buccal surface score	Lingual surface score	Mesial surface score	Distal surface score
18					
19					
20					
21					
22					
23					
24					
25					
26					
27					
28					
29					
30					
31					

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