Title

Comparing Salivary and Plaque Microbiomes in Fixed vs Removable Orthodontic Treatment

Author

Pal, Arvin

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Comparing Salivary and Plaque Microbiomes in Fixed vs Removable Orthodontic Treatment

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

by

Arvin Pal

2022

ABSTRACT OF THE THESIS

Comparing Salivary and Plaque Microbiomes in

Fixed vs Removable Orthodontic Treatment

by

Arvin Pal

Master of Science in Oral Biology

University of California, Los Angeles 2022

Professor Renate Lux, Chair

In this study, we analyze the salivary and plaque microbiomes of patients undergoing orthodontic treatment using either traditional metal braces or removable clear aligners over a 12 month period. In addition to clinical plaque and gingival health measurements, next generation sequencing of the V1 to V3 region of the 16S rRNA gene from DNA extracted from the saliva and plaque samples and microbial community analysis was performed. During treatment, plaque, saliva, and tray plaque remained distinct in terms of beta diversity analysis, and saliva and plaque became more differentiated as treatment time increased. Each group appears to have a different genus-level bacterial community profile that changes as orthodontic treatment progresses. Over time, beta diversity analysis shows that plaque and tray plaque microbiomes become more distinct, and salivary microbiomes appear to represent an intermediary between the two.

The thesis of Arvin Pal is approved.

Jimmy K. Hu

Nini C. Tran

Renate Lux, Committee Chair

University of California, Los Angeles

2022

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INTRODUCTION

The Oral Microbiome

The variety of microorganisms residing in the oral cavity were first described in 1892 by WD Miller as so numerous and complex, that their quantification and identification was simply impossible [1]. Today, we know that the oral microbiome is the second most abundant microbiome in the human body, after the gastrointestinal tract, and comprises a delicate ecosystem which consists of hundreds of different predominantly commensal but also pathogenic bacteria [2]. The average adult can harbor as many as 100 billion bacteria in their mouth, and the accessibility of the oral cavity makes this massive microbiome uniquely convenient for scientific investigation [3]. The anatomy of the oral epithelium, teeth and gingival sulci offer aerobic as well as anaerobic bacteria the chance to thrive, and the proximity of these communities to epithelial tissues also provides under inflammatory conditions the opportunity for these microbes to enter systemic circulation if their biofilm is disrupted [4]. Therefore, microorganisms living in the oral cavity have local and systemic effects on health: ranging from caries and periodontal disease in the mouth, to atherosclerosis and endocarditis in the heart [4; 5].

Local Effects of Oral Microorganisms

In this study, we focus on the local effects of bacteria living in the oral cavity. The most common pathologies associated with oral microorganisms are dental caries and periodontal disease [6; 7]. Caries is a disease process which results in the decay of hard tooth structure, while periodontal disease affects the soft tissue and bone supporting teeth [8]. The initiation of both disease processes are marked by increased complexity

and dysbiosis of the oral microbiome [9]. For example, literature suggests that patients with periodontitis have greater numbers of *Porphyromonas gingivalis,* a keystone pathogen, than healthy patients [9; 10]. This is one of many pathogens that are known to be associated with periodontal disease[10]. Similarly, dental caries has often been linked to disproportionately high levels of *Streptococcus mutans* in the oral microbiome [9; 11]. However, contemporary literature suggests that no singular pathogen is responsible for either disease state – both caries and periodontitis result from disturbances among various constituents of oral microbial communities, which can trigger dysbiosis[9]. Numerous studies have shown common microbial shifts associated with oral disease, but it is not clearly exactly which bacteria are involved in these shifts, especially in patients with orthodontic appliances.

Importance of Gingivitis and White Spot Lesions

White spot lesions and gingivitis are early indicators that foreshadow the more serious conditions of dental caries and periodontitis, respectively [12]. White spot lesions present on tooth surfaces as initial signs of demineralization, which will progress to dental caries if left untreated [13]. Gingivitis, which is most often caused by plaque accumulation around the gingival margin of teeth, is marked by inflammation and bleeding of gingival tissues, [14]. If gingivitis is unchecked, gingival inflammation will eventually spread to the underlying alveolar bone and cause periodontitis, or bone loss [15]. Further progression of these disease states is the leading cause for tooth loss worldwide [14].

Orthodontic Appliances and the Oral Microbiome

Orthodontic treatment presents a new twist to the oral microbial community because it necessitates the placement of foreign materials in the mouth. Orthodontic brackets, bands, elastics, and aligners all provide new surfaces for bacteria to adhere to and propagate in novel ways [16]. While metal braces have been used since the inception of modern orthodontics [17], clear aligners were invented in 1944 but were rarely utilized until reintroduced as *Invisalign*® by Align Technologies in 1998, and have gained a significant market share ever since [18].

Regardless, both clear aligners and fixed metal braces introduce inflammation and new surfaces for colonization into the mouth and disrupt the balanced intraoral microbial ecosystem during orthodontic treatment [19]. As a result, all orthodontic patients are at increased risk of white spot lesions, caries, and periodontal disease (Figure 1) [20]. Clear aligners offer an aesthetic option which has drawn more adults to orthodontic offices than ever before [21]. Unlike children, whose main risk is for caries during orthodontic treatment, adults also present significant risk of periodontal disease [21]. This shift in patient population requires further understanding of how new treatment modalities compare with traditional methods in terms of oral health. In this study, we focus on the differences in oral microbial changes between patients using fixed metal braces and clear aligners.

Figure 1: Left: Healthy dentition and gingiva after removing orthodontic brackets and appliances. No demineralization or staining is present on the crowns, and gingival is non-edematous and coral pink. Right: White spot lesions and gingivitis after orthodontic treatment. Note the demineralization which occurred around the bracket sites [22].

Saliva as a Diagnostic Marker

Humans have three major salivary glands – the parotid, submandibular and sublingual glands. The proteins and peptides contained in the saliva these glands produce aid in balancing the microbial ecosystem of the oral cavity [23]. Because of its importance in oral biofilm formation and host defense, saliva may also have a significant role in the establishment and progression of oral diseases [23]. Saliva has immense potential as a diagnostic tool in detecting microbes involved with oral diseases, since it provides uniquely fast and noninvasive collection of biomarkers [24; 25; 26] which may be representative of the entire oral microbiome, unlike regional plaque which may only reflect the subgingival, supragingival, or interproximal areas they were collected from. In 2019, Lundmark et al reported that “salivary microbial community composition differed significantly between patients with chronic periodontitis and healthy controls” [27], and in

2021 Diao et al concluded that “free salivary pathogens might play an important role in the recolonization of bacteria as well as the prognosis and recurrence of periodontal diseases” [28]. These studies foreshadow the potential clinical significance of the salivary microbiome to the screening and early diagnosis of patients with caries and periodontal disease.

Previous Studies on Orthodontic Patients

Studies have shown that 26% of patients with traditional braces [29] but only 1.2% of patients with clear aligners [30] develop white spot lesions during orthodontic treatment. Additionally, current literature suggests that fixed appliances are associated with poorer periodontal health and increased periodontopathic bacteria in comparison to clear aligners [21; 31; 32]. Conversely, another study focused on saliva noted significant differences in *Firmicutes* and *TM7* at the phylum level and *Neisseria* at the genus level between the aligners and braces; however, these microbial differences were not correlated with consistent clinical manifestations between patient groups [19; 33]. The inferred microbial function of the aligner group suggested this group would actually be more predisposed to periodontal diseases, which is not observed clinically [19].

Another study which analyzed the saliva of orthodontic patients found increased levels of cariogenic mutans streptococci and lactobacilli after the delivery of orthodontic appliances [34], but the study was limited only to treatment with metal brackets. Furthermore, another study compared metal braces and aligner patients and found no differences in the salivary counts of *S. mutans* or *L. acidophilus* among adolescent

patients treated for 1 month [33]. However, patients treated with aligners had lower salivary levels of *S. sanguinis* compared to those treated with braces [33].

While both treatment modalities result in a change in oral health, it remains unclear if the discrepancy is due to microbiological differences, or simply the increased convenience of oral hygiene with clear aligners [19]. Identifying the actual differences in bacterial populations between the two groups would help clarify the underlying reasons behind these observations.

Study Outline

In this study, we analyze the salivary microbiome of patients undergoing orthodontic treatment using either traditional metal braces or removable clear aligners. The salivary microbiome of these patients is compared to the respective microbiomes found in the tooth-associated plaque above the gingival margin, as well as the plaque found on the clear aligner trays themselves, throughout the first 12 months of orthodontic treatment. Clinical data, such as plaque levels and gingival health, are also compared with changes in salivary microbial composition throughout treatment, in order to identify a potential relationship between microbial composition and oral health during orthodontic treatment.

OBJECTIVES & SPECIFIC AIMS

A healthy oral cavity contains a delicate ecosystem of microbial species which has the potential to become pathogenic if disrupted. Tooth-plaque studies have shown that orthodontic appliances, chiefly metal braces or clear aligners, cause shifts towards potential dysbiosis to the oral microbiome. However, the relationship of these plaque changes to the salivary microbiome, which bathes nearly every surface of the oral cavity, remains unclear. Our hypothesis is that similar changes occur to plaque and saliva during orthodontic treatment, and these changes become more dramatic as treatment time increases, and more remarkably in fixed appliances than with clear aligners. There are three specific aims this study will use to test this hypothesis:

Aim 1: Determine the relationship between plaque and salivary microbiomes.

a) Analyze all T0 patients to determine the relationship between plaque and salivary microbiomes without any orthodontic appliances.
b) Compare salivary microbiome with supragingival tooth-associated plaque, and clear aligner tray plaque.

Aim 2: Analyze how plaque and salivary microbiomes change over time depending on the orthodontic appliance used.

a) CA vs FA patients at 0 months (to establish a baseline), 3 months, 6 months, and 12 months.

Aim 3: Determine how clinical signs of oral health or disease during orthodontic treatment is reflected in changes in plaque and salivary microbiomes.

a) Compare saliva microbiome in patients with plaque score > 0 and = 0.

b) Compare plaque and saliva microbiome in patients with gingival score > 0 and = 0.

c) Observe the correlation of plaque index with microbial composition of saliva.

d) Observe the correlation of gingival index with microbial composition of saliva.

MATERIALS & METHODS

Study Design

Using IRB #16- 001258, patients who presented to the UCLA Orthodontics Clinic were recruited to enroll in the study. Ultimately, 20 new patients enrolled into the study. 10 patients were planning on starting orthodontic treatment with either fixed metal braces, or clear aligners, respectively. No distinction was made for patients being treated with fixed metal braces between self-ligating and traditional twin bracket designs, because previous studies have shown no significant difference between the two in terms of plaque adherence or gingival inflammation [35]. Patients were excluded from the study if they initially presented with active caries, advanced periodontal disease, chronic systemic diseases, or xerostomia. Additionally, patients were excluded if they used any antibiotic medications in the last 30 days or had any history of radiation therapy to the head or neck region.

Data Collection

Clinical data, saliva, and plaque samples were collected at the following orthodontic treatment timepoints: 0 months, 1 month, 3 months, 6 months, and 12 months. Saliva and plaque samples were collected at each timepoint at the beginning of the appointment, before any elastic ligature ties were removed from their braces, to avoid disturbance of the intraoral bacterial load. Clinical data included measuring plaque levels and gingival health. Plaque levels were quantified using Turesky et al. Modified Quigley-Hein Plaque Index (PI), which evaluates supragingival tooth plaque on a scale from 0-5 after the patient uses a plaque disclosing solution (Figure 2) [36; 37]. Gingival

health was measured using the Löe and Silness Gingival Index (GI), which relies upon the two most common clinical signs of inflammation: swelling and bleeding (Figure 3) [38]. This index is qualitative rather than quantitative, as it does not consider periodontal probing measurements [39].

Figure 2: This image displays the clinical appearance of each score on the Turesky Modification of the Quigley-Hein Plaque Index (PI). 0 = No plaque present; 1 = Separate flecks of plaque at the cervical margin; 2 = A thin continuous band of plaque (up to 1 mm) at the cervical margin; 3 = A band of plaque wider than 1 mm but covering less than 1/3 of the surface; 4 = Plaque covering at least 1/3 but less than 2/3 of the surface; 5 = Plaque covering more than 2/3 of the surface [40].

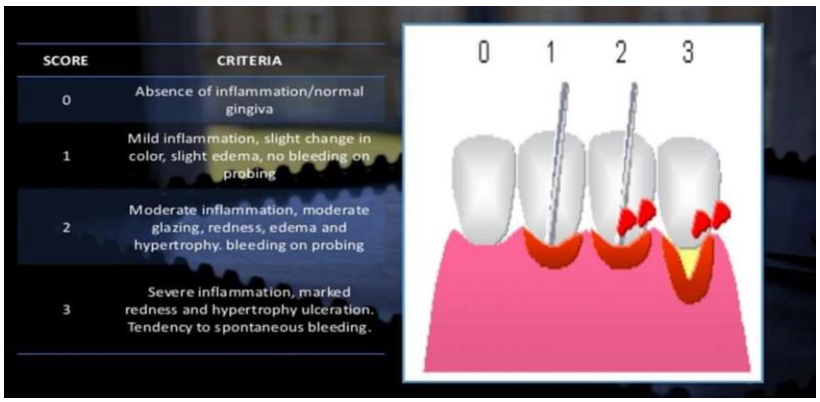


Figure 3: Löe and Silness Gingival Index (GI): 0 = Normal coral pink gingiva with no evidence of inflammation. 1 = Mild Inflammation with slight changes in color and slight

edema, but no bleeding on probing. 2 = Moderate inflammation redness, edema, glazing and bleeding upon probing. 3 = Severe inflammation with marked redness and edema, ulceration, and a tendency to bleed spontaneously [41].

Saliva samples were obtained by having patients expectorate into disposable test tubes containing 15% glycerol in phosphate buffered saline, which were then labeled and stored in a freezer at -20ºC until further analysis was completed [42]. Sterilized periodontal scalers were used to collect supragingival plaque from both anterior and posterior teeth. Plaque was sampled from the gingival 1/3 of the buccal and lingual surfaces of the central incisors (anterior plaque sample) and first or second premolars (posterior plaque sample) as some patients did not have first premolars if their orthodontic treatment included extractions. Additionally, tray plaque (TP) was obtained using interproximal brushes on the patient's most recent clear aligner trays. Tray and tooth plaque samples were deposited into individual sterile collection tubes containing 15% glycerol in phosphate buffered saline [42].

DNA Extraction and 16S rRNA Sequencing

Next generation sequencing of the V1 to V3 region of the 16S rRNA gene from DNA extracted from the saliva and plaque samples and microbial community analysis was performed using the MiSeq platform (Illumina) available at the UCLA Microbiome Core [43]. Bioinformatic data analysis was completed with the following steps: once barcodes were demultiplexed and trimmed, sequences with > 3% uncertain base pairs and low 8 quality sequences containing bases with Phred quality values < 20 were removed. Using 16S

rRNA sequences, operational taxonomic units were clustered at a 98% similarity level using QIIME 2 [44] and compared to the Human Oral Microbiome Database [45] for taxonomic assignment.

Data and Statistical Analysis

Alpha diversity using the Shannon Index, Beta diversity using Weighted UniFrac, and principal coordinate analyses (PCoA) were calculated in QIIME 2. PcoA was performed to calculate “coordinates” for each sample, which allowed charting each sample relative to other samples. The closer a sample clusters to others on either the x-axis (PcoA1) or y-axis (PcoA2), the more similar the samples [49]. Power was calculated using the G*Power statistical analysis program [50]. Data normality was calculated utilizing the Shapiro-Wilk analysis [51]. The statistical significance for PI data was calculated using t-tests, and the Mann Whitney U-test was used to calculate significance for the GI data ($p \leq 0.05$).

RESULTS

Demographics and Time Points

The average age of the fixed appliance group (23.0 ± 13.6 years) was lower than the average age of the clear aligner group (30.9 ± 12.3 years), but the difference was not significant (p = 0.201). The fixed appliance group was comprised of 6 females and 4 males, while the clear aligner group had 7 females and 3 males (Table 1). Baseline (0 months) samples were collected for all 20 patients, but due to differing treatment schedules and failed appointments, some patients did not have samples collected at one or more of the 1, 3, 6 or 12 month(s) recalls (Supplemental Tables 1 & 2).

	Fixed Appliance	Clear Aligner
Patients	10	10
Female	6	7
Male	4	3
Average Age (years)	23.0	30.9
St. Dev. (years)	13.6	12.3

Table 1: Gender and age values for fixed appliance and clear aligner groups.

Clinical Data Analysis

Shapiro-Wilk analysis was used to test the normality of the data to a threshold of p = 0.05 [51]. It was found that the plaque index data followed a normal distribution, and the gingival index data did not follow a normal distribution (Table 2).

	Plaque Index	Gingival Index
n	72	72
Mean	1.92	0.47
St. Dev.	0.82	0.24
W	0.9804	0.8559
Distribution	Normal	Not Normal

Table 2: Shapiro-Wilk statistical analysis results shows PI values follow a normal distribution while GI values were not normally distributed. Each analysis is calculated using site specific values, which results in two values per appointment per patient (n=72).

The G*Power statistical analysis software was used to calculate implied α and power of the study [50]. The implied power of the PI data was 0.9676 and the effect size was calculated as 0.0670. The implied power of the GI data was 0.7105 and the effect size was calculated as 0.1893. Plaque and gingival index average values are displayed with standard deviation for each group and time point in Table 3. The same data are graphically represented in Figures 4 & 5. A significant difference exists between 0 to 6 months and 0 to 12 months ($p < 0.05$) in the PI of the FA group; however, there was no significant difference between any CA PI timepoints. Since GI scores did not follow a normal distribution, the Mann-Whitney U-test was used to calculate significance. There was a significant difference in GI scores ($p < 0.05$) between 0 to 6 months and 0 to 12 months in the FA group, and between 1 to 3 months in the CA group. Figure 6 compares PI and GI between both patient groups. There is no significant difference between FA and CA groups in PI or GI measurements in the 0, 1, and 3 month(s) time points; however, both PI and GI display a significant difference between FA and CA groups at the 6 and 12 month time points.

Appliance	Time Point (Months)	n	PI (Avg ± St. Dev)	GI (Avg ± St. Dev)
Fixed Appliances	0	10	1.52 ± 0.61	0.36 ± 0.48
	1	3	1.65 ± 0.70	0.33 ± 0.58
	3	9	2.17 ± 0.75	0.55 ± 0.50
	6	7	2.57 ± 0.51	1.12 ± 0.29
	12	8	2.70 ± 0.65	0.92 ± 0.52
Clear Aligners	0	10	1.59 ± 1.00	0.23 ± 0.22
	1	3	1.17 ± 1.15	0.07 ± 0.12
	3	7	2.03 ± 0.68	0.43 ± 0.32
	6	8	1.66 ± 0.74	0.23 ± 0.37
	12	7	1.74 ± 0.61	0.27 ± 0.37

Table 3: Average ± standard deviation of PI and GI values for both patient groups at each time point. The number of samples collected at each time point is shown as "n."

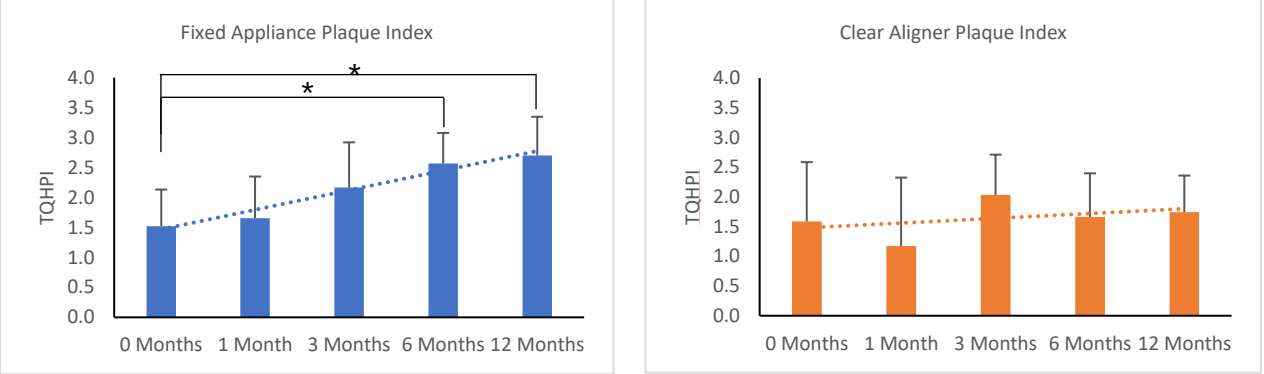


Figure 4: Fixed appliance (left) and clear aligner (right) plaque index measurements for each timepoint between 0 and 12 months. Trendlines are indicated as dotted lines. There is a significant difference ($p < 0.05$) between 0 to 6 months and 0 to 12 months in the FA group. No significant difference is noted between any CA timepoints.*

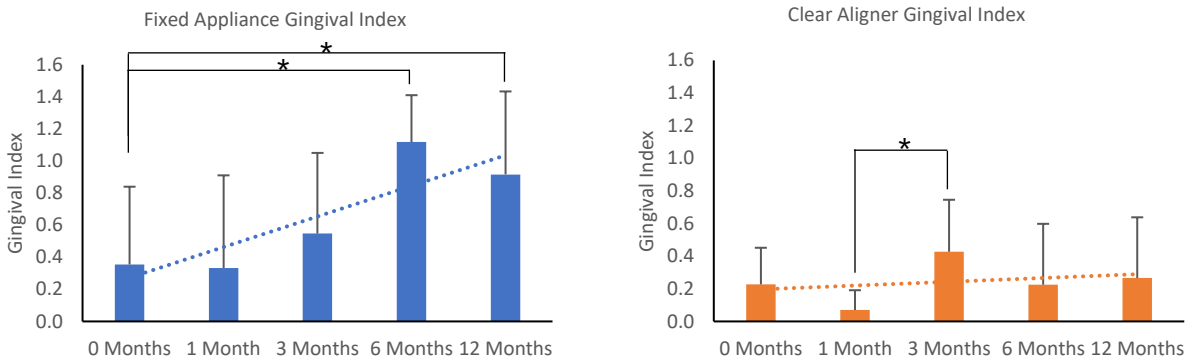
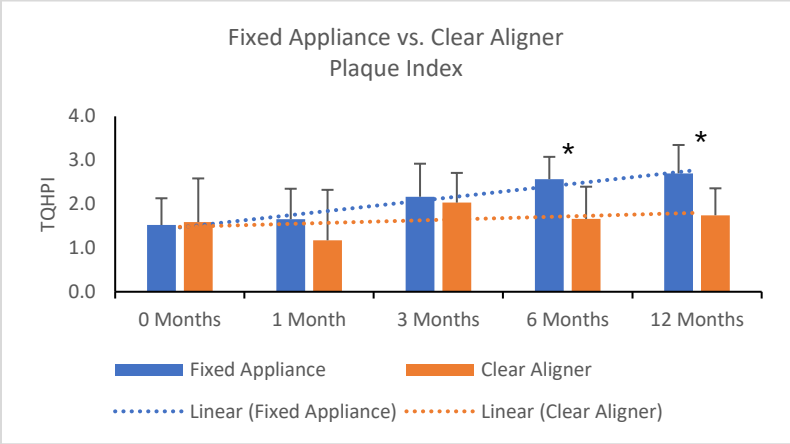


Figure 5: Fixed appliance (left) and clear aligner (right) gingival index measurements for each timepoint between 0 and 12 months. Trendlines are indicated as dotted lines. There is a significant difference (= $p < 0.05$) between 0 to 6 months and 0 to 12 months in the FA group, and between 1 to 3 months in the CA group.*



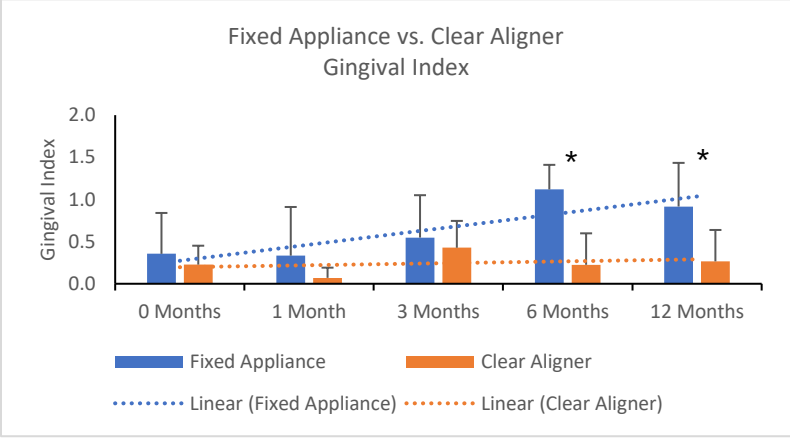


Figure 6: (Top): Comparison of PI between FA and CA patient groups. There is a significant difference (= $p < 0.05$) between the groups at both 6 and 12 months. (Bottom): Comparison of GI between FA and CA patient groups. Again, there is a significant difference between the groups at both 6 and 12 months. Trendlines show that FA patients increase in PI and GI over time, while the CA group appears stable.*

Baseline Microbial Analysis

At baseline (0 months – directly prior to receiving the respective appliances), there is no significant difference in Alpha diversity using the Shannon Index between saliva and plaque microbial communities between FA or CA groups (Figure 7). Beta diversity, as measured by Weighted UniFrac and visualized using principal coordinate analysis plots (Figure 8), shows that saliva and plaque form two distinct clusters at baseline (0 months). There is no discernable distinction in beta diversity between saliva and plaque of FA or CA groups at 0 months.

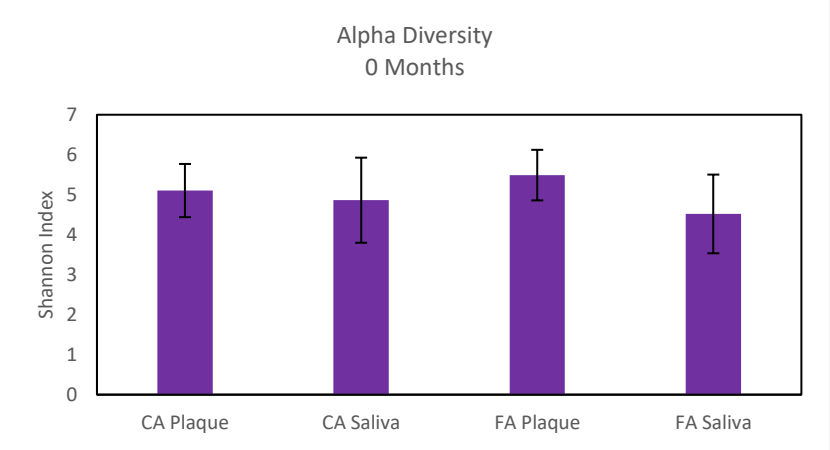


Figure 7: Alpha diversity analysis, based on the Shannon Index, at 0 months shows no significant difference between saliva and plaque microbial communities in FA or CA groups.

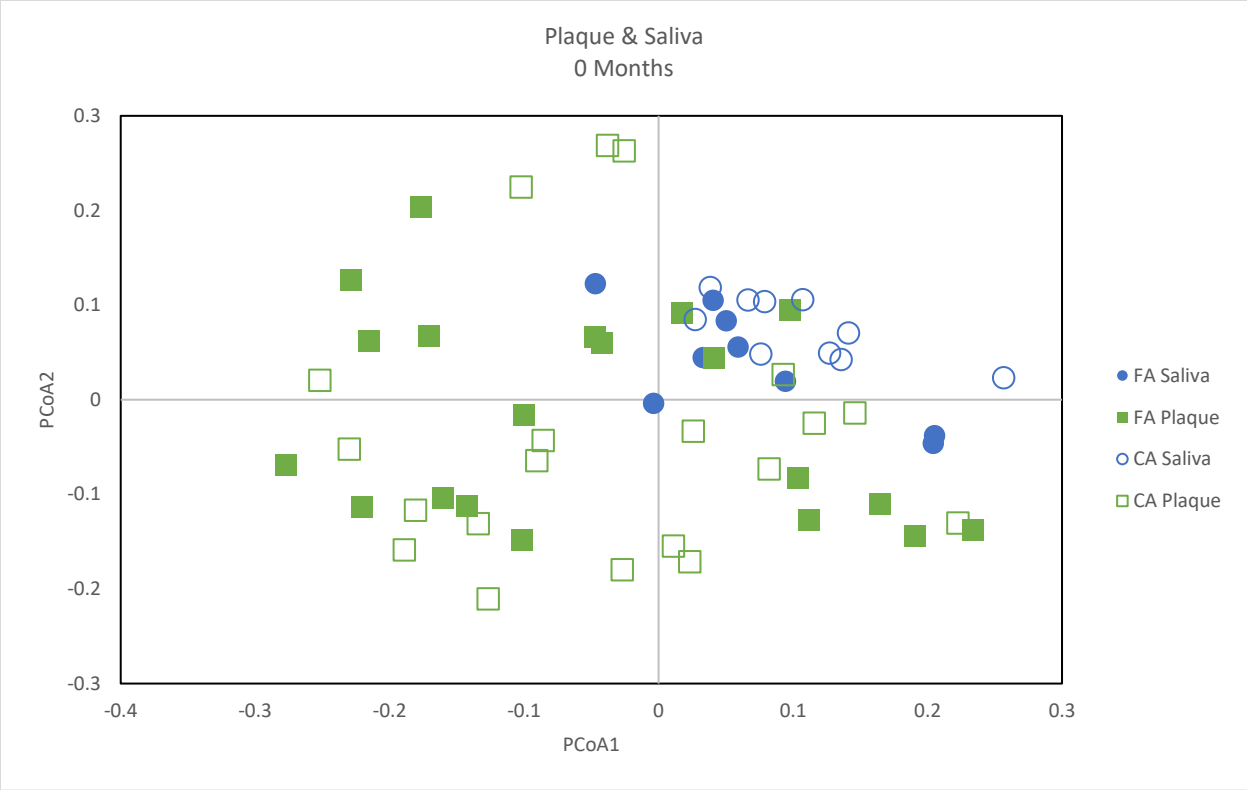


Figure 8: Weighted UniFrac Beta diversity analysis of saliva and plaque microbes based on principal coordinates one and two (PCoA1 & PcoA2). At 0 months both FA and CA saliva cluster together, and FA and CA plaque is spread similarly in a broader cluster.

Alpha Diversity Analysis

Alpha diversity calculations were completed for CA plaque, saliva, and tray plaque, as well as FA saliva and plaque at each time point (Figure 9). The 1-month data are not displayed due to an insufficient number of successfully sequenced samples. The only significant difference ($p < 0.05$) found in the CA data was found between the plaque from 3 and 12 months. Tray plaque did not exist at 0 months, as trays were first delivered at that appointment. For the FA group, there was a significant difference ($p < 0.05$) between saliva and plaque at both 0 months and 12 months, but not 3 or 6

months. There was also a significant difference between salivary microbial composition between 0 and 6 months, as well as between 6 and 12 months. No other significant differences in Alpha diversity between time point or location were present in the FA or CA groups.

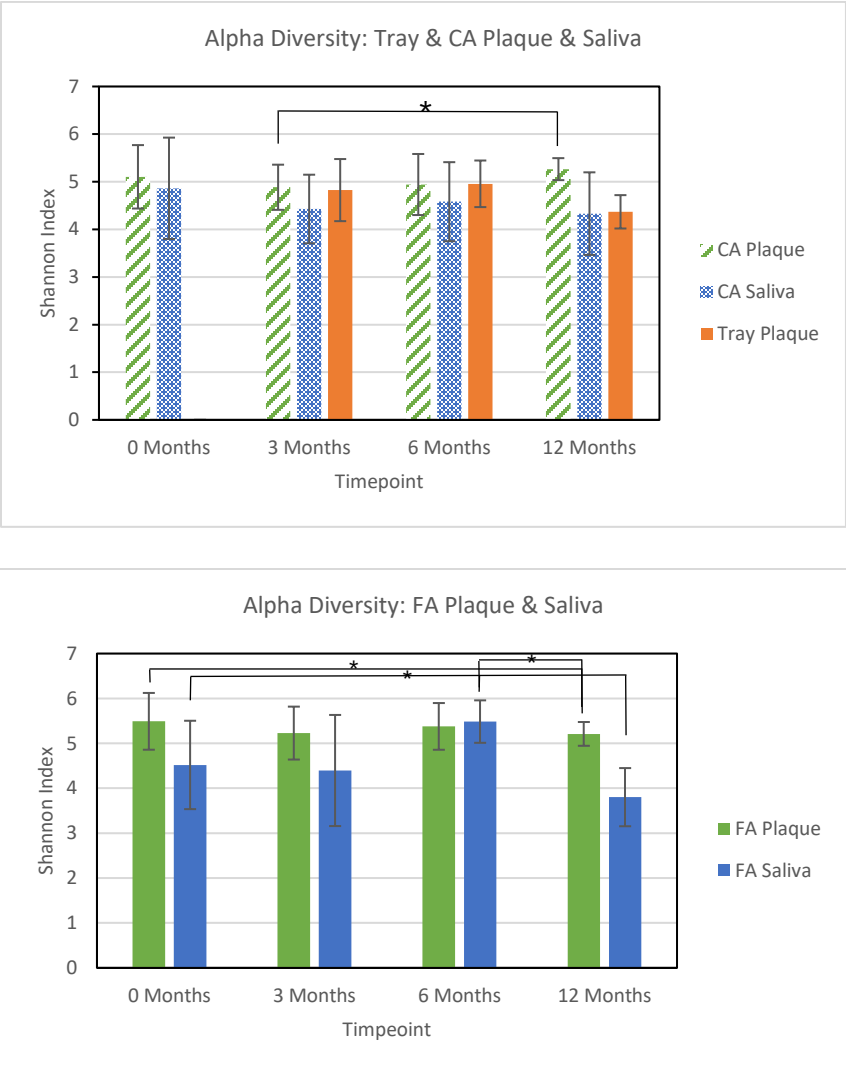


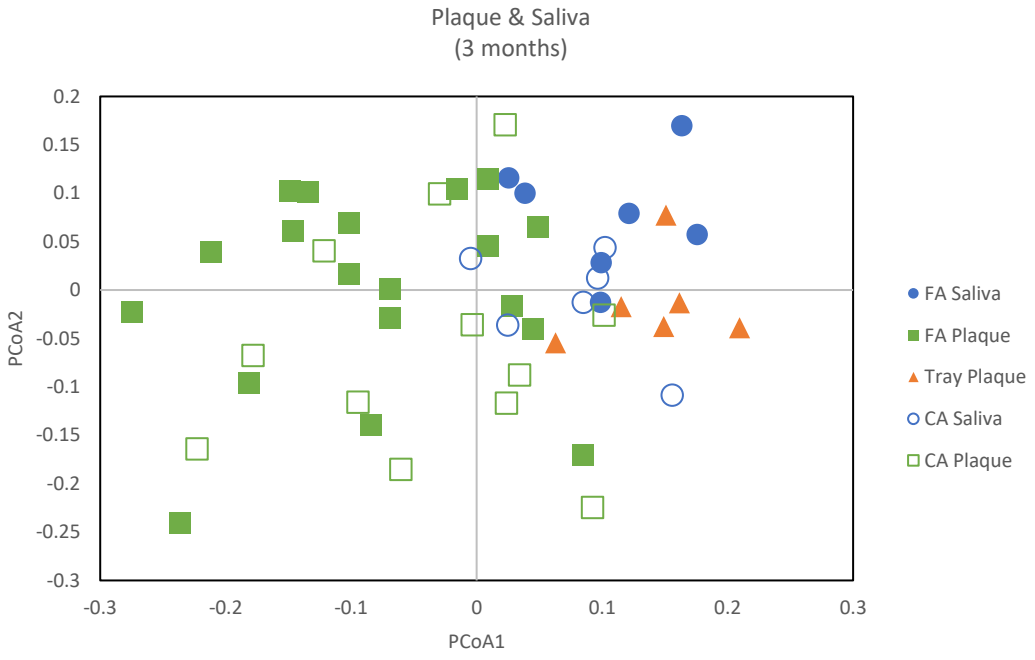
Figure 9: Alpha diversity analysis of saliva, plaque, and tray plaque.

*(Top): CA plaque, saliva, and tray plaque is compared at each time point. The only significant difference (* = p < 0.05) found is between CA plaque at 3 and 12 months.*

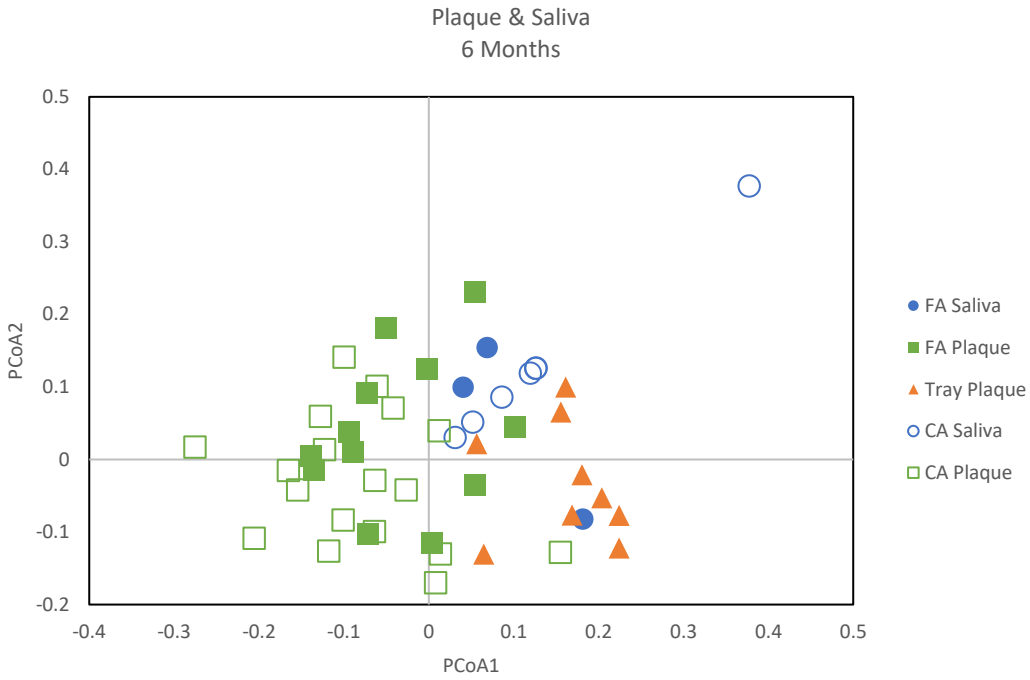
(Bottom): FA plaque and saliva is compared at each time point. A significant difference was noted between saliva and plaque microbiomes at both 0 and 12 months, as well as between saliva at 0 and 6 months, and 6 and 12 months.

Beta Diversity Analysis

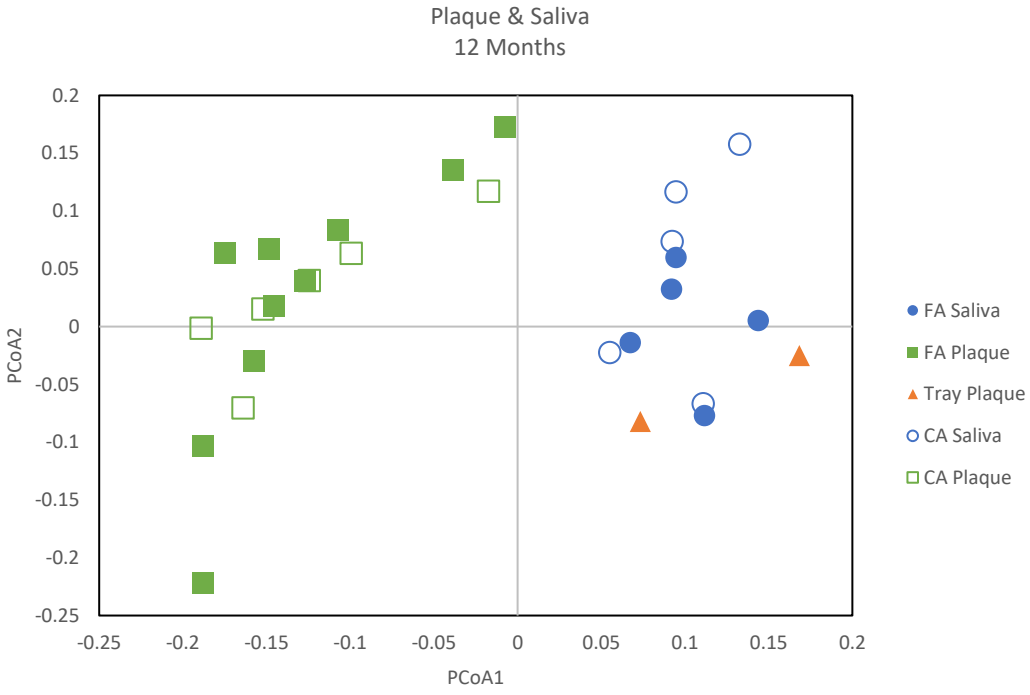
Beta diversity using principal coordinate analysis plots of Weighted UniFrac distances at each time point during treatment is shown in Figure 10. At each time point, saliva and plaque appear in distinct clusters apart from each other. Similarly, tray plaque appears in its own cluster between the distinct plaque and saliva groups. The beta diversity analysis from 12 months shows the greatest disparity between saliva and plaque for both FA and CA groups.



A


Plaque & Saliva
6 Months
PCoA2
PCoA1
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.4
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
FA Saliva
FA Plaque
Tray Plaque
CA Saliva
CA Plaque

B

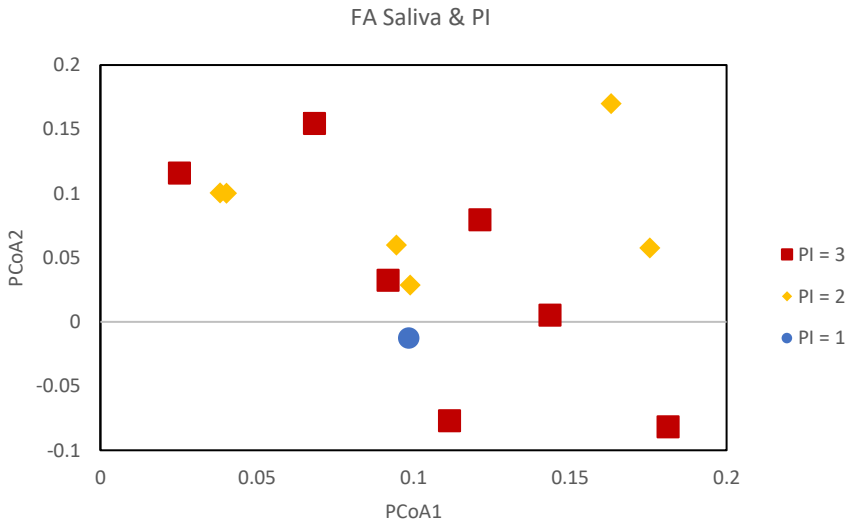

Plaque & Saliva
12 Months
PCoA2
PCoA1
0.2
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
-0.2
-0.25
-0.25
-0.2
-0.15
-0.1
-0.05
0
0.05
0.1
0.15
0.2
FA Saliva
FA Plaque
Tray Plaque
CA Saliva
CA Plaque

C

Figure 10A-C: Beta diversity analysis of saliva, plaque, and tray plaque. (From top to bottom): 3, 6, and 12 months beta diversity analysis. Plaque, saliva, and tray plaque remain in distinct clusters through each time point. The largest discrepancy between plaque and saliva for both FA and CA groups was found at 12 months.

Comparison of Clinical and Microbial Findings

To determine if there was a difference in microbial composition based on clinical findings, beta diversity analysis was completed by aggregating all time points per group and organizing the data points based on PI or GI scores. FA and CA analyses are shown in Figures 11 and 12, respectively. No significant differences were present in the comparison of FA saliva based on PI scores; however, FA plaque based on PI, and FA saliva and plaque based on GI appear to have semi-distinct clusters. In the CA group, saliva compared to both GI and PI present semi-distinct groups along the gradient of scores, but CA plaque did not present conclusive differences in either PI or GI scoring group.



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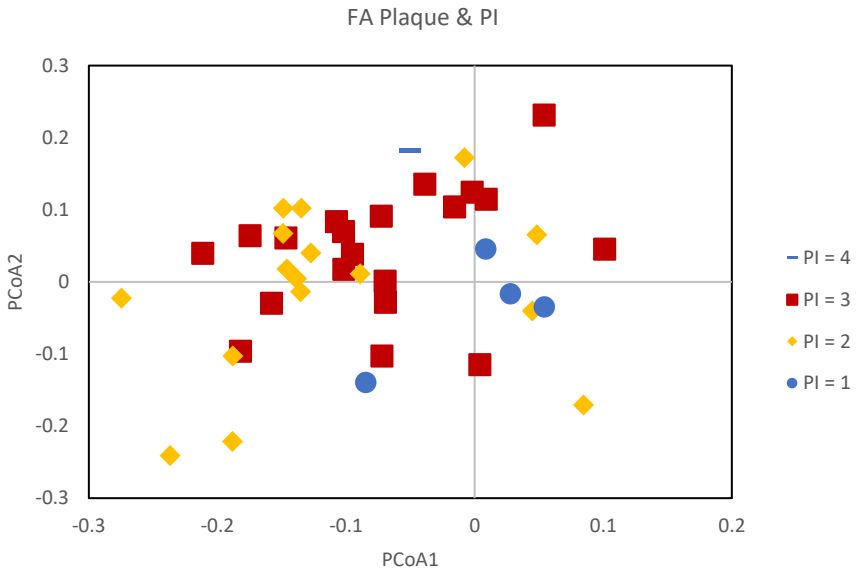
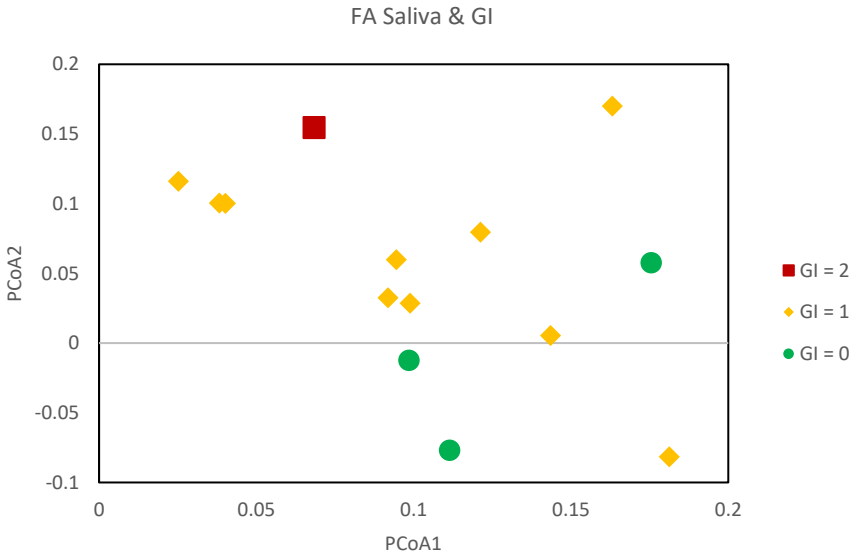
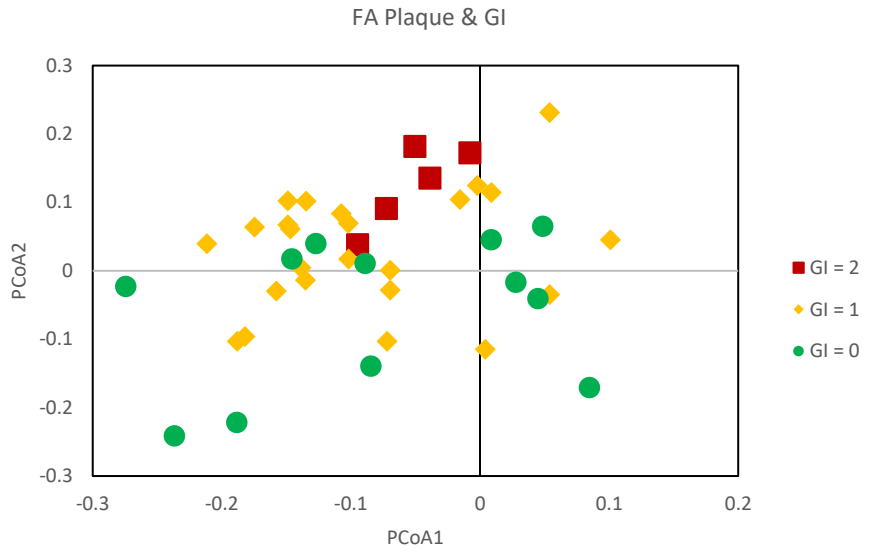

FA Plaque & PI
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
PCoA2
-0.3
-0.2
-0.1
0
0.1
0.2
PCoA1
PI = 4
PI = 3
PI = 2
PI = 1

B

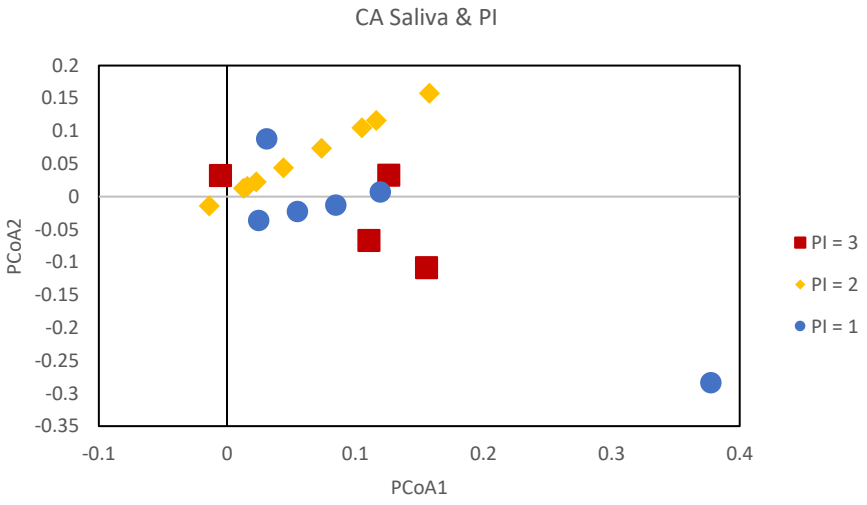

FA Saliva & GI
0.2
0.15
0.1
0.05
0
-0.05
-0.1
PCoA2
0
0.05
0.1
0.15
0.2
PCoA1
GI = 2
GI = 1
GI = 0

C

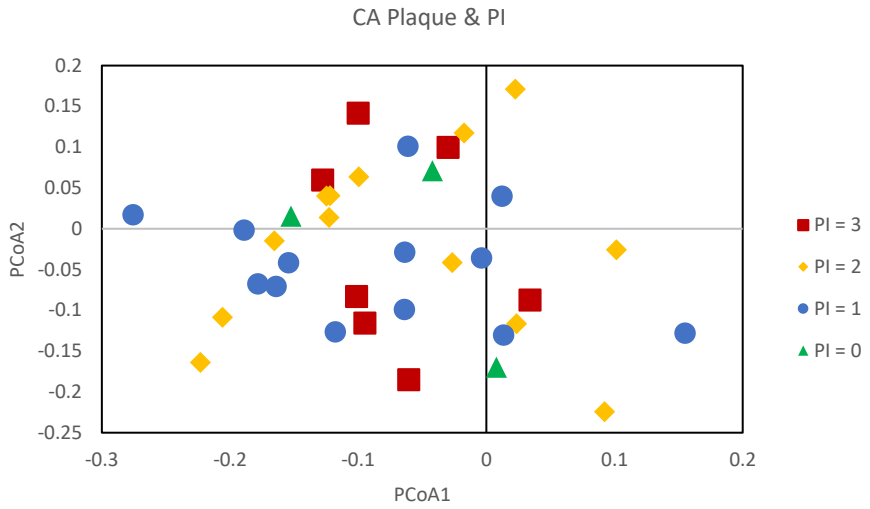


D

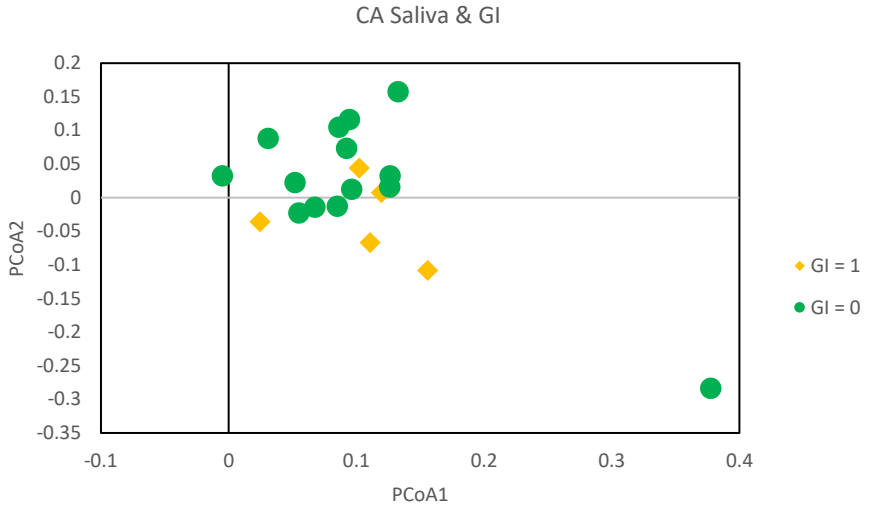
<u>Figure 11A-D</u>: Beta diversity analysis of FA saliva and plaque in comparison to clinical findings. A) FA saliva based on PI. B) FA plaque based on PI. C) FA saliva based on GI. D) FA plaque based on GI.



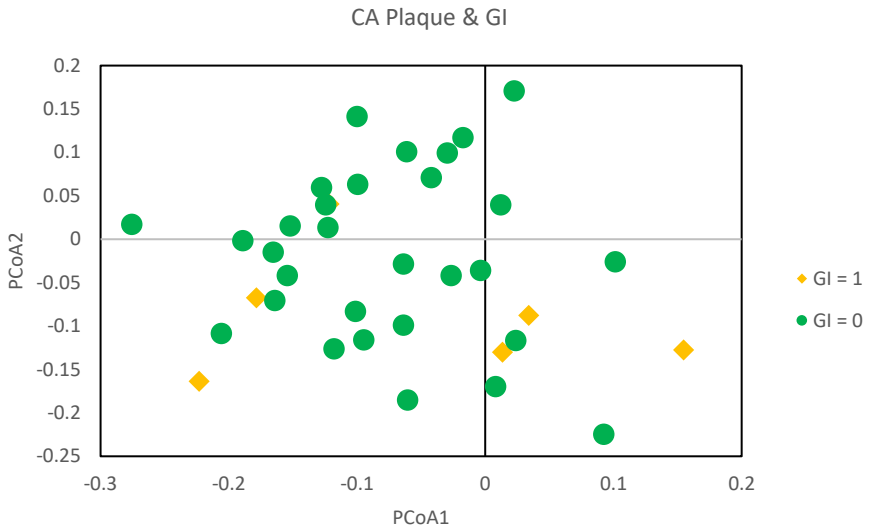
A


CA Plaque & PI
PCoA2
PCoA1
0.2
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
-0.2
-0.25
-0.3
-0.2
-0.1
0
0.1
0.2
PI = 3
PI = 2
PI = 1
PI = 0

B


CA Saliva & GI
PCoA2
PCoA1
0.2
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
-0.2
-0.25
-0.3
-0.35
-0.1
0
0.1
0.2
0.3
0.4
GI = 1
GI = 0

C

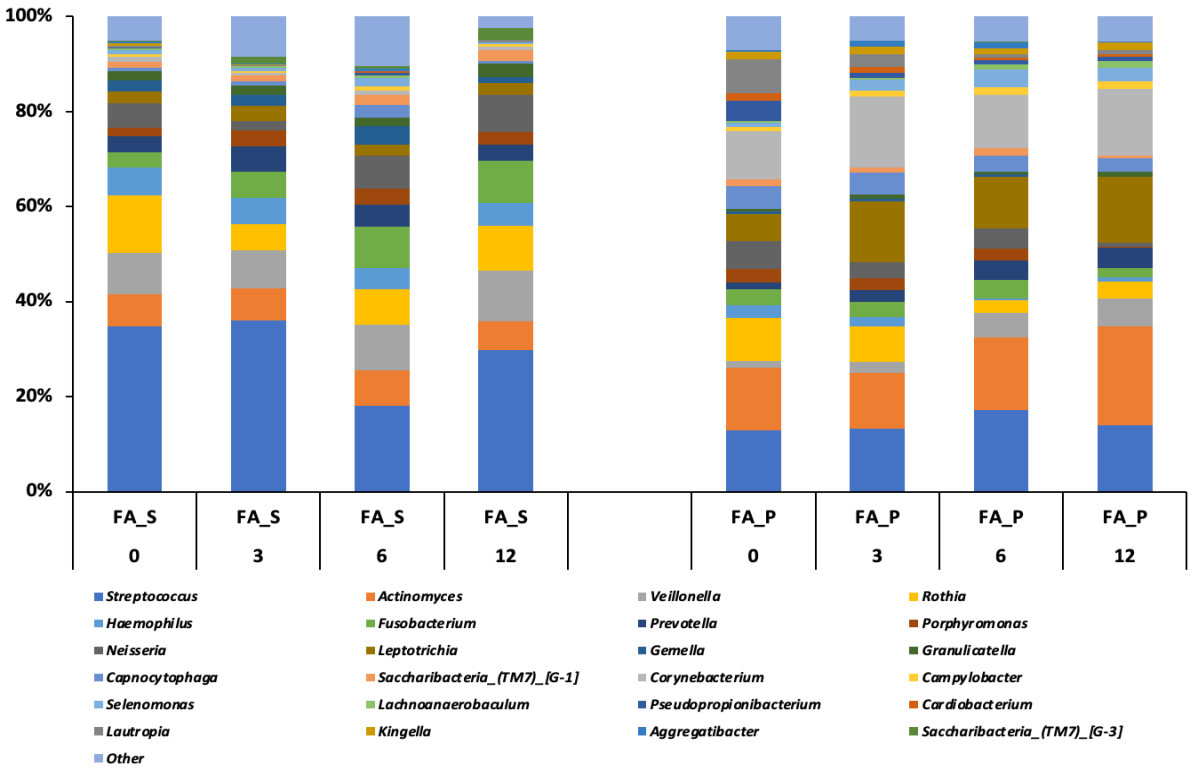


D

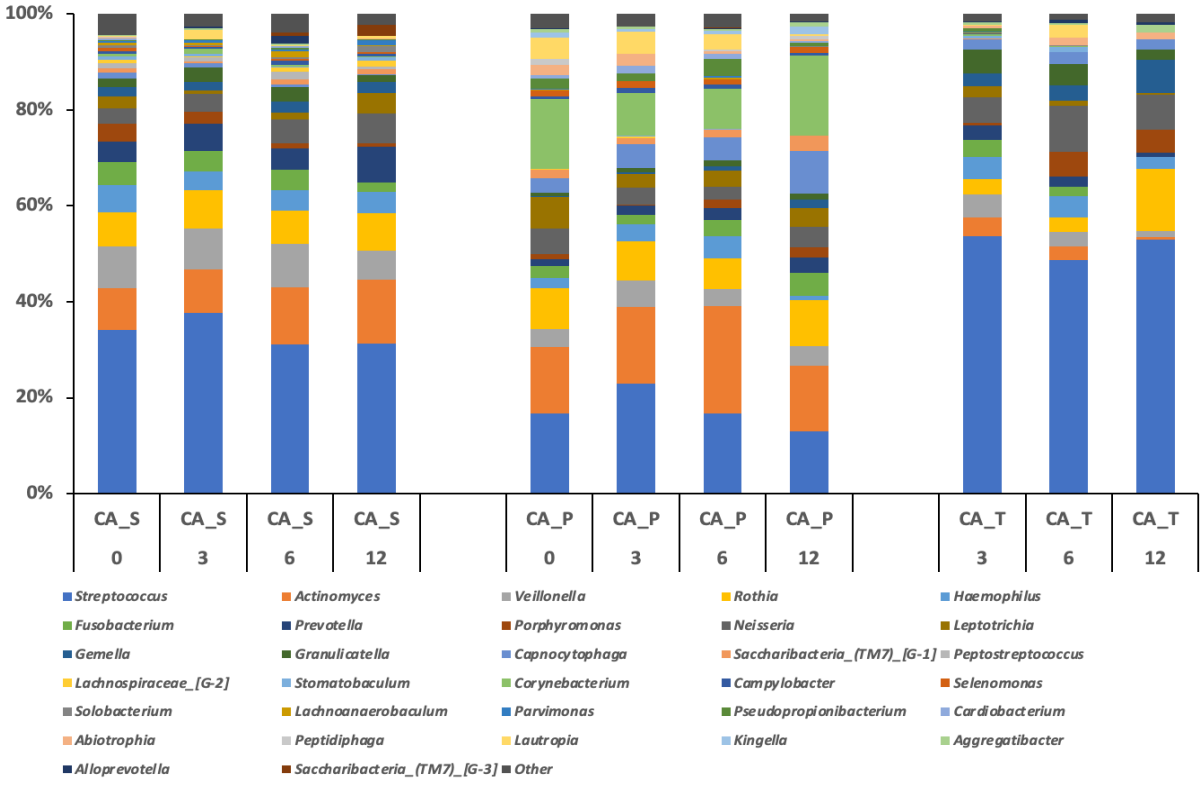
Figure 12A-D: Beta diversity analysis of CA saliva and plaque in comparison to clinical findings. A) CA saliva based on PI. B) CA plaque based on PI. C) CA saliva based on GI. D) CA plaque based on GI.

Community Composition and Genus Level Analysis

Each group appears to have a different bacterial community profile that changes as orthodontic treatment progresses. Figure 13A shows that FA saliva and plaque have different microbial community composition at the beginning of treatment. Figure 13B shows that CA tray plaque contains > 50% *Streptococcus* and is distinct from the profile of both CA saliva and plaque microbial communities, which are also distinct from each other. FA and CA saliva and plaque, as well as CA tray plaque, all experience individual genera fluctuations as treatment progresses, but the largest components remain relatively stable between time points. The major elements saliva and plaque of CA and FA patients appear relatively similar throughout treatment.


100%
80%
60%
40%
20%
0%
FA_S 0
FA_S 3
FA_S 6
FA_S 12
FA_P 0
FA_P 3
FA_P 6
FA_P 12
Streptococcus
Actinomyces
Veillonella
Rothia
Haemophilus
Fusobacterium
Prevotella
Porphyromonas
Neisseria
Leptotrichia
Gemella
Granulicatella
Capnocytophaga
Saccharibacteria_(TM7)_[G-1]
Corynebacterium
Campylobacter
Selenomonas
Lachnoanaerobaculum
Pseudopropionibacterium
Cardiobacterium
Lautropia
Kingella
Aggregatibacter
Saccharibacteria_(TM7)_[G-3]
Other

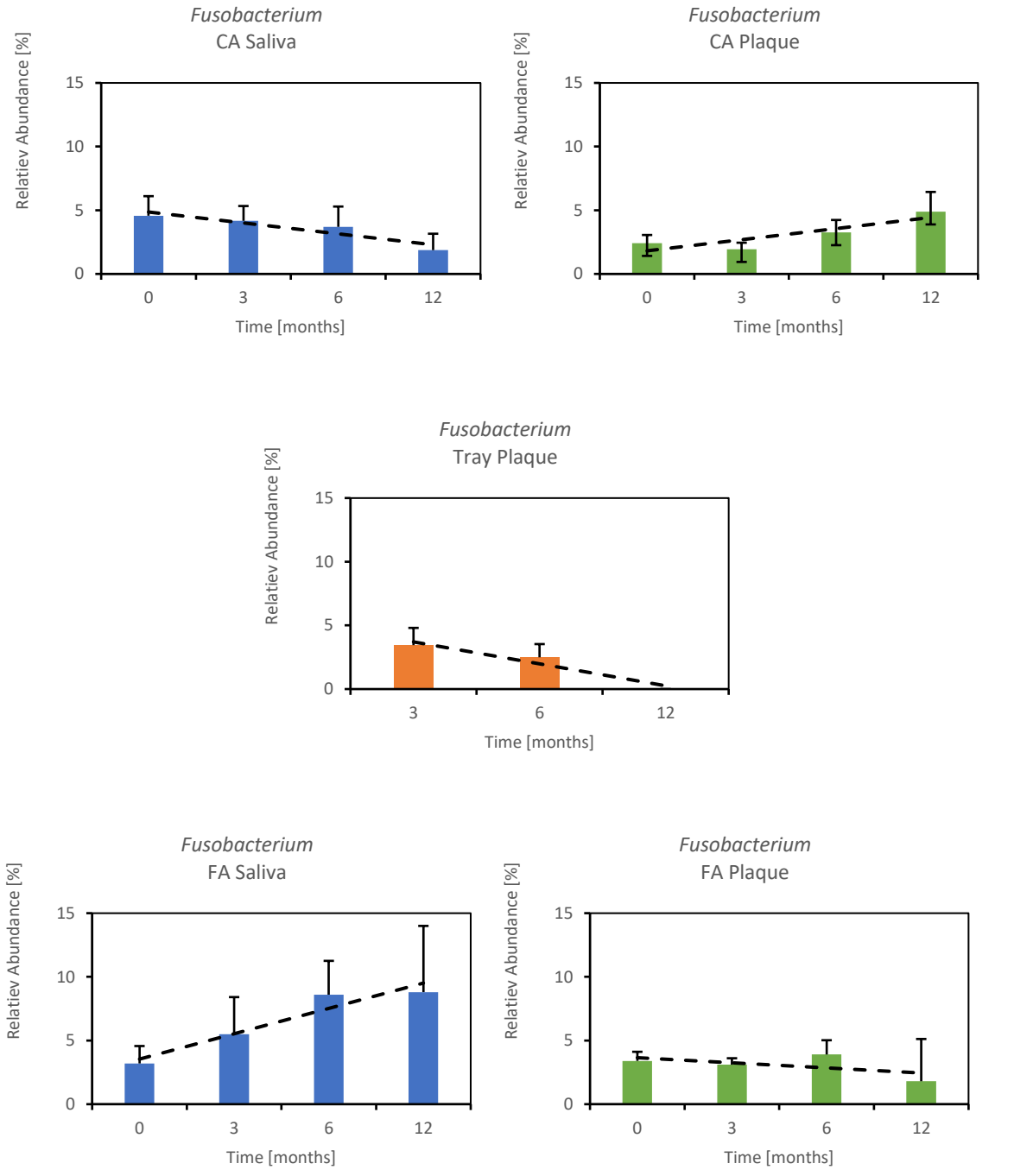
A


100%
80%
60%
40%
20%
0%
CA_S 0
CA_S 3
CA_S 6
CA_S 12
CA_P 0
CA_P 3
CA_P 6
CA_P 12
CA_T 3
CA_T 6
CA_T 12
Streptococcus
Actinomyces
Veillonella
Rothia
Haemophilus
Fusobacterium
Prevotella
Porphyromonas
Neisseria
Leptotrichia
Gemella
Granulicatella
Capnocytophaga
Saccharibacteria_(TM7)_[G-1]
Peptostreptococcus
Lachnospiraceae_[G-2]
Stomatobaculum
Corynebacterium
Campylobacter
Selenomonas
Solobacterium
Lachnoanaerobaculum
Parvimonas
Pseudopropionibacterium
Cardiobacterium
Abiotrophia
Peptidiphaga
Lautropia
Kingella
Aggregatibacter
Alloprevotella
Saccharibacteria_(TM7)_[G-3]
Other

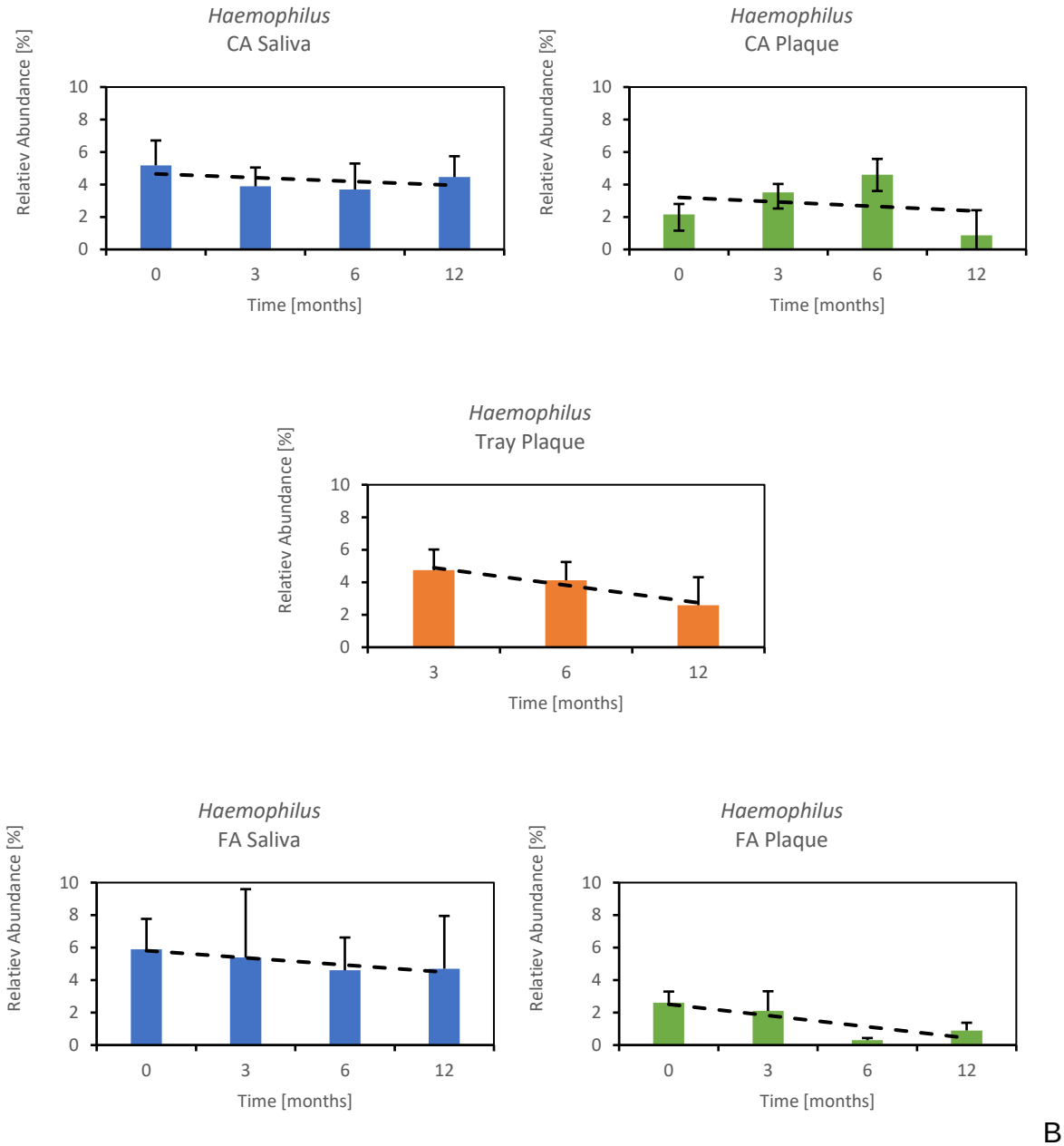
B

Figure 13A-B: Microbial community composition taxa analysis. A) Fixed appliance community analysis for saliva (left) and plaque (right) at each time point. B) Clear aligner community analysis for saliva (left), plaque (middle), and tray plaque (right).

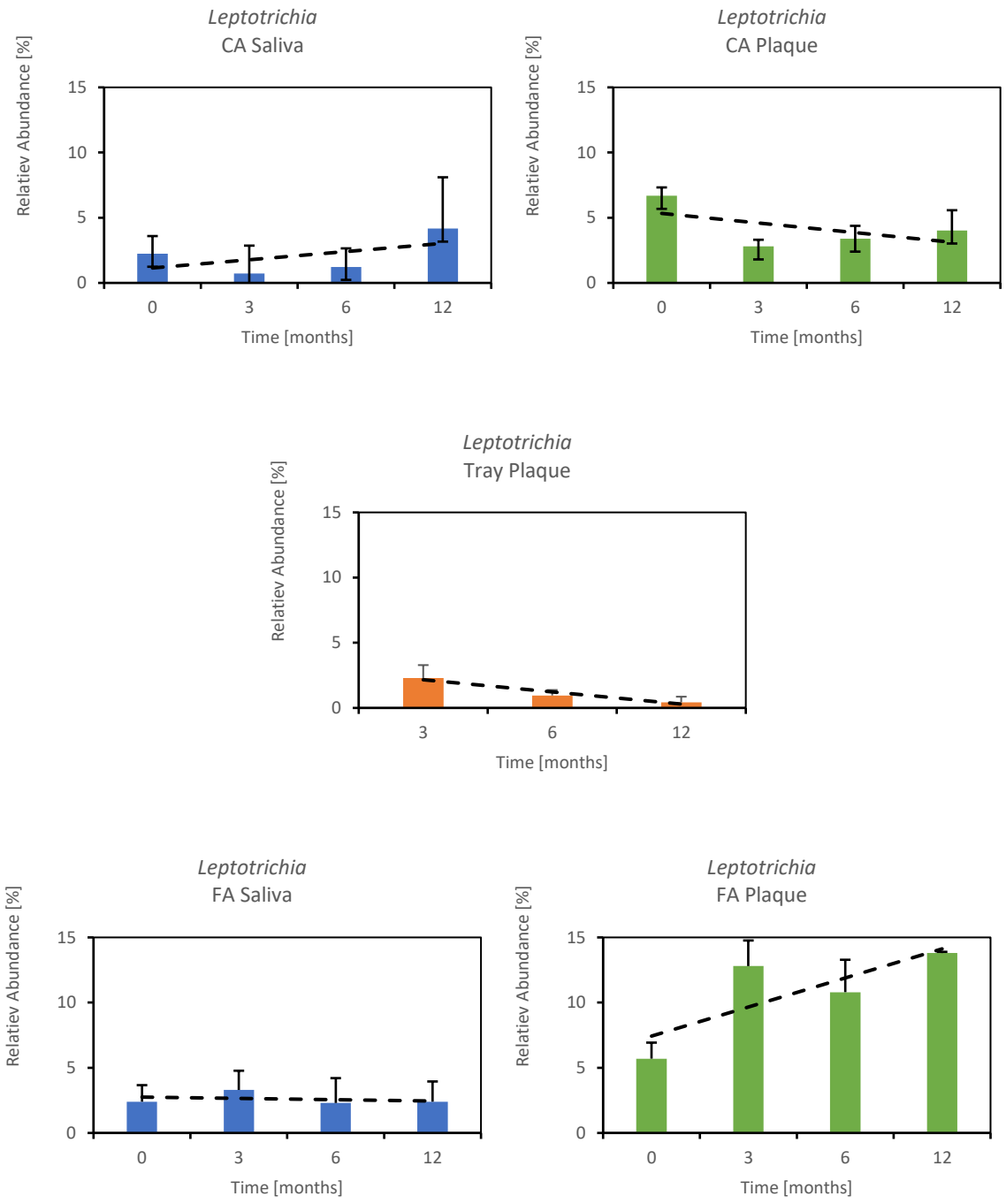
Figure 14A-C shows genus level analysis from the 16S rRNA sequencing data of selected significantly changed individual genera. During treatment, *Fusobacterium* decreases in CA Saliva and Tray Plaque, but increases in CA Plaque. It remains relatively unchanged in FA Plaque but increases in FA Saliva. *Haemophilus* remains stable over time in CA Saliva and Plaque but decreases in Tray Plaque during treatment. In FA, *Haemophilus* slightly decreases in both Saliva and Plaque communities. Lastly, *Leptotrichia* increases in CA Saliva, but decreases in CA Plaque and Tray Plaque. *Leptotrichia* is stable in FA Saliva but increases in FA Plaque during the 12 months of treatment.


Fusobacterium
CA Saliva
Relatiev Abundance [%]
15
10
5
0
0
3
6
12
Time [months]
Fusobacterium
CA Plaque
Relatiev Abundance [%]
15
10
5
0
0
3
6
12
Time [months]
Fusobacterium
Tray Plaque
Relatiev Abundance [%]
15
10
5
0
3
6
12
Time [months]
Fusobacterium
FA Saliva
Relatiev Abundance [%]
15
10
5
0
0
3
6
12
Time [months]
Fusobacterium
FA Plaque
Relatiev Abundance [%]
15
10
5
0
0
3
6
12
Time [months]

A


Haemophilus
CA Saliva
Relatiev Abundance [%]
10
8
6
4
2
0
0
3
6
12
Time [months]
Haemophilus
CA Plaque
Relatiev Abundance [%]
10
8
6
4
2
0
0
3
6
12
Time [months]
Haemophilus
Tray Plaque
Relatiev Abundance [%]
10
8
6
4
2
0
3
6
12
Time [months]
Haemophilus
FA Saliva
Relatiev Abundance [%]
10
8
6
4
2
0
0
3
6
12
Time [months]
Haemophilus
FA Plaque
Relatiev Abundance [%]
10
8
6
4
2
0
0
3
6
12
Time [months]

B



C

Figure 14A-C: Genus level analysis of relative abundance in CA Saliva, CA Plaque, Tray Plaque, FA Saliva, and FA Plaque based on 16S rRNA sequencing data of the following genera: A) Fusobacterium, B) Haemophilus, and C) Leptotrichia.

DISCUSSION

Importance of the Study

Fixed appliances and clear aligners both introduce new surfaces in the oral cavity for bacterial colonization, which necessitates diligent oral hygiene routines by patients to maintain good oral health. Fixed appliances utilize a variety of metals and elastics in the form of brackets, wires, bands, elastomeric o-rings, and interarch rubber bands, each of which present a unique environment for microbial growth, especially because of the inherent brushing and flossing difficulties introduced with these appliances. On the other hand, clear aligners require bonded composite attachments and full coverage of all dentition for the prescribed time of 22 hours per day during treatment, but their ease of removal allows for easier hygiene access in comparison to fixed appliances. Several studies have shown greater plaque accumulation and microbiome dysbiosis in fixed appliances than clear aligners, but relatively few studies have investigated the impact of each treatment modality on salivary microbiome composition, and even fewer utilized next generation 16s rRNA sequencing [21; 31; 32]. As the use of clear aligners continues to grow in the field of orthodontics [18], and fixed appliance therapy continues its decades-long association with white spot lesions and gingival inflammation - a more thorough understanding of how each treatment affects both saliva and plaque is necessary to understand the entire oral environment. By investigating saliva and plaque in both treatment modalities, we come closer to fully understanding microbial changes in the oral cavity caused by orthodontic treatment.

Plaque and Gingival Indices

Fixed appliance patients exhibited a significant average increase in plaque index score of 1.18 ($p < 0.05$) between 0 and 12 months, compared to the clear aligner mean increase of 0.15, which was not significant (Table 3 & Figure 4). Between specific time points, there was a significant increase in PI for the fixed appliance group from 0 to 6 months and 0 to 12 months, ($p < 0.05$), however, no significant changes in PI between any time points in the clear aligner group was observed (Figure 4). Consequently, this study concurs with previous research in showing that fixed appliance patients accumulate more supragingival plaque than clean aligner patients. Similarly, gingival index scores in the FA group significantly increased ($p < 0.05$) between 0 to 6 months by 0.76 and from 0 to 12 months by 0.56, but CA patients only had a significant increase in GI scores of 0.27 between 1 and 3 months (Table 3 & Figure 5). However, this difference should be approached with caution given the small sample size (n=3) of the 1 month time point. Besides the 1 to 3 month time point comparison, there was no significant change in the GI scores of CA patients throughout the first 12 months of treatment. Akin to the PI scores, this study concludes that gingival health worsens during fixed appliance therapy but stays relatively unchanged with clear aligner therapy (Figure 6). The lower plaque and gingival index scores may be attributed to having fewer difficult to reach “nooks and crannies” in clear aligners compared to fixed appliances where plaque can accumulate, and the ease of plaque debridement for clear aligner patients who can completely remove their appliances daily.

The Relationship Between Saliva, Plaque, and Tray Plaque

It has been confirmed through this study, and many others, that plaque accumulation increases with orthodontic therapy; however, few studies have evaluated how this change is reflected on the salivary or tray plaque microbiome. Firstly, to establish the baseline relationship between saliva and plaque, alpha and beta diversity analyses were completed (Figures 7 & 8). Alpha diversity can be described as the observed richness (number of taxa) or evenness (the relative abundances of those taxa) of an average sample [46]. Beta diversity can be defined as the variability in taxonomic composition among sampling units for a given area [48]. Figure 7 shows no significant difference in alpha diversity between plaque and saliva for either patient group at the beginning of treatment, which indicates similar individual microbial diversity within samples. Conversely, Figure 8 shows that at baseline there is already a distinction between saliva and plaque microbiomes, as they occupy different regions on the chart. Plaque is broadly spread across, while saliva samples are more densely gathered in one area – indicating that the communities differ in their taxonomic composition and/or abundance of microbes.

As treatment progressed the only significant differences ($p < 0.05$) in Shannon Index alpha diversity were found in CA plaque composition between 3 and 12 months, and both FA saliva and plaque microbial composition from 0 to 12 months (Figure 9). This data suggests that there is no significant change in CA saliva or tray plaque throughout treatment, but FA plaque and saliva experience a significant shift between baseline and 12 months. This shift may once again be attributed to the inherent difficulties of

maintaining good oral hygiene with fixed appliances in comparison to clear aligners. Figures 10A-C demonstrate the change in beta diversity exhibited by saliva, plaque, and tray plaque for both FA and CA patients at 3, 6, and 12 months. Similar to the 0-month data in Figure 8, saliva and plaque microbes remain distinguishable by the broad spread of plaque and the dense cluster of salivary samples. For CA patients, tray plaque also presents as a dense cluster distinct from both saliva and plaque communities. It appears that plaque and tray plaque microbes have minimal overlap in beta diversity, but saliva represents some middle ground between the two. Theoretically, this is a logical finding since saliva is constantly bathing the entire oral cavity and may act as an intermediary between tray and supragingival tooth plaque. This difference is most dramatic in the final 12-month time point (Figure 10C) where a large diastema exists between plaque and tray plaque communities, with salivary communities found in between. Overall, these results imply that saliva, plaque, and tray plaque microbiomes becoming gradually more distinct in both fixed appliance and clear aligner patients as treatment time increases.

Community Composition and Genus Level Shifts During Treatment

The bacterial community dysbiosis that occurs during orthodontic treatment, as described in previous studies [28] was also confirmed in the results of this study (Figure 13A-B). Many changes occur to the oral microbiome as treatment begins and progresses, which is evident in all saliva, plaque, and tray plaque groups. Although Figure 13A-B demonstrate that the most prevalent microbial community members in each group remain relatively stable throughout treatment, many changes occur in the

relative abundance of smaller population groups. Each appears to have a unique growth or decline of certain bacterial genera after beginning orthodontic treatment. More specifically, Figure 14A shows *Fusobacterium*, a genus which is considered commensal but whose members can act as an opportunistic pathogens in periodontal diseases [52]. The genus declines during treatment in both CA Saliva and Tray Plaque, increases in CA Plaque & FA Saliva, yet remains unchanged in FA Plaque. Decreases in CA Saliva and Tray Plaque *Fusobacterium* relative abundance could possibly be attributed to a variety of factors, including more attentive oral hygiene during orthodontic treatment, or perhaps the genus finds clear aligner plastic less hospitable for growth than teeth without plastic coverage. Conversely, *Fusobacterium* becomes relatively more abundant in CA Plaque and FA Saliva communities over time, and shows no change in FA Plaque. Again, this shift is subject to many variables, but further studies on *Fusobacterium* would be necessary to determine if the genus displays an affinity toward adherence on teeth or metal, over plastic surfaces, for proliferation.

Haemophilus is a health-associated bacterial genus [53] that was presented in Figure 14B. While there was no notable change in the genus for CA Saliva or Plaque, there was an observable reduction in relative abundance within Tray Plaque, FA Saliva, and FA Plaque communities. This finding supports the clinical data which shows poorer clinical signs of oral health (Table 3) in patients with fixed appliances, which worsened as treatment progressed (Figures 4 & 5). Interestingly, Tray Plaque demonstrated a reduction in relative abundance of this health-associated genus which was not reflected in the saliva, plaque, or clinical presentations of the same patients. It is possible that

Haemophilus symbiotically thrives within a healthy oral microbiome, which many CA patients maintained, but is ineffective under inflammatory conditions (like the FA microbiome) where other pathogens may proliferate more rapidly.

Lastly, Figure 14C displays the genus *Leptotrichia*, another disease-associated genus commonly found in the oral cavity. While there are no significant shifts in relative abundance of the genus in CA Saliva, Tray Plaque, or FA Saliva – a divergence occurs between CA and FA Plaque communities. *Leptotrichia* abundance decreases during treatment in CA Plaque, but increases in FA Plaque communities. It is possible that this genus is partially responsible for the poorer clinical health outcomes of FA patients, but further investigation would be required to ascertain the extent.

Before drawing any conclusions from these results, it must be considered that as a genus level analysis, there is a significant possibility that very different species of the same genus are involved in the observed changes.

The Relationship Between Clinical and Microbial Changes

Since both clinical and microbial data was collected on each patient group, a combination of these data sets has the potential to provide insight into how increases in plaque or gingival indices may be reflected in microbiome diversity shifts. Figures 11A-D and 12A-D display beta diversity based on PI and GI scores for FA and CA patients, respectively. FA plaque based on PI, and FA saliva and plaque based on GI appear to have semi-distinct clusters; however, there were no significant differences in the comparison of FA saliva based on PI scores. The results of this analysis are

inconclusive, perhaps due to sample size, and warrant further consideration in future studies.

Study Limitations

Several factors in the design of this study had the potential to influence the results. Firstly, saliva samples were stored in glycerol to enable regrowth of the bacteria; however, glycerol can also interfere with DNA extraction and may have affected sequencing. This may explain why the initial 16S rRNA sequencing resulted in a significant portion of samples having insufficient strands to analyze. Delays caused by lab closures during the COVID-19 pandemic combined with deadlines for master's thesis defense precluded the ability to re-run samples after the initial sequencing. Because this study relied on clinical data collection, and patients missed a variety of appointments throughout their treatment, there are a range of samples missing from each time point – for example, very few samples were collected at the 1-month time point. Furthermore, although data were collected for 12 months, most orthodontic treatment lasts between 24-36 months followed by an indefinite retention period, so the time points presented here only provide a snapshot into changes which occur during the first year of treatment. Future studies may consider focusing on patients further along in treatment, or even in retention, to see if the changes observed in this experiment are sustained. Similarly, it was impossible to standardize oral hygiene practices among patients, so it is conceivable that certain patients took more or less time and effort in brushing or flossing during treatment. Lastly, a large shortcoming of this study was the small sample size of 10 patients per group. The results in this study would have been

more conclusive with a larger sample size. For example, the correlation of clinical and microbial data would have benefitted from larger patient groups, as there were insufficient data points to distinguish between potential clusters in this study.

CONCLUSION

Patients who undergo orthodontic treatment with fixed appliances accumulate more plaque and have poorer gingival health as treatment progresses over the first 12 months, but patients who wear clear aligners experience no significant difference in plaque levels or gingival health during the same interval. At baseline, patients without any orthodontic appliances have no significant difference in alpha diversity between plaque and saliva microbial communities but form distinct clusters in beta diversity analysis. Over time, beta diversity analysis shows that plaque and tray plaque microbiomes become more distinct, and salivary microbiomes appear to represent an intermediary between the two. Future studies are needed to develop a more comprehensive understanding of the relationship between saliva, plaque, and tray plaque. Using a larger sample size of patients with more long term and post-appliance removal timepoints will provide the opportunity to assess if the changes observed in salivary and plaque microbiome composition continue to change in treatment beyond 12 months, and if the changes are sustained after the completion of treatment.

SUPPLEMENTAL TABLES & FIGURES

Appliance	Baseline	1 Month	3 Months	6 Months	12 Months	#visits	Age	Gender
FA	1	0	1	1	1	4	21	Male
FA	1	1	1	0	0	3	40	Male
FA	1	0	1	1	1	4	12	Female
FA	1	0	1	1	1	4	17	Male
FA	1	0	1	1	1	4	14	Female
FA	1	1	1	0	1	4	50	Female
FA	1	1	1	0	0	3	11	Female
FA	1	0	1	1	1	4	15	Female
FA	1	0	0	1	1	3	15	Female
FA	1	0	1	1	1	4	35	Male
Total	**10**	**3**	**9**	**7**	**8**	**37**		

Appliance	Baseline	1 Month	3 Months	6 Months	12 Months	#visits	Age	Gender
CA	1	1	0	1	1	4	38	Female
CA	1	0	1	1	1	4	27	Female
CA	1	1	1	0	1	4	56	Male
CA	1	0	1	1	0	3	23	Female
CA	1	0	1	1	1	4	25	Female
CA	1	0	1	0	0	2	51	Female
CA	1	0	0	1	1	3	24	Male
CA	1	0	0	0	1	2	19	Male
CA	1	0	1	0	0	2	23	Female
CA	1	0	1	0	0	2	23	Female
Total	**10**	**2**	**7**	**5**	**6**	**30**		

<u>Supplemental Tables 1 & 2</u>: Display of the number of samples collected at each time point for the FA and CA groups, respectively. Only 3 samples were collected at the 1-month time point for the FA group, and only 2 at the same time point for the CA group. Overall, more data was collected in the FA group (37) than the CA group (30).

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