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Alterations in the subgingival microbiome during orthodontic treatment

by

Isaac Lucas Chen, DDS

THESIS

Submitted in partial satisfaction of the requirements for the degree of

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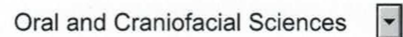

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Dedication and Acknowledgements

I would like to thank Dr. Andrew Jheon, Dr. Gerald Nelson and Dr. Pia Lodberg for their support and contribution to my master thesis. I want to specially thank Dr. Andrew Jheon for without him I wouldn't have been able to complete this project.

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ABSTRACT

Alterations in the subgingival microbiome during orthodontic treatment

Isaac Chen, DDS

Full fixed appliance orthodontic treatment commonly called braces increases plaque accumulation and risk of periodontal disease. Previously, microbial analysis was limited to cultivable bacteria using Sanger sequencing often assaying for candidate species. Considering the enormity of the oral microbiome and the restrictions of prior studies, we have utilized 16S rRNA next-generation sequencing (NGS) to catalog global changes in subgingival microbiomes at distinct mandibular tooth sites in subjects before and after orthodontic treatment over 12 weeks. Subjects with braces showed significant increases and maintenance in microbial richness 6 and 12 weeks after treatment. The increase in richness was accompanied by a decrease in Streptococcus, the most abundant genera found across all samples. An increase in shared OTUs was also observed at 6-12 weeks demonstrating the tendency of subgingival microbiomes to become more uniform with orthodontic treatment. We confirmed the presence of candidate bacteria associated with periodontal diseases, and also identified numerous novel species in subgingival microbiomes that showed significant changes with orthodontic intervention. We have generated for the first time, global profiles of subgingival microbiomes during orthodontic treatment and demonstrate that traditional orthodontic treatment may predispose subjects to periodontal diseases such as gingivitis regardless of oral hygiene.

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INTRODUCTION

In 2007, the National Institutes of Health (NIH) funded the human microbiome project (HMP) to contribute to the understanding of how the microbiome associated with our bodies correlates with human health by mapping the normal microbial makeup of healthy humans.[1] It was discovered that nearly everyone routinely carries pathogens and microorganisms known to cause illnesses. However, in healthy individuals pathogens cause no disease and simply coexist with their host and the rest of the microbiome.[1] Recently, Sender et al. (2016) published a revised estimate of bacteria to human cell ratio to be nearly 1:1, an estimated 38 trillion bacteria to 30 trillion human cells.[2] Each human body contains a personalized microbiome that is essential to maintaining health but also capable of eliciting disease. For example, there is a concentration of 10^{11} bacteria/ml in the gut that provide both beneficial and harmful effects.[2] This gut flora enhances our immune system, helps absorb vitamins, and utilizes the food consumed. Conversely, the gut flora may also be associated with obesity, intestinal and systemic inflammation, cancer, and autism.[3] The oral pathogens may be important players in systemic diseases, such as diabetes,[4] infective endocarditis,[5] bacterial pneumonia,[6] adverse pregnancy outcomes,[7] inflammatory bowel disease,[8] pancreatic cancer,[9] and colon cancer.[10] Despite its substantial impact on overall human health, the complexities of the oral bacterial community and how its composition changes when shifting between the healthy and diseased states are far from being fully understood.

The oral cavity or mouth includes several distinct microbial habitats, such as the gingival sulcus, attached gingiva, tongue, cheek, lip, hard palate, soft palate, and teeth.

Contiguous with the oral cavity are the tonsils, pharynx, esophagus, eustachian tube, middle ear, trachea, lungs, nasal passages, and sinuses. The human oral microbiome is defined as all microorganisms found on or in the human oral cavity and its contiguous extensions (stopping at the distal esophagus).[11] Studies have shown that different oral structures and tissues are colonized by distinct microbial communities,[12] and it has been estimated that less than half of the bacterial species present in the oral cavity can be cultivated using anaerobic microbiological methods and that there are likely 500 to 700 common oral species.[13]

Oral Microbiome and Orthodontics

The past three decades have seen greater focus on dentofacial esthetics in the adult population with an increasing demand for orthodontic treatment in appearance-conscious adults.[14] The primary motivating factor has been reported to be a desire to improve dental appearance.[15] And while fixed orthodontic treatment is capable of correcting malocclusions and improving dental appearance, it has also been correlated with periodontal disease. Aligned teeth can be more easily cleaned, which can benefit the periodontal condition in the long term.[16,17] However, plaque accumulation is also common during orthodontic treatment which can lead to gingival inflammation, including bleeding, swelling, and hyperplasia.[18]

Since plaque is the primary etiologic factor in gingivitis,[19] it is possible that orthodontic treatment could increase the risk of gingivitis or even periodontitis. The etiology of gingivitis and periodontitis is microbial infection, which leads to an imbalance between the host and the microorganism, and a change in the subgingival microbiota.[20] Due to the patient’s inability to adequately clean around fixed appliances the subgingival

microbial environment changes by increasing plaque accumulation and deepening gingival sulcus.[21–23] Orthodontic appliances generally increased the level of periodontopathogens in subgingival plaques,[24–27] especially *Lactobacillus.*[28] Similarly, 2- to 3-fold increases in both clinical indexes and numbers of motile organisms have been reported at gingival sites 6 months after appliance placement,[29] as well as early increases in anaerobes and *Prevotella intermedia*, and a decrease in facultative anaerobes.[30] This shift in the subgingival microflora to a periodontopathogenic population is similar to the microflora at periodontally diseased sites.[31] However, several other studies have reported no significant differences,[32] or a decrease in the level of periodontopathogens during orthodontic treatment due to metal corrosion, which imposed toxic effects on the microorganism.[33] Thus, previous studies regarding changes in periodontopathogens during orthodontic treatment have been inconsistent.

The combination of orthodontic intrusion and periodontal treatment has been shown to improve compromised periodontal conditions, if oral hygiene is maintained and tissues are healthy.[34] However, root resorption of 1-3 mm has been associated with intrusion of incisors in adult patients showing marginal bone loss and deep overbite. Re et al. (2000), in a 12-year report, showed orthodontic treatment is no longer a contraindication in the therapy of severe adult periodontitis.[35] In such cases, orthodontic treatment might enhance the possibilities of saving and restoring a deteriorated dentition.

The main short-term effects of orthodontic bands on the periodontium are gingivitis and gingival enlargement. Gingival enlargement occurs after placement of a fixed appliance. This condition rapidly improves within 48 hours after the appliance is

removed.[36] The increase in probing depth during orthodontic treatment has also been attributed to this enlargement.[37–39] Because this gingival enlargement is also seen in patients with good oral hygiene, mechanical irritation caused by the band or cement, in addition to trapped plaque, may be implicated.[29,37] When such iatrogenic irritations are inevitable, the risk of loss of attachment can be anticipated.[39]

Animal studies have shown that in dentitions with reduced periodontia, orthodontic forces and tooth movements do not induce gingivitis without plaque.[6] Adults and adolescents have similar risks of developing periodontal disease after orthodontic treatment.[29] The most important factor in the initiation, progression, and recurrence of periodontal disease in a reduced periodontium is microbial plaque.[40,41] Clinical studies have demonstrated that, with adequate plaque control, teeth with reduced periodontal support can undergo successful tooth movement without compromising their periodontal situation.[29,42]

In a cross-sectional study, radiographic crestal bone levels in 104 adults, who had completed orthodontic therapy at least 10 years previously, were shown to be no different from 76 matched control subjects.[43] However, another study in adolescents indicated that up to 10% of the 38 children had significant loss of attachment (mean, 1-2 mm) in 2 years.[44] In a recent study, 267 adults who had severe periodontal disease with pathologic migration of the anterior teeth had combined orthodontic-periodontal treatment.[35] Before orthodontic treatment began, 129 patients underwent surgical periodontal treatment (i.e., modified Widman flap), and the remainder nonsurgical treatment with hand instruments or ultrasonic devices. The patients were followed for varying periods (2-12 years) after the end of treatment. The results of this study suggest

that orthodontic treatment is not a contraindication in adult patients with severe periodontitis, and in such cases, may improve the possibilities of saving and restoring a deteriorated dentition.[35]

Guo *et al.* (2017) reviewed the changes in periodontopathogens in patients undergoing orthodontic treatment with facial metal brackets and/or bands.[32] The frequency of periodontopathogens in the subgingival plaques and the changes in the microbial composition were summarized. In the studies reviewed, four common periodontopathogens that are highly related to periodontal diseases were selected for qualitative and quantitative analysis: *Aggregatibacter actinomycetemcomitans (Aa), Porphyromonas gingivalis (Pg), Prevotella intermedia (Pi)* and *Tannerella forsythia (Tf)*. Quantitative analysis revealed that in the short-term (e.g., 1-3 months) *Aa, Pg, and Pi* did not have a statistically significant difference in the first molar region before and after appliance placement, but *Pi* did have a statistically significant different at the incisor region. On the other hand, *Tf*, a member of the red complex which are associated with severe periodontitis, showed significant differences before and after appliance placement.[21,45,46] However, when compared to quantitative analyses, the results of qualitative analyses were inconsistent. For example, five out of six studies reported that *Aa* remained unchanged after appliance placement,[21,22,45–47] but one study demonstrated a significant increase one month after appliance placement and maintained a high detection rate after 3 months.[48] For *Pg*, two studies stated no significant changes,[21,45] two reported a significant reduction,[46,49] and two showed a significant increase.[18,22] For *Tf*, two found a significant increase,[18,21] but three studies found an insignificant increasing trend.[22,45,46] For *Pi*, two studies reported an increase

after placement followed by a decrease several months later,[47,50] two other studies reported significant increases 3 months after placement,[18,48] and four other studies found insignificant increasing trends.[21,22,45,46] Besides those four periodontopathogens, *Fusobacterium nucleatum (Fn), Prevotella nigrescens (Pn) and Campylobactor rectus (Cr)* also increased after appliance placement. [21]

Three studies monitored long-term changes (e.g., >6 months) in subgingival microorganisms.[21,47,50] Two studies reported transient changes.[47,50] One study reported an increase in pathogens during the first 6 months followed by a return to pretreatment levels after 12 months.[21] Kim *et al.* (2011) showed a rising trend in the frequency of several periodontopathogens in the first six months,[21] but another study reported an increasing trend in *Pi* frequency after three months and a decreasing trend after six months.[50] Guo et al. (2017) speculated that these inconsistencies may be due to relatively short observation times.[32]

Six studies also followed subgingival microbial changes after orthodontic appliance removal.[21,46,47,51,52] Four of these studies reported no significant difference in *Aa* frequency after orthodontic appliance removal,[46,47,51,52] but two studies reported a significant reduction in *Aa* levels.[21,53] Five studies reported no significant difference in *Pg* frequency,[46,47,51–53] but one reported a significant reduction.[49] Three studies showed no significant difference for *Tf* frequency,[47,51,52] but one showed a significant reduction after appliance removal.[42,67] Five studies reported no apparent changes in *Pi* levels after appliance removal.[46,47,51–53] Four studies showed that the colonization of microorganisms on teeth varied during orthodontic treatment.[21,46,50,51] Sandic et al. found that only the frequency of *Tf* significantly decreases after appliance removal on

the first molar, and that the frequency of *Tf* on incisors showed no significant difference. Guo *et al.* (2017) concluded that the transient increase in subgingival microorganisms can be explained by the imbalance to the host-microorganisms when orthodontic appliances and forces are placed.[32] After several months when the host immunity improves and the host-microorganisms adapt and re-establish, the levels of periodontopathogens return to pretreatment levels. Despite knowing that periodontopathogen levels return back to pretreatment levels after appliance removal, it is beneficial to the patient to stress the importance of oral hygiene and periodontal maintenance during the early stages of orthodontic treatment when the levels of periodontopathogens are higher.[32]

The inconsistent and frequent contradictory results highlighted by Guo *et al.* (2017) underscore the difficulty of profiling the microbiome during orthodontic treatment.[32] Some experimental limitations include a lack of large and high-quality randomized controlled trials (RCTs), small number of relevant research articles and patients, and observation times were relatively short. In addition, sample collection methods, sample detection methods, teeth selection, and sample pooling were inconsistent in all the studies and hence difficult to perform a meta-analysis. Regarding sample collection, some studies used sterile paper points and others used sterile curettes. Five different techniques were used for sample detection: 1) PCR method to detect the 16S rRNA gene, 2) quantitative PCR (qPCR), 3) reverse transcriptase PCR, 4) DNA probe method, and 5) culture method. Different teeth might have different microbial flora, but only four of the included studies analyzed their results based on the tooth, whereas the other nine studies pooled all the samples together regardless of

tooth-specific differences. Hence, further clinical trials with adequate methodologies and reliable analyses of the microbial changes during orthodontic treatment are needed.[32]

Plaque accumulation, metal corrosion, hormonal levels, the microbial baseline, host immunity and tooth movement are factors that cause changes in the subgingival microorganisms during orthodontic treatment.[30,54–57] Orthodontic appliances lead to plaque accumulation, which may lead to periodontal disease. Tezal *et al.* (2006) discovered that a high presence of supragingival plaque accumulation would lead to a high presence of subgingival periodontopathogen plaque.[58] Orthodontic tooth movement such as tipping and intrusion are also factors that can cause changes in the subgingival plaque composition as the supragingival plaque moves into the subgingival sulcus. Every individual has a microbial baseline in which host-microorganisms are in equilibrium with periodontophatogenic microorganisms, when factors such as hormone imbalance, particularly in adolescents or pregnant patients, change the content in the microorganisms it can lead to a dis-equilibration of the microbial baseline resulting in periodontal inflammation.[59] Nickel ions released from the brackets and archwires could also result in toxic effects on bacteria.[33]

Only one other study has used 16S rRNA gene sequencing to look at the microbial changes during clear aligner treatment (CAT). How CAT affects the subgingival microbial community still lacks a lot of research. There can be many possible factors affecting how CAT changes the microbial community during treatment. Guo *et al.* (2018) postulated that the gingival margin of the clear aligner, that is an undercut, could cause extensive biofilm accumulation that can influence the supra- and sub- gingival bacteria.[60] Clear aligners made from thermoplastic appliance are prone to

salivary film adherence,[61] and are not completely smooth, which may also facilitate bacterial adhesion.[62] Therefore, changes in the oral microbiota caused by CAT can affect the subgingival microbial community. Guo *et al.* (2018) revealed a slightly decreasing microbial diversity with a significant change of microbial structure during the first three-months of CAT in 10 female patients.[61] However, the patients were free from periodontal diseases with stable levels of periodontal microorganisms and core microorganisms. Their preliminary findings indicated that clear aligners induced nonpathogenic changes of the subgingival microbiome in the first three-months of treatment. The five genera, Streptococcus, Neisseria, Actinomyces, Fusobacterium and Rothia were relatively abundant. Among these, Streptococcus (11.95%), Neisseria (7.29%) and Actinomyces (7.27%) showed high abundances at T0, while Actinomyces (13.10%), Streptococcus (11.09%) and Fusobacterium (8.34%) manifested high abundances at T1, and Rothia (11.36%), Actinomyces (10.53%) and Fusobacterium (8.36%) had highest abundances at T2. The relative abundances of eight predominant genera that could cause periodontal disease were found to have no significant difference during the first three-months of CAT.[60] At the species level, the periodontal pathogens investigated were Aggregatibacter actinomycetemcomitans (Aa), Prevella intermedia (Pi), Campylobacer rectus (Cr), Fusobacterium nucleatum (Fn) and Treponema denticola (Td). The relative abundance of Pi slightly decreased at T2 (0.55%) and T1 (0.80%) compared with T0 (1.15%). A slightly increased relative abundance of Aa at T2 (0.17%) was found. However, the changes in relative abundances of these five putative periodontal pathogens were not significantly different during the first three-months of CAT.[60]

Technological Advancements in Microbiome Research

The gingival sulcus is a natural space found between a tooth and the gingiva that surrounds the tooth. Within this sulcus resides all forms of resident and transient bacteria that form a complex ecosystem. This polymicrobial population of bacteria have a key role in the etiology of chronic periodontitis.[63] However, the role of individual species and their complex interactions with the host was not very well understood. Advancement in bacteria identification techniques have revealed that gram-negative bacteria can be associated with health and that gram-positive bacteria can also play a role in disease.[64]

Traditional cultivation methods have been used to isolate and characterize over 280 oral bacterial species.[12] However, a large proportion of oral bacteria cannot be cultivated *in vitro*, which impedes the thorough and in depth understanding of the natural microbial community residing in dental plaques.[13] The use of molecular biological techniques, such as denaturing gradient gel electrophoresis,[65] quantitative real-time polymerase chain reaction,[66] microarray chips,[67] checkerboard hybridization,[68] and cloning sequencing of DNA,[69] enable the identification and classification of uncultured oral bacteria. However, all these approaches have remarkable biases and drawbacks that do not facilitate the comprehensive study of bacterial diversity, as high-abundance bacteria are predominantly identified and low-abundance species are left undetected.[70] Therefore these techniques are limited and have only been able to implicate minor constituents of the subgingival community, the gram-negative bacteria *Porphyromonas gingivalis, Tannerella forsythia, and Treponema denticola*.[63]

16S ribosomal RNA (rRNA) -based, high-throughput, next generation sequencing (NGS) is able to catalog the composition of the subgingival microbial community.[71] Human and microbial DNA are purified from each collected specimen and analyzed using DNA sequencing platforms. The genome sequence data are then sorted to identify specific bacterial species via the variable regions of 16S rRNA. Bacterial 16S rRNA helps form the cellular structures for protein translation.[1] In short, this method can analyze the microbial composition and community structure, providing an unprecedented amount of information. The high-throughput and cost-effective technique simplifies the exploration of sophisticated microbial diversity.[72] Investigators of oral bacteria using 16S rRNA sequencing deposited more than 700 orally derived 16S sequences in GenBank, but less than half are from species that have been identified by culture technique.[64]

Oral Microbiome and Periodontitis

Kumar *et al.* (2005) studied the biofilms associated with chronic periodontitis and periodontal health and identified candidate pathogens and beneficial species.[64] Fifteen subjects with moderate to severe generalized chronic periodontitis, between the ages of 42 to 80 years were recruited, as well as 15 healthy age- and sex-matched control subjects. Samples from the mesial sulcus of every tooth were collected and pooled using sterile endodontic paper points in all subjects but those with chronic periodontitis were separated into two groups: samples from deep pocket depth (greater than or equal to 6mm) and samples from shallow pocket depth (less than or equal to 3mm). Specimens were collected, bacterial DNA amplified using PCR, cloned into plasmids,

and analyzed using 16S rRNA Sanger sequencing. The classes *Clostridia* and *Bacilli* of the phylum *Firmicutes* of the domain *Bacteria* accounted for 75% of all clones, dominating the subgingival flora in both health and periodontitis. Both *Clostridia* and *Deferribacteres* showed significant association with periodontitis, whereas *Bacilli* was significantly associated with periodontal health. Within the *Clostridia* class the most numerous genera were *Peptostreptococcus, Veillonella,* and *Selenomonas*, whereas *Streptococcus* and *Gemella* were the more numerous genera in the *Bacilli* class. Gram-positive but not gram-negative bacteria showed significant differences in relation to periodontal health status. Gram-positive facultative bacteria accounted for a greater fraction of total bacteria in healthy subjects than in subjects with periodontitis, whereas gram-positive anaerobic species were more common in subjects with periodontitis than in healthy subjects. Furthermore, uncultivable bacteria were claimed to comprise 59.9% of the total bacteria species. For example, the genera *Deferribacteres, Megasphaera, Desulfobulbus,* and *Lachnospira* were composed entirely of uncultivated phylotypes. Uncultivated phylotypes were predominant within the genera *Selenomonas, Veillonella,* and *Peptostreptococcus.* Other genera such as *Campylobacter, Gemella, Streptococcus,* and *Neisseria* were composed predominantly of cultivable species. The distribution of the 22 most common bacterial genera in relation to disease status (Figure 1) and the species or phylotypes that showed an association with disease or health (Table 1) are presented. The ranking of these species indicates their relative prevalence among all clones.[64]

Statistically significant associations of bacteria with periodontitis and health were identified at the genera level.[64] Surprisingly, more associations between gram-positive

bacteria and disease were identified although gram-negative bacteria are usually thought to be important in disease.[64] There was strong association between gram-positive anaerobic cocci (GPAC; commonly known as peptostreptococci) and periodontitis. GPAC were far more numerous although as mentioned, gram-negative anaerobes are commonly associated with periodontitis. Limitations of the culturing method could explain why these potential pathogens were previously not identified.[64] GPAC have been isolated from a wide range of human infections and appear to synergistically interact with other bacteria.[73] Previous epidemiologic evidence, though limited, have linked *Peptostreptococcus micros* with odontogenic infections.[74,75] *P. micros* demonstrates both adhesion to epithelium and coaggregation with other species such as *P. gingivalis* and *F. nucleatum* mediated by extracellular polysaccharides.[76,77] Elevated levels of *P.* micros were found in advanced chronic periodontitis,[78] and mobile teeth.[79] Peptostreptococci isolated from chronic skin ulcers have been shown to inhibit keratinocyte and fibroblast proliferation and re-populate wounds in tissue culture model systems.[80] These data suggest that *P. micros* may play a role in preventing wound healing in chronic disease and may be important in the physical structure of a disease-associated biofilm.[64]

Gram-positive rod *Filifactor alocis,* previously associated with both chronic periodontitis,[81] and endodontic lesions,[82] were also found to be significantly elevated in subjects with disease (Table 1). Several gram-negative bacterial species were also associated with periodontitis, but in much smaller quantities compared to the gram-positive, disease-associated species. *Megasphaera* oral clones BB166, MCE3_141,

and BS073 were associated with disease.[64] *Megasphaera* clone BB166 has been previously associated with chronic periodontitis.[81]

Desulfobulbus CH031 and R004 were significantly associated with deep sites. *Desulfobulbus* species have been previously detected in the gingival sulcus,[13,81] and the human gut.[83] *Desulfobulbus* are sulfate-reducing bacteria and have been frequently detected in aquatic environmental samples. *Campylobacter sputorum* subsp. *sputorum* and *Campylobacter* strain BB120 were strongly associated with disease. Taken as a whole the genus *Campylobacter* was associated with health, but this association was accounted for by the highly prevalent species *C. gracilis* and *C. showae*.

Many clones of *Selenomonas* were detected, most from the cultivable species *S. sputigena*, *S. infelix*, and *S. noxia*. None of these were associated with disease, although *S. noxia* has been previously linked to active periodontitis.[84] The less numerous and uncultivated *Selenomonas* phylotypes D0-042, EY047, and AH132 were associated with disease, and, in contrast, *Selenomonas* strain DS051 was detected more frequently in healthy subjects. *Dialister pneumosintes* and *Dialister* phylotype ME_134 were associated with periodontitis. *D. pneumosintes* has been previously linked to periodontitis,[78,85] and to endodontic infections.[86] *Deferribacteres* phylotypes W090 and BH007 were associated with periodontitis, and W090 has been previously linked to disease.[81] In addition, uncultivated phylotypes of *Catonella*, *Streptococci*, *Atopobium*, *Eubacterium*, and *Treponema* were also significantly associated with disease (Table 1).

P. gingivalis, *T. denticola*, and *T. forsythia* were rarely detected and of these, only *T. forsythia* was associated with disease. Strong associations with disease have

been observed for these species in many prior studies, but when quantitative results have been reported, they have comprised only a small fraction of the total bacteria. The sample size in the study by Kumar *et al.* (2005) did not provide adequate power to detect association for minor species. More numerous bacteria did show strong associations with disease however, indicating that potentially important bacteria have been overlooked in previous studies due to technical challenges.

Kumar *et al.* (2005) found *Streptococcus* and *Veillonella* spp. in high numbers in all samples and at a significantly greater percentage of the microbial community in healthy subjects than in those with periodontitis. At the species level both *S. sanguis* and *S. mutans* were associated with periodontal health, as was the overall most abundant species, *Veillonella* sp. oral clone X042. Haffajee *et al.* (1998) and Tanner *et al.* (1998) have associated both *Streptococcus* and *Veillonella* with periodontal health.[84,87] The parallel fluctuations of *Streptococci* and *Veillonella* levels are not surprising because *Veillonella* utilizes short-chain acids such as lactates that are secreted by gram-positive facultatives such as streptococci,[88] and it has been shown that *veillonella* will not colonize tooth surfaces without streptococci.[89]

The microbial profile of periodontal health also included the less abundant genera *Campylobacter*, *Abiotrophia*, *Capnocytophaga*, *Gemella*, and *Neisseria*. This confirms earlier studies linking *Capnocytophaga*[84,87,90] and *Campylobacter gracilis*[91] to health.

Levels of the genera *Streptococcus* and *Veillonella* were more similar between shallow and deep sites in individuals with periodontitis than between healthy individuals and those with periodontitis. A similar phenomenon was observed for many health- and

disease-associated species (Table 1) - many more differences were observed between healthy and diseased subjects than were found between shallow and deep sites in individuals with disease. Kumar *et al.* (2005) concluded that disease may involve a disruption in the microbial ecology of the entire dentition. Furthermore, transitions between health and chronic periodontitis may be associated with shifts in the relative proportions of major bacteria.

The elucidation of microbial shifts associated with disease onset and progression has obvious clinical implications, but the discovery of species associated with stable, healthy ecosystems may be equally or more important in the development of therapies for periodontitis. For example, colonization stability has been studied in the gastrointestinal tract, where colonization with normal flora decreases susceptibility to infections by exogenous pathogens such as *Salmonella* or *Clostridium* spp.[92,93]. These studies have contributed to the clinical management of gastrointestinal infections using probiotics or microbial replacement therapy.[94]

Kumar *et al.* (2006) conducted a follow-up study to examine the stability of bacterial colonization in the gingival crevice and to identify microbial shifts associated with changes in periodontal health, which allows the examination of the relationship between changes in health status and changes in the predominant subgingival flora, for both cultivated and uncultivated species.[95] Subjects whose clinical status worsened often showed conservation of less than half the bacterial community, and in one extreme case only 16% of the flora was conserved, but no subject exhibited complete replacement of subgingival bacteria during the 2-year observation period.[95]

No significant relationship was observed between the total number of species present or clinical stability or disease status, although a much larger range was observed among subjects whose periodontal health worsened.[95] This does not lend support to the model, based on cultivation, that a healthy periodontium is associated with flora of low complexity whereas more diverse flora are seen in disease. [19,31,96] However, Kumar *et al.* (2006) noted that only 100 clones from each sample were identified, but by no means were all species present in the samples.[95]

Altogether, these findings suggest that changes in periodontal health are accompanied by shifts within oral bacterial communities, as well as the corollary that a stable clinical state is reflected in a stable oral microbiota. An understanding of this relationship could potentially be useful for the development of diagnostic and prognostic tests for periodontitis. Understanding of the triggers for these microbial shifts will be important for the development of therapeutic approaches. [95]

Veillonella sp. oral clone X042 was the most common bacterium detected overall, accounting for 9.5% of all clones. Levels of this phylotype increased when periodontal health improved and decreased when the clinical status worsened.[95] This phylotype was the most prevalent bacterium in a previous study using quantitative clonal analysis as well, and higher levels were significantly associated with health in that study[64]. This strong association with health suggests that monitoring of the levels and changes in levels of this phylotype may prove clinically useful. Exploration of the role of this apparently beneficial phylotype in the plaque biofilm could also have important implications for microbial replacement therapy or probiotics. In other infectious diseases, a shift from health to disease is associated not only with an increase in levels

of pathogenic species but also with a decrease in levels of protective species.[94] When large numbers of resident bacteria saturate an ecological niche, they create resistance to colonization by exogenous pathogens. This “colonization resistance” conferred by the presence of beneficial species is considered an important barrier function of gut commensals and may be important in the gingival crevice as well.

The purpose of our study was to track for the first time the changes in subgingival bacterial composition in individual tooth sites before and during the first three months of orthodontic treatment in patients undergoing traditional fixed braces using 16S rRNA NGS. Previous studies of oral microbiomes in orthodontic patients were limited to what was cultivable using Sanger sequencing, but with 16S rRNA NGS it is possible to look at the full microbiome and more accurately report the shifts in microbiome during the first 3 months of orthodontic treatment. Furthermore, each sample was not pooled but analyzed separately to observe site-specific changes.

MATERIALS AND METHODS

Subjects

Seventeen subjects (9 Females and 8 Males; Age 12.1 -29.3 years) were selected among patients who arrived for orthodontic treatment at the Department of Orthodontics, University of California San Francisco. Nine bracketed and two invisalign patients completed the 12-week study. Five control subjects also completed the 12-week study. The study design was approved by the UCSF Ethics Committee (IRB 15-17868). Subjects were enrolled according to the following criteria: (1) no known systemic disease; (2) no use of antimicrobial, antifungal or anti-inflammatory drugs within 3 months before the baseline examination; (3) no craniofacial anomalies; (4) not-pregnant; and (5) would be undergoing treatment with fixed orthodontic appliances in the mandibular arch. Five subjects (4 female and 1 male) not undergoing orthodontic treatment were selected as a control group.

Orthodontic bonding

All subjects received oral hygiene instructions by the same clinician in the form of both verbal instructions and an instructional video by Aquarium 3 (Dolphin Imaging & Management Solutions). Subjects were instructed not to immediately brush prior to their appointment, but to brush as they would routinely do.

All subjects received orthodontic therapy with fixed buccal appliances. The teeth were prepared etched and primed with Transbond Plus Self Etching Primer (3M Unitek), then metal brackets (3M Unitek, GAC, Opal or GAC innovation) were bonded directly with Transbond XT Light Cure Adhesive Paste (3M Unitek) onto incisors and premolars.

Bands (Unitek and GAC) were cemented with polyacid-modified composite resin (Ultra Band-Lok) onto molars. The arch wires were tied using o-chains (RMO) and in one case self-ligated. Six patients failed to complete the entire study.

Collection of subgingival microbiota

Subgingival crevicular fluid samples were collected from the straight buccal gingival crevice of the lower right first molar (Tooth 1), lower right central incisor (Tooth 2), lower left first premolar (Tooth 3) and the straight lingual of lower left first premolar (Tooth 4). In 2 cases, the lower left first premolar was extracted for treatment, in those cases the lower left second premolar was sampled instead.

Samples were collected at three different time points: before appliance placement (tp1), 6 weeks (tp2) and 12 weeks (tp3) into treatment. Samples of the control group not undergoing orthodontic treatment were also collected at three different time points. Sampling sites were isolated using High Heat Cheek Retractors (Ortho Technology) and the tongue held back with a mouth mirror when sampling for Tooth 4. Then two sterile absorbent paper points size 30 (META) were inserted into the gingival crevice and were left in situ for 60 seconds. These paper points were transferred immediately into Eppendorf tubes containing 250 mL of buffer and frozen at -80°C.

Periodontal Assessment

Immediately after sampling the sites, the probing depths (PD), GM-CEJ measurement, clinical attachment loss (CAL), and bleeding on probing (BOP) were measured and recorded in Axium periodontal charting system for all three time points. A sterilized periodontal probe was used to measurement PD and GM-CEJ. BOP was

observed and classified as present or not present after the periodontal measurements. All samplings and measurements were performed by the same clinician. Once all the samples were collected, they were sent to Jackson Laboratory in Farmington, CT for 16S rRNA gene sequencing and analysis.

16S rRNA gene sequencing

Metagenomic DNA from the sampled paper points will be extracted using a PowerSoil ® DNA Isolation Kit. 16S V1-3 regions from the metagenomic DNA will be amplified using primers 27F and 534R (27F:5'-AGAGTTTGATCCTGGCTCAG-3' and 534R: 5'- ATTACCGCGGCTGCTGG-3'). Sequencing reads will be processed by removing the sequences with low quality (average qual <35) and ambiguous nucleotides (N's). Chimeric amplicons will be removed using UChime software. The processed reads will be used for OTU generation by our automated pipeline. Each OTU will be classified from phylum to genus level using the most updated RDP classifier and training set. A taxonomic abundance table will be generated with each row as bacterial taxonomic classification, each column as sample ID and each field with taxonomic abundance. The abundance of a given taxon in a sample will present as relative abundance (the read counts from a given taxon divided by total reads in the sample).

Statistical Analysis

Each sample was subsampled to the lowest number of read counts among samples in the dataset and rarefied to 5000 read counts. The abundance of a taxon in a sample was indicated as the relative abundance, which was calculated by dividing the

number of reads for a taxon by the total read counts of the sample. Alpha diversity indices including richness and Shannon diversity were calculated. To examine difference between control and bracket samples, we performed non-metric multidimensial scaling (NMDS) plots with Bray-curtis distance. Microbiome stability in both groups was determined by calculating the Bray-curtis dissimilarity from the same samples across different time points. For sequencing data, differences in the relative abundances of taxa between the groups were determined with the Mann-Whitney test. A p-value ≤0.05 was considered significant. Differences in Bray Curtis distances between the groups were analyzed with analysis of variance (adonis). A p-value ≤0.05 was considered statistically significant. Differences in richness and Shannon diversity were analyzed with student's t-test. A p-value ≤0.05 was considered statistically significant.

RESULTS

Description of metadata and sequence data

A total of 16 subjects completed the study. Subgingival samples were obtained from 9 bracket subjects, 5 control subjects, and 2 clear aligner subjects at three timepoints (tp1 = 0 day, tp2 = 6 weeks, tp3 = 12 weeks) and at four different sites in the mouth (i.e., tooth1 = buccal of lower right 1st molar (30B), tooth2 = buccal of lower right central incisor (25B), tooth3 = buccal of lower left 1st premolar (21B), and tooth 4 = lingual of lower left 1st premolar (21L). The average ages for each group were: control group, 30 years [range 25.2-33.5]; bracket group, 15 years [range 12.1-16.9]; and clear aligner group, 23 years [range 17.1-29.3].

We performed clinical exams to check for signs of gingivitis by measuring gingival pocket depths, clinical attachment loss, plaque, and bleeding on probing. In the control group, no subjects showed signs of gingivitis but one subject developed gingivitis at tp2 and tp3. In the bracket group, one subject showed gingivitis at tp1, 8 at tp2 and 9 at tp3. (Figure 2A) In the invisalign group, both subjects showed gingivitis at tp1, but none at tp2 and tp3.

The variable regions 1 and 3 (V1-V3) of 16S rRNA were sequenced and generated a total of 4,784,134 after quality filtering, with an average of 24,021 ± 11,065 (range: 3 to 64,486) sequences per sample (Figure 2B). Amongst 192 samples (i.e., 16 subjects x 4 tooth locations x 3 tp), we identified 420 unique operational taxonomic units (OTUs), which represents 128 genera. All analyses were done with rarefied data at 9,367 sequences, which excluded 17 samples due to low sequence counts.

Inter-subject variation

Samples from each subject at baseline were analyzed at the genus level. The top 15 most abundant genera collectively made up on average 91.5 ± 7.8% of each sample taken from baseline. There was high inter-subject variation as demonstrated by the large ranges of standard deviations in Shannon diversity (Tooth1: 1.22 ± 0.58, Tooth2: 1.66 ± 0.63, Tooth3: 1.27 ± 0.65, Tooth4: 2.01 ± 0.48) (Figure 3A) and Bray Curtis dissimilarity (Tooth1: 0.56 ± 0.21, Tooth2: 0.66 ± 0.19, Tooth3: 0.60 ± 0.19, Tooth4: 0.70 ± 0.41) (Figure 3B). The relative abundance of the top 15 bacterial species from subjects whose samples resulted in sequencing data showed large variation (Figure 3C).

Different sites in the mouth harbor different microbiomes

Tooth site differences were analyzed using non-metric multidimensional scaling (NMDS) plots of the Bray Curtis values and were calculated based upon relative abundance of bacterial species in all sample at the OTU level (Figure 4A). There was clear cluster separation in 3 out of the 4 tooth sites with the exception of tooth1 (red, 30B) and tooth3 (blue, 21B) seeming to cluster similarly. Adonis testing of site differences was significant, with a p-value at 0.004. Interestingly, the microbiomes identified for tooth3 (21B) and tooth4 (21L) were significantly different indicating distinct bacterial communities even though the samples were collected from the same tooth albeit different locations (Figure 4A).

Similarity of microbiomes after orthodontic bracket intervention

Before delivery of orthodontic brackets at tp1, distinct clusters of samples were observed at each tooth site that were confirmed to be significantly different by adonis testing (p = 0.041) (Figure 4B). Upon delivery of orthodontic brackets at 6 weeks (i.e., tp2), we observed clusters that tended to overlap suggesting that the microbiomes at various tooth sites became increasingly similar with bracket treatment. At 12 weeks after bracket delivery (i.e., tp3), the trend in increased similarity between tooth sites remained with the exception of tooth4, which was interestingly the only site where samples were collected from the lingual side. The increasing similarity of oral microbiomes after bracket intervention was supported by the increase in the number of OTUs shared among the different tooth sites at tp2 and tp3 as observed from Venn diagrams – at tp1, there were fewer shared OTUs (i.e., 136) compared to shared OTUs at tp2 (i.e, 217) and tp3 (i.e, 220).

Changes in microbiomes with orthodontic bracket intervention

NMDS plots of Bray Curtis values based on timepoints showed that samples at tp1 housed similar microbiomes compared to control group samples (Figure 5A). Although adonis testing showed significant differences, this was likely due to higher dispersion of the bracket group samples compared to control group samples. The microbiomes of the bracket group were altered after bracket delivery (i.e., tp2 and tp3 vs. tp1) as suggested by the bracket clusters migrating towards the left (Figure 5A). These differences were significant as tested by adonis (p-value = 0.001 and 0.002). The control samples appeared to house similar bacterial communities over 12 weeks (i.e.,

tp1, tp2, tp3), as expected. When comparing the dissimilarities of samples from the same subject between timepoints, the bracket subjects overall had significantly higher dissimilarities between tp1 and tp2 (p-value < 0.01) and between tp1 and tp3 (p-value < 0.01) when compared to control subjects using Mann-Whitney testing (Figure 5B). Taken together, our data suggest that microbiomes at tp1 are significantly altered at tp2 and tp3.

Alpha diversity: Richness & Shannon diversity

Richness, as calculated by the number of OTUs present in a sample, were similar in control subjects at tp1, tp2, tp3, as well as bracket subjects at tp1 (Figure 5B). At tp2 and tp3, richness was overall significantly higher in the bracket group and there were significant differences between specific teeth/sites. Our data demonstrate an increase in the number of different bacterial species 6 weeks after bracket intervention, and these changes appeared to be maintained at 12 weeks (Figure 5B).

The Shannon diversity plot, a measure of diversity in a sample that takes into consideration richness and evenness, showed trends similar to richness suggesting an increase in bacterial species but the relative abundances of these species were low after bracket intervention - bracket samples showed significant increases in Shannon diversity at tp2 and tp3 when compared to control samples (Figure 5B).

Taxa bar plot

The relative abundance of the top 15 most abundant OTUs showed an overall decrease after bracket intervention at 6 weeks especially in Tooth1 and Tooth2 (Figure

5C). Tooth3 showed changes in the relative abundance values of the top 15 OTUs after intervention but the overall relative abundance stayed at similar baseline values. Tooth 1, 2, and 3 had the biggest change in the relative abundance of the most abundant OTU: OTU_1: Streptococcus mitis, which was decreased from tp1 to tp2. Interestingly, the microbiomes in Tooth4 where specimens were collected from the lingual surface did not change with bracket treatment. Over 150 OTUs were considered "core" in each of the 4 tooth sites in the bracket group (Figure 5D). More OTUs were common at the various sites between tp2 and tp3 than with tp1.

Significant OTU differences

We determined specific OTUs that were significantly different between the control and bracket groups using Mann-Whitney testing (Figure 5E). Many OTUs were identified to be significantly different between control and bracket samples at tp2 and tp3 (Figure 5E, left panel). However, there were no significantly different OTUs after false discovery rate (FDR) correction.

We also determined the OTUs that were significantly different within the bracket group at the three timepoints (Figure 5E, right panel). Not surprisingly, more OTUs were significantly different between tp1 and tp2 (tp1 v 2), and tp1 and tp3 (tp1 v 3) than between tp2 and tp3 (tp2 v 3). However, after FDR correction, none of these OTUs were significant. We identified 10 OTUs that were significantly and consistently different from baseline across multiple sites (Figure 5F).

DISCUSSION

Changes in the oral microbiome during orthodontic treatment has been reported previously but these studies have been limited due to the use of culture systems to expand bacterial species[50], candidate gene targeted approaches to identify specific bacteria[47], and the use of Sanger sequencing[13]. To our knowledge, our study is the first to globally profile the oral microbiome longitudinally in orthodontic patients without culture expansion and utilizing NGS. Subjects were either treated with full-fixed orthodontic appliances (BRACKET group) or received no treatment (CONTROL group). BRACKET subjects received orthodontic bands on their molars and brackets on the buccal crown surface of all remaining teeth. We evaluated changes in subgingival microbiomes at 4 distinct sites in each BRACKET subject before and after delivery of fixed orthodontic appliance over a period of 3 months. We collected samples from the subgingiva of mandibular teeth - the 1st molar (Tooth1), central incisor (Tooth2), and 1st premolar (Tooth3 and Tooth4). All samples were collected from the buccal side with the exception of Tooth4, where samples were collected from the lingual side. Samples were collected in CONTROL subjects at the same sites and times.

The placement of orthodontic bands and brackets on teeth has previously shown to increase supragingival plaque accumulation.[29,37] Prolonged accumulation of supragingival plaque leads to alterations in the subgingival microbiome[97]. Additionally, orthodontic leveling and aligning displaces teeth and all intrusive or tipping forces will re-locate supragingival plaque into the gingival pocket and alter bacterial equilibrium.[98] Furthermore, plaque accumulation has been shown to lead to gingival inflammation and

gingivitis.[58] A large majority of BRACKET subjects exhibited bleeding on probing (BOP) after 6 and 12 weeks of appliance placement (Figure 2), an indication of gingivitis or periodontits, whereas only 1 CONTROL subject exhibited bleeding on probing. The presence of orthodontic bands and brackets makes oral hygiene more challenging. However, one of the subjects that exhibited BOP at timepoint 2 did not show BOP at tp3 demonstrating an improvement of their oral hygiene, and the reason why orthodontic treatment may not be contraindicated for subjects with severe periodontal disease.[99]

At tp1, there was high inter-subject variation as demonstrated by the large range of standard deviations in Shannon diversity (Figure 3A) and Bray Curtis dissimilarity (Figure 3B) analyses. Non-metric multidimensional scaling (NMDS) plots of the Bray Curtis values in all tooth sites (i.e., CONTROL and BRACKET) at tp1 showed significantly different and distinct clusters of three out of four sites, with overlapping clusters or similarity between Tooth1 and Tooth3, both of which are posterior teeth (Figure 4A). Interestingly, Tooth3 and Tooth4 appeared to be significantly different even though specimens were collected from the same tooth albeit different surfaces (buccal vs. lingual). This may be due to mechanical disruptions of the supragingival plaque by the tongue or because brackets were bonded to the buccal surface. Regardless, our data highlights the vast differences in microbiomes at each tooth site and subject. These site-specific differences are also underscored by differences in microbiomes at tp1 in CONTROL and BRACKET specimens (Figures 5A, 5C). The high site-specific variations in microbiomes, which were also reported previously, led to our longitudinal experiment design where tp1 would serve as the baseline microbiome for each subject. Thus, although comparing CONTROL and BRACKET subjects resulted in some

interesting results (discussed below), high site- and subject-specific variability of microbiomes forces us to compare within the same subjects and sites over time.

We observed large changes in the microbiome with bracket intervention. At tp1 the microbiomes were significantly different (p=0.041) but at 6 weeks and 12 weeks after intervention, the clusters were more overlapped underscored by p values of 0.347 and 0.339 at tp2 and tp3 significantly (Figure 4B). This demonstrates that the microbiomes became more uniform with brackets. This uniformity of microbiomes after bracket intervention was supported by an increase in the number of shared OTUs among the different tooth sites after bracket intervention as shown in the Venn diagrams (Figure 4C).

Although bracket specimens tended to become more uniform after bracket intervention, microbiomes at tp2 and tp3 were significantly different from control samples as tested by adonis (Figure 5A). Increased uniformity at tp2 and tp3 in bracket specimens was also demonstrated with increases in shared OTUs at tp2 and tp3 compared to tp1 at every tooth site (Figure 5D).

Richness and Shannon diversity analysis revealed that Shannon diversity had trends similar to richness but were less significant. At tp2 and tp3, richness was overall significantly higher in the bracket group showing an increase in the number of different species at 6-12 weeks after intervention (Figure 5B). Thus, orthodontic brackets appear to play a large role in altering the composition of subgingival microbiomes. This suggests that maintenance of good oral hygiene during orthodontic treatment may not be the biggest factor in subjects developing gingivitis and periodontal disease.

A number of OTUs were identified (Figure 5F). The top two OTUs were OTU_18: Candidatus Saccharibacteria and OTU_117: Selenomonas sputigena, the latter previously having been associated in periodontal diseases[100]. Candidatus Saccharibacteria is a major lineage of the Bacteria domain, the existence of which until recently was only known through environmental 16S rRNA sequencing as no species had been grown in the lab, a requirement for taxonomy.[101] We also observed significantly increased levels of Megasphaera, Selenomonas, Dialister, Atopobium, Treponema in multiple sites, these were previously reported to be elevated in disease sites.[64] We observed Prevotella denticola and Prevotella oulorum to be significantly increased but not Prevotella intermedia and Prevotella nigrescens as previous studies have found.[18] Actinomyces was elevated as previous studies have shown.[60] These newly identified candidates outnumbered *Porphyromonas gingivalis* and other species previously implicated as periodontopathogens, and it is not clear if newly identified and more numerous species may play a more important role in pathogenesis.

Presently, no new treatment modalities can be concluded from this study, however a clearer understanding of the microbial shift during orthodontic treatment may one day prevent periopathogenic bacteria from successfully colonizing subgingivally and instead strengthen and maintain a more robust healthy microbiome. The use of 16S rRNA NGS to study the shifts in microbiome is an improvement to previous methods. Future studies should continue using this method, secure larger sample sizes of both braces and clear aligners, and continue sampling the patient until the completion of treatment and during retention.

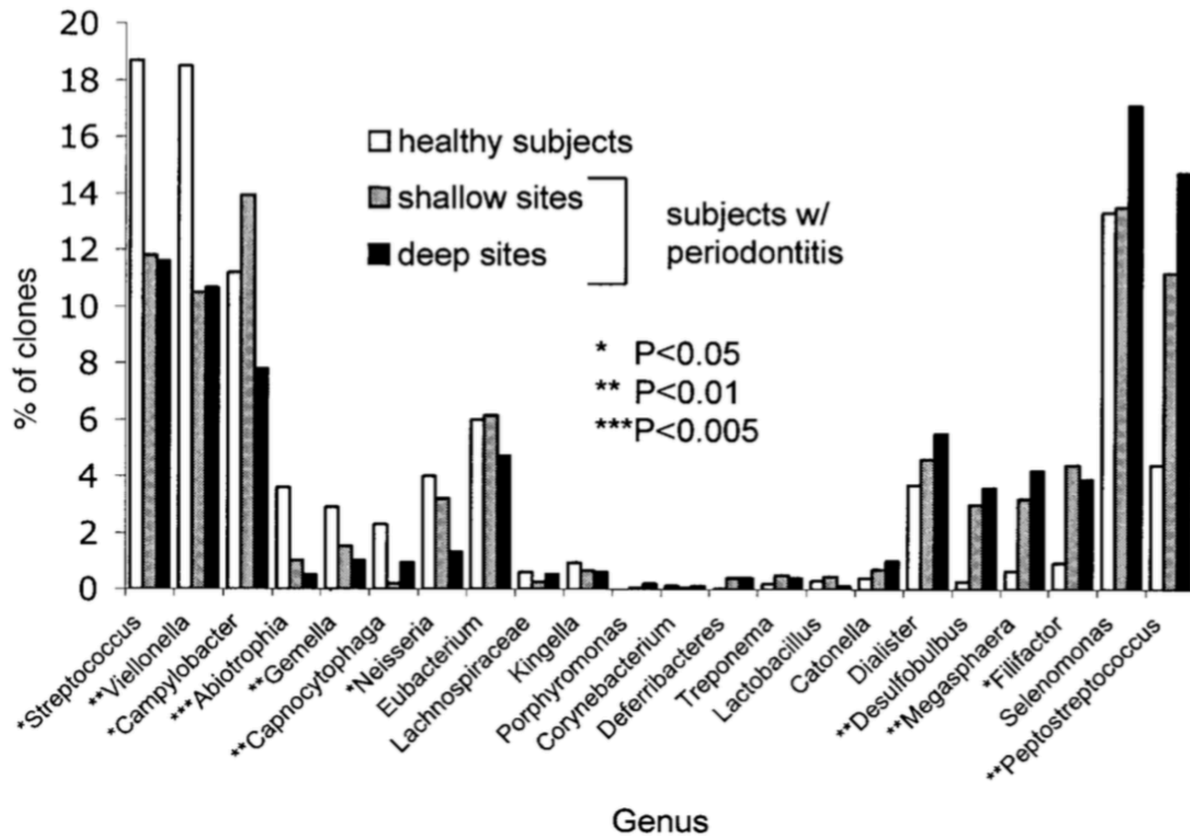


Figure 1. Distribution by health status for genera accounting for >0.025% of total bacteria are shown. The genera are arranged in a gradient from those predominant in health shown on the left to those predominant in periodontitis shown on the right.

TABLE 1. Species and phylotypes significantly associated with disease and health ($P < 0.1$) [2]

Clinical status and overall rank	Species and/or phylotype	*P*		
		Levels		Presence (between subjects[c])
		Between sites[a]	Between subjects[b]	
Disease				
6	*Peptostreptococcus* sp. oral clone BS044		0.07	
7	*Filifactor alocis*		0.04	
15	*Peptostreptococcus* sp. oral clone CK035	0.06	0.05	0.06
16	*Megasphaera* sp. oral clone BB166		0.01	0.009
17	*Desulfobulbus* sp. oral clone CH031		0.03	
19	*Dialister pneumosintes*		0.01	0.002
20	*Campylobacter sputorum sputorum*		0.008	0.06
24	*Desulfobulbus* sp. oral clone R004		0.006	0.001
27	*Campylobacter* sp. oral clone BB120		0.03	0.008
36	*Selenomonas* sp. oral clone D0042		0.01	0.002
50	*Deferribacteres* sp. oral clone W090		0.03	0.008
53	*Megasphaera* sp. oral clone MCE3_141		0.003	0.0003
54	*Selenomonas* sp. oral clone EY047	0.031		
90	*Dialister* sp. oral clone MCE7_134		0.06	0.01
100	*Catonella* sp. oral clone BR063		0.01	0.002
118	*Tannerella forsythia* (*Bacteroides forsythus*)			0.03
119	*Deferribacteres* sp. oral clone BH007		0.01	
123	*Streptococcus* sp. oral strain 9F		0.05	0.01
129	*Atopobium* sp. oral clone C019			0.03
140	*Peptostreptococcus anaerobius*			0.03
151	*Eubacterium* sp. oral strain A35MT			0.04
153	*Megasphaera* sp. oral clone BS073		0.01	0.002
154	*Selenomonas flueggei*-like sp. clone AH132			0.03
165	*Treponema* sp. strain 6:H:D15A-4			0.04
172	*Streptococcus* sp. oral clone DP009		0.04	0.008
Health				
1	*Veillonella* sp. oral clone X042		0.0008	
2	*Campylobacter gracilis*	0.04	0.02	
21	*Abiotrophia adiacens*		0.003	0.007
31	*Eubacterium saburreum*		0.0009	0.005
38	*Campylobacter showae*		0.05	0.02
42	*Gemella* sp. strain 1754-94		0.009	0.002
44	*Streptococcus sanguis*		0.01	0.002
49	*Capnocytophaga gingivalis*		0.02	0.05
81	*Streptococcus mutans*		0.02	0.003
133	*Abiotrophia* sp. oral clone P4PA_155 P1			0.03
145	*Rothia dentocariosa*			0.03
150	*Eubacterium* sp. oral clone OH3A			0.04
155	*Selenomonas* sp. oral clone DS051			0.03

[a] Comparison of levels between deep and shallow sites in subjects with periodontitis by Wilcoxon signed-rank test.
[b] Comparison of levels between healthy and subjects with periodontitis by Kruskal-Wallis analysis of variance.
[c] Comparison of presence or absence of species by chi-square test.

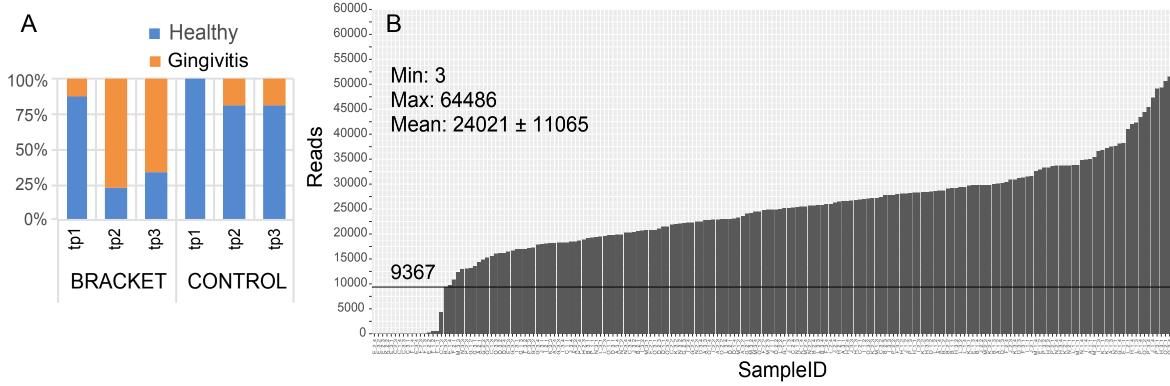


Figure 2. A. Periodontal Status. B. Read Distribution. 16S rRNA sequencing. Mean: 24021 ± 11065. Range: 3 – 64486. All samples were rarefied at 9367 for analysis, which excluded 17 samples.

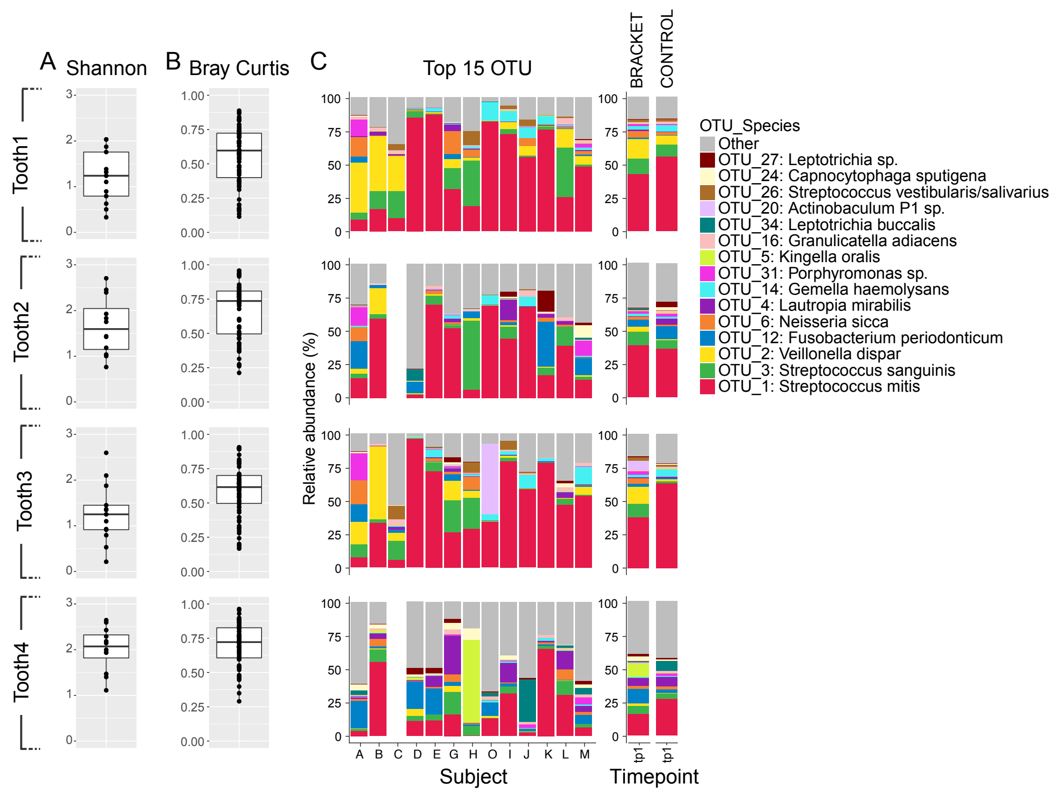


Figure 3. Inter-subject variation. A. Shannon diversity box plots. B. Bray Curtis dissimilarity box plots. C. Relative abundance bar plots of the top 15 most abundant species of all subjects at Timepoint 1. Gray represents “Other”, aggregate of all other genus.

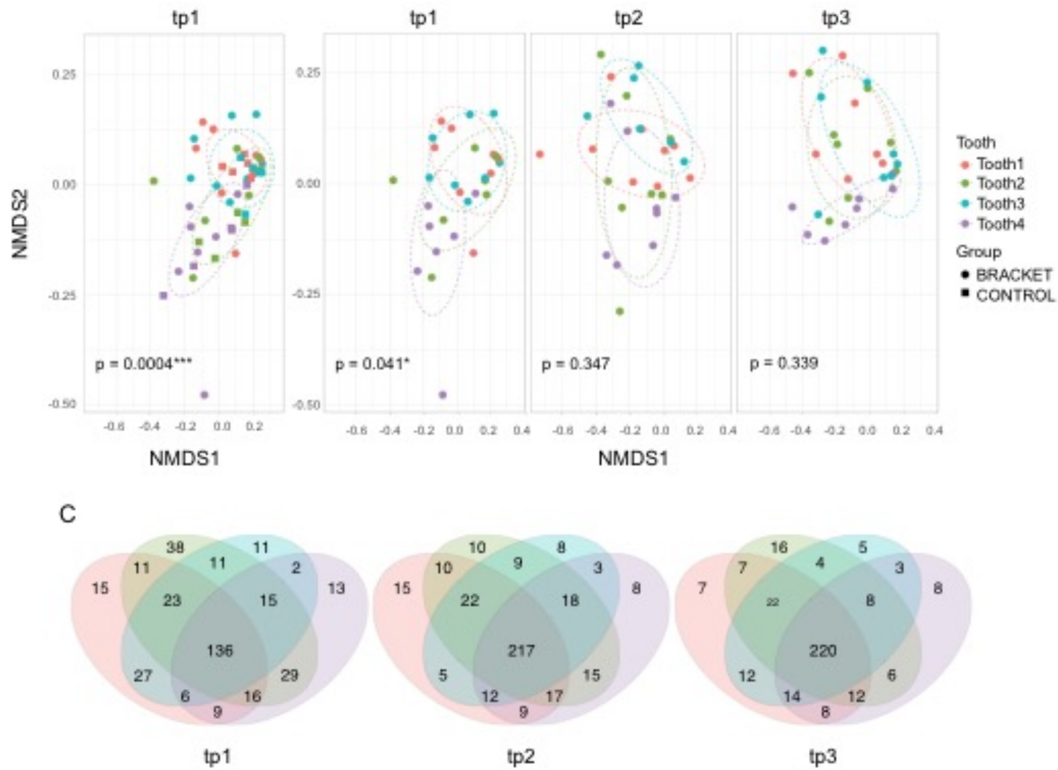


Figure 4. Tooth site differences. A. Non-metric multidimensional scaling (NMDS) plots of all samples in Timepoint 1. Bray-Curtis dissimilarity index was calculated based on relative abundances in each sample at the OTU level. Colors by Tooth site, shapes by Group. Significant tooth sites differences determined by adonis testing. Ellipses represent one standard deviation from the center. B. NMDS plot of Bracket samples over time. Colors by Tooth site, shapes by Group. Significant tooth sites differences determined by adonis testing. Ellipses represent one standard deviation from the center. C. Venn diagrams of OTUs shared and unique among each tooth site over time.

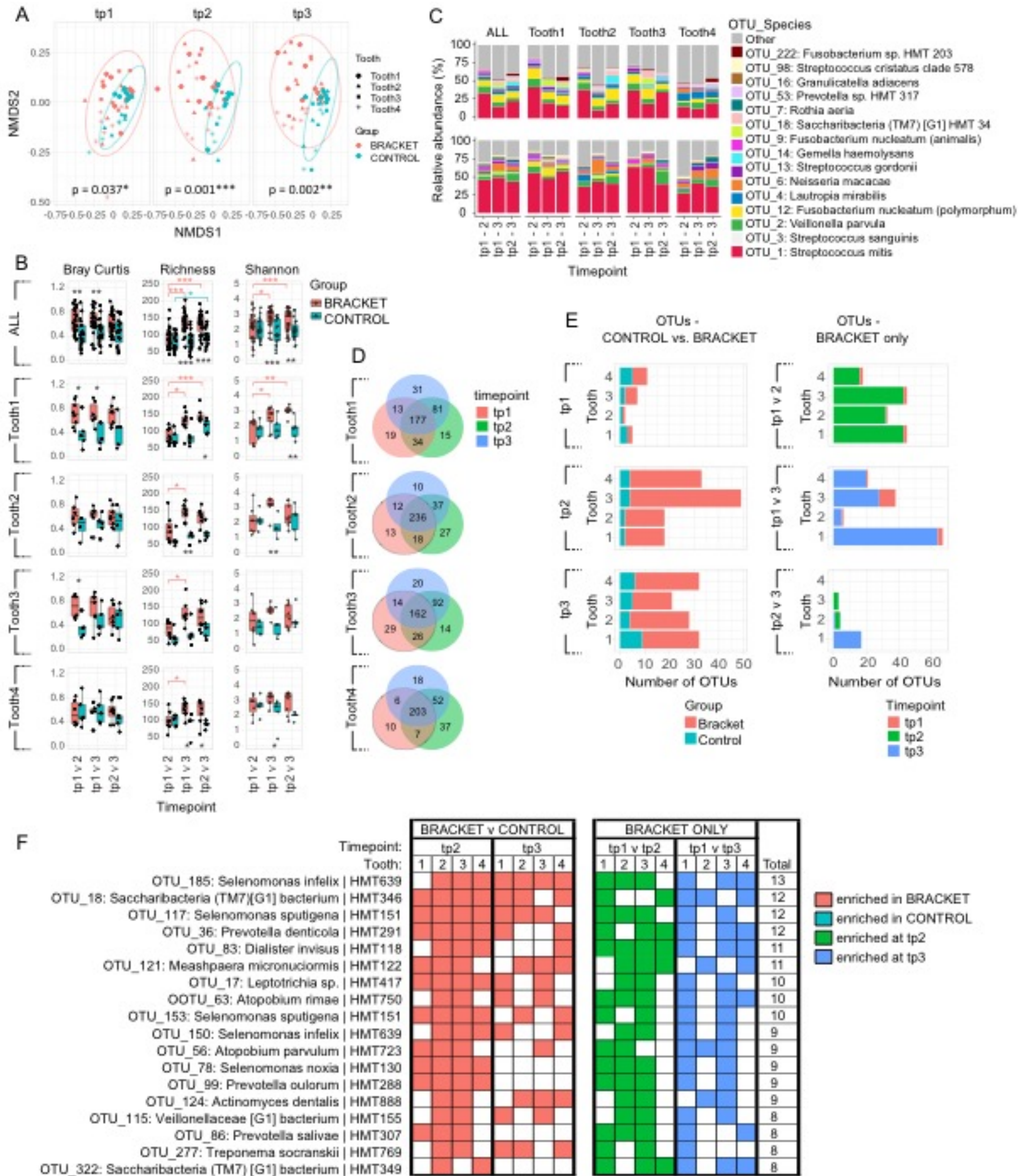
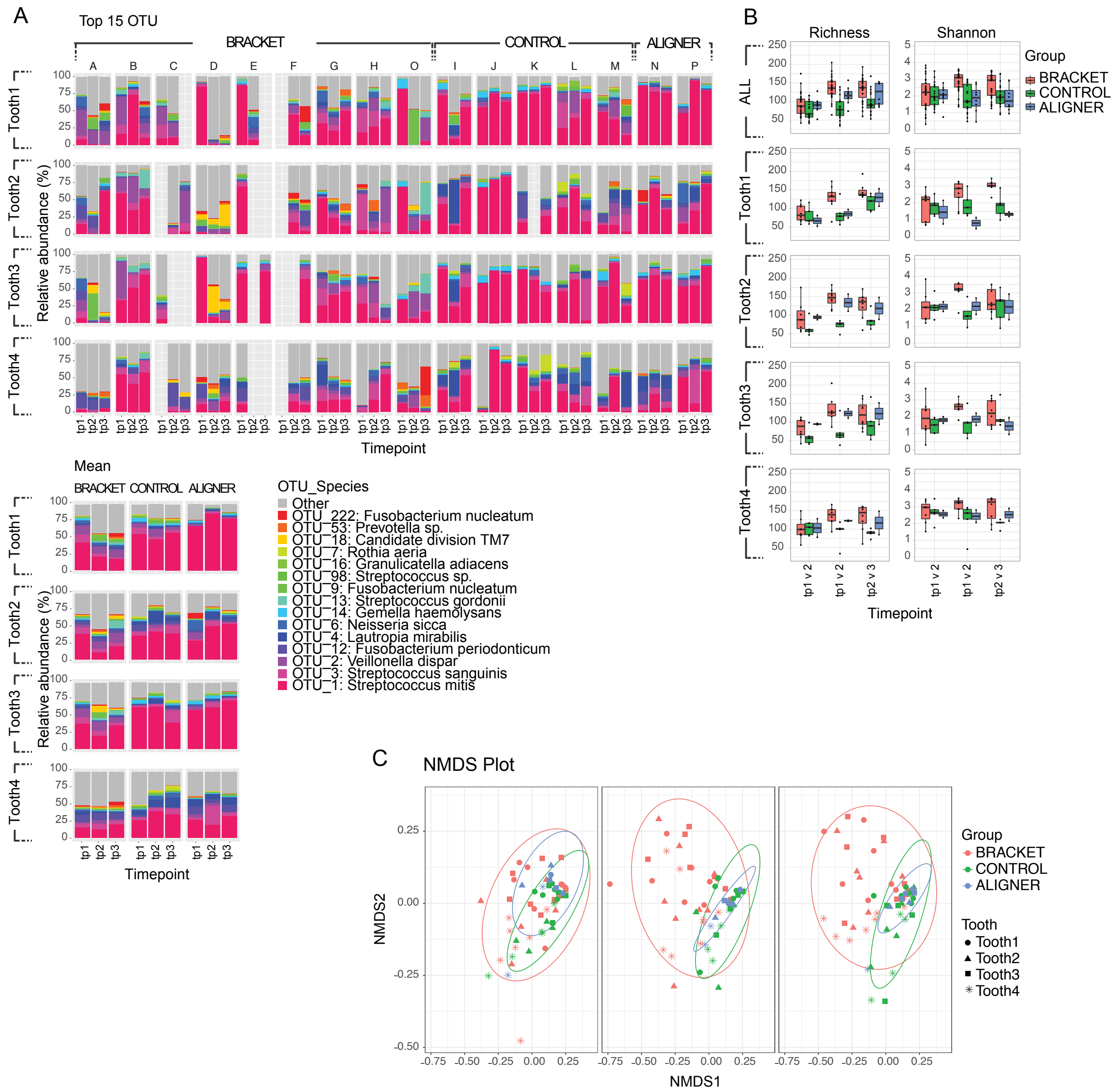


Figure 5**.** Intervention differences. A. Non-metric multidimensional scaling (NMDS) plots of all samples in tp1 over time based on Bray-Curtis dissimilarity index. Colors by Group, Shapes by site. Ellipses represent 95% confidence level. B. Bray Curtis dissimilarity, Richness, and Shannon diversity of samples over time, aggregated by group and separated by tooth site. Colors by Group. Significance by Mann-Whitney testing. Significance: red = between bracket samples, blue = between control samples, purple = bracket vs. control samples. C. Taxa bar plots of top 15 most abundant OTUs over time, separated by site. Gray represents all other OTUs. D. Venn diagrams of shared and unique OTUs between timepoints separated by site. E. Number of significantly different OTUs at each site and timepoint. F. OTUs that are significantly different at multiple sites.



Supplemental Figure 1.

Supplemental Table 1.

Patient	AGE (y.m)	SEX	Tooth	Bracket type	Pocket Depth	Clinical Attachment Loss	Bleeding On Probing	Plaque
1	13.4	Female	1	Unitek 018	2	2	no	yes
	13.4	Female	2		2	2	no	yes
	13.4	Female	3		2	1	no	yes
	13.4	Female	4		2	1	no	yes
	13.5	Female	1		2	2	yes	yes
	13.7	Female	2		2	1	no	no
	13.7	Female	3		3	2	yes	yes
	13.7	Female	4		2	1	yes	yes
	13.7	Female	1		4	4	yes	yes
	13.8	Female	2		2	1	no	no
	13.8	Female	3		3	2	yes	yes
	13.8	Female	4		3	2	yes	yes
2	14.2	Female	1	GAC 022	2	1	no	no
	14.2	Female	2		2	1	no	no
	14.2	Female	3		2	3	no	no
	14.2	Female	4		2	1	no	no
	14.5	Female	1		2	2	no	yes
	14.11	Female	2		1	0	no	yes
	14.11	Female	3		2	1	no	yes
	14.11	Female	4		3	2	yes	yes
	14.6	Female	1		3	3	yes	yes
	15	Female	2		1	0	no	no
	15	Female	3		2	1	no	no
	15	Female	4		2	1	no	no
3	16	Female	1	Opal 022	2	3	no	no
	16	Female	2		2	2	yes	no
	16	Female	3		2	3	no	no
	16	Female	4		2	1	no	no
	16.2	Female	1		2	3	yes	no
	16.3	Female	2		1	0	yes	yes
	16.3	Female	3		2	1	no	no
	16.3	Female	4		1	0	no	no
	16.3	Female	1		4	3	no	no
	16.5	Female	2		1	0	no	no
	16.5	Female	3		2	1	no	yes
	16.5	Female	4		2	1	no	yes
	12.1	Male	1		2	1	no	no
	12.1	Male	2		2	1	no	yes
	12.1	Male	3		2	1	no	no

Patient	AGE (y.m)	SEX	Tooth	Bracket type	Pocket Depth	Clinical Attachment Loss	Bleeding On Probing	Plaque
4	12.1	Male	4	GAC 022	2	1	no	no
	12.11	Male	1		3	2	yes	yes
	12.11	Male	2		2	1	yes	yes
	12.11	Male	3		3	2	no	no
	12.11	Male	4		3	2	no	yes
	13	Male	1		3	2	yes	yes
	13	Male	2		2	1	yes	yes
	13	Male	3		4	2	yes	yes
	13	Male	4		3	2	no	yes
5	13.11	Female	1	Unitek 018	2	2	no	no
	13.11	Female	2		2	1	no	yes
	13.11	Female	3		2	1	no	no
	13.11	Female	4		2	1	no	no
	14	Female	1		3	2	no	yes
	14	Female	2		1	0	no	yes
	14	Female	3		3	1	no	no
	14	Female	4		3	1	no	no
	14.1	Female	1		3	2	no	yes
	14.1	Female	2		1	0	no	yes
	14.1	Female	3		2	1	no	no
	14.1	Female	4		3	1	no	no
6	13.5	Male	1	Unitek 018	1	0	no	no
	13.5	Male	2		1	1	yes	yes
	13.5	Male	3		1	0	no	no
	13.5	Male	4		1	0	no	no
	13.6	Male	1		3	2	no	yes
	13.6	Male	2		1	0	yes	yes
	13.6	Male	3		3	2	no	yes
	13.6	Male	4		3	2	no	yes
	13.8	Male	1		2	2	no	yes
	13.8	Male	2		1	1	yes	yes
	13.8	Male	3		2	1	yes	yes
	13.8	Male	4		2	1	yes	yes
7	16.3	Male	1	Unitek 018	3	3	no	yes
	16.3	Male	2		2	1	no	yes
	16.3	Male	3		2	1	no	yes
	16.3	Male	4		2	1	no	yes
	16.5	Male	1		4	4	yes	yes
	16.5	Male	2		2	1	yes	yes

Patient	AGE (y.m)	SEX	Tooth	Bracket type	Pocket Depth	Clinical Attachment Loss	Bleeding On Probing	Plaque
7	16.5	Male	3	Unitek 018	2	1	yes	yes
	16.5	Male	4		2	1	no	yes
	16.6	Male	1		4	4	No	Yes
	16.6	Male	2		2	1	No	No
	16.6	Male	3		3	2	Yes	Yes
	16.6	Male	4		2	1	Yes	Yes
	16.5	Male	1		2	2	No	No
	16.5	Male	2		1	1	Yes	Yes
	16.5	Male	3		2	1	No	No
	16.5	Male	4		2	1	No	No
	16.7	Male	1		3	3	No	Yes
8	16.7	Male	2	GAC Innovation 022	1	1	Yes	Yes
	16.7	Male	3		2	1	Yes	Yes
	16.7	Male	4		2	1	No	Yes
	16.9	Male	1		2	1	No	No
	16.9	Male	2		1	0	Yes	No
	16.9	Male	3		2	1	No	Yes
	16.9	Male	4		2	1	No	No
	16	Female	1		2	1	No	No
	16	Female	2		2	1	No	No
	16	Female	3		2	1	No	No
	16	Female	4		2	1	No	No
	16.2	Female	1		3	3	No	No
9	16.2	Female	2	Unitek 018	1	0	No	No
	16.2	Female	3		2	1	No	No
	16.2	Female	4		3	2	No	No
	16.3	Female	1		3	2	No	Yes
	16.3	Female	2		1	0	No	Yes
	16.3	Female	3		2	1	No	Yes
	16.3	Female	4		2	1	No	Yes
	17.1	Male	1		2	3	No	No
	17.1	Male	2		2	1	Yes	No
	17.1	Male	3		1	0	No	No
	17.1	Male	4		2	1	No	No
	17.3	Male	1		2	2	No	No
10	17.3	Male	2	INVISALIGN	1	0	No	No
	17.3	Male	3		1	0	No	No
	17.3	Male	4		2	1	No	No
	17.5	Male	1		2	2	No	No

Patient	AGE (y.m)	SEX	Tooth	Bracket type	Pocket Depth	Clinical Attachment Loss	Bleeding On Probing	Plaque
	17.5	Male	2		1	0	No	No
	17.5	Male	3		1	0	No	No
	17.5	Male	4		2	1	No	No
11	28.12	Female	1	INVISALIGN	2	1	No	No
	28.12	Female	2		2	1	Yes	No
	28.12	Female	3		2	2	No	No
	28.12	Female	4		3	1	No	Yes
	29.2	Female	1		3	4	No	No
	29.2	Female	2		1	0	No	Yes
	29.2	Female	3		1	0	No	No
	29.2	Female	4		3	2	No	Yes
	29.3	Female	1		3	2	No	No
	29.3	Female	2		2	1	No	No
	29.3	Female	3		2	1	No	No
	29.3	Female	4		2	1	No	No
12	32.2	Female	1	CONTROL	4	3	No	No
	32.2	Female	2		2	1	No	No
	32.2	Female	3		2	1	No	No
	32.2	Female	4		3	2	No	No
	32.3	Female	1		2	3	No	No
	32.3	Female	2		1	1	No	No
	32.3	Female	3		1	1	No	No
	32.3	Female	4		2	1	No	No
	32.5	Female	1		3	3	No	No
	32.5	Female	2		1	1	No	No
	32.5	Female	3		2	3	No	No
	32.5	Female	4		2	1	No	No
13	29.2	Female	1	CONTROL	2	4	No	No
	29.2	Female	2		1	0	No	No
	29.2	Female	3		2	1	No	No
	29.2	Female	4		2	1	No	No
	29.3	Female	1		2	4	No	No
	29.3	Female	2		1	1	No	No
	29.3	Female	3		1	3	No	No
	29.3	Female	4		2	1	No	No
	29.5	Female	1		2	3	No	No
	29.5	Female	2		1	1	No	No
	29.5	Female	3		1	3	No	No
	29.5	Female	4		2	1	No	No

Patient	AGE (y.m)	SEX	Tooth	Bracket type	Pocket Depth	Clinical Attachment Loss	Bleeding On Probing	Plaque
14	27.1	Male	1	CONTROL	2	4	No	No
	27.1	Male	2		2	3	No	No
	27.1	Male	3		2	3	No	No
	27.1	Male	4		2	1	No	No
	27.3	Male	1		2	3	No	No
	27.3	Male	2		1	2	Yes	No
	27.3	Male	3		2	3	No	No
	27.3	Male	4		1	1	No	No
	27.4	Male	1		2	3	No	No
	27.4	Male	2		1	1	Yes	No
	27.4	Male	3		1	1	Yes	No
	27.4	Male	4		2	2	No	No
15	25.2	Female	1	CONTROL	2	3	No	Yes
	25.2	Female	2		1	0	No	No
	25.2	Female	3		2	2	No	No
	25.2	Female	4		3	1	No	No
	25.4	Female	1		2	4	No	No
	25.4	Female	2		1	0	No	No
	25.4	Female	3		1	0	No	No
	25.4	Female	4		3	1	No	No
	25.5	Female	1		3	4	No	No
	25.5	Female	2		1	1	No	No
	25.5	Female	3		1	2	No	No
	25.5	Female	4		2	1	No	No
16	33.3	Female	1	CONTROL	2	3	no	yes
	33.3	Female	2		2	1	no	no
	33.3	Female	3		2	1	no	no
	33.3	Female	4		2	1	No	No
	33.4	Female	1		3	4	No	No
	33.4	Female	2		1	0	Yes	No
	33.4	Female	3		1	0	No	No
	33.4	Female	4		3	1	No	No
	33.5	Female	1		2	3	No	Yes
	33.5	Female	2		1	1	No	No
	33.5	Female	3		1	2	No	No
	33.5	Female	4		1	0	No	Yes

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