MICROBIOME SHIFTS IN THE SUPRAGINGIVAL BIOFILM IN PATIENTS UNDERGOING ORTHODONTIC TREATMENT WITH FIXED APPLIANCES: A PILOT STUDY

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ABSTRACT

Ashley D. Morgenstern: Microbiome Shifts in the Supragingival Biofilm in Patients Undergoing Orthodontic Treatment with Fixed Appliances: A Pilot Study (Under the direction of Tate Jackson)

Oral health and disease are now best understood as dysbiotic shifts in the oral microbiome, yet little is known about specific microbial-ecological events that precede the development of disease. Orthodontic treatment is an elective dental procedure known to be a risk-increasing intervention for dental caries and periodontal disease. In this pilot study, we used next-generation sequencing methods to characterize longitudinal metagenomic changes in the supragingival microbiome of adolescents undergoing fixed orthodontic treatment.

The study cohort comprised 10 healthy orthodontic patients (aged 13-15) scheduled for treatment with fixed appliances. One trained and calibrated examiner recorded clinical measures of bleeding on probing, gingival index, and plaque index. Supragingival plaque samples were collected from facial surfaces of maxillary incisor and mandibular premolar index teeth using sterile curettes at four-time points: before bonding of orthodontic appliances and at 1, 6, and 12 weeks after bonding. Samples were placed in sterile buffer solution and snap-frozen until analysis. DNA was isolated using a QIAamp® Microbiome Kit and a 16S rRNA amplicon library was prepared to carry out pair-ended, 150bp Illumina sequencing. After bioinformatics processing using the QIIME pipeline, microbial diversity was determined by PD Whole Tree and Shannon diversity indices, and significant differences between ethnic groups determined by Kruskal-Wallis tests.
We obtained 3.6 million high-quality reads across all time points that identified 98 genera and 216 species. Biofilm diversity decreased (phylogenetic diversity, whole tree, Shannon index) from baseline (pre-treatment) to one week (post-treatment). This ecological shift was primarily due to a 73% relative decrease in *Corynebacterium matruchotii* and relative increases in Actinobacteria: *Lautropia Mirabilis* (109%) and *Veillonella* (27%). By 12 weeks, diversity had regressed to nearly baseline levels.

These results provide initial proof-of-principle that an elective oral disease risk-increasing intervention, fixed orthodontic appliances, can induce a dysbiotic shift in the supragingival oral biofilm of healthy patients.
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# TABLE OF CONTENTS

LIST OF TABLES.............................................................................................................................. viii
LIST OF FIGURES ............................................................................................................................ ix
LIST OF ABBREVIATIONS ................................................................................................................ x
LIST OF SYMBOLS ........................................................................................................................... xi

REVIEW OF THE LITERATURE ........................................................................................................ 1
  Bacterial Association with Disease ................................................................................................. 1
  Orthodontic Appliances as a Risk Factor ....................................................................................... 6
  Oral Microbiome Dysbiosis ............................................................................................................. 9
  Technology Advances in DNA Sequencing .................................................................................... 9

MICROBIOME SHIFTS IN THE SUPRAGINGIVAL BIOFILM IN PATIENTS UNDERGOING FIXED ORTHODONTIC TREATMENT WITH FIXED APPLIANCES: A PILOT STUDY ........................................................................................................ 12
  Introduction ................................................................................................................................... 12
  Materials and Methods .................................................................................................................. 14
    Sample ......................................................................................................................................... 14
    Data Collection ............................................................................................................................ 15
    DNA Isolation ............................................................................................................................... 16
    16S rRNA Amplicon Sequencing ................................................................................................. 16
    Sequencing Data Analysis ........................................................................................................... 17
  Statistical Analysis ........................................................................................................................ 18
  Results ...........................................................................................................................................
Discussion .................................................................................................................. 19
Conclusions ................................................................................................................ 21
Tables ........................................................................................................................ 23
Figures ...................................................................................................................... 24
References ................................................................................................................ 27
LIST OF TABLES

Table 1 – Demographic data and periodontal status changes baseline to 3 months after fixed orthodontic treatment .............................................. 23
LIST OF FIGURES

Figure 1 – Alpha Diversity T0 through T3, n=10 ........................................................................... 24
Figure 2 – Alpha Diversity T0 through T3, n=7 ........................................................................... 24
Figure 3 – Hyperplastic gingival response ..................................................................................... 25
Figure 4 – Bacterial Composition in African American Participants, T0 and T1 ......................... 26
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOP</td>
<td>Bleeding on Probing</td>
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<tr>
<td>CDC</td>
<td>Center for Disease Control</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<td>MS</td>
<td>Mutans Streptococci</td>
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<td>NGS</td>
<td>Next-Generation Sequencing</td>
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<td>NHANES</td>
<td>National Health and Nutrition Examination Survey</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PD</td>
<td>Phylogenetic Diversity</td>
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<td>PGM</td>
<td>Personal Genome Machine</td>
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<td>QIIME™</td>
<td>Quantitative Insights Into Microbial Ecology</td>
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<td>rRNA</td>
<td>Ribosomal Ribonucleic Acid</td>
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<tr>
<td>tRNA</td>
<td>Transfer Ribonucleic Acid</td>
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<td>US</td>
<td>United States</td>
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<tr>
<td>WSL</td>
<td>White Spot Lesion</td>
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LIST OF SYMBOLS

®
Registered Trademark

™
Trademark Symbol
A REVIEW OF THE LITERATURE

Bacterial Association with Disease

Periodontal diseases and caries are the most common oral diseases. It’s important to put into perspective how many people these diseases affect. Gingivitis is defined by inflammation of the gingiva without bone loss. Studies indicate that the prevalence of gingivitis in the average population varies but is fairly high. Brown found that in a sample of working US adults, 44% of participants had gingivitis at an average of 2.7 sites/participant.\(^1\) In another study, Horning sampled patients in a military dental clinic and recorded 37% had gingivitis without periodontitis.\(^2\) In the National Survey of Oral Health in US Schoolchildren, gingivitis was observed in 60% of children ages 14-17 years.\(^3\) While other studies found that more than 70% of children over the age of 7 have gingivitis.\(^4,5\)

Periodontal disease is defined as gingivitis with associated destruction of the periodontal supporting structures, including alveolar bone. Chronic periodontal disease affects adults more than children and the NHANES study from 2009-2012 estimating that 46% of adults experience the disease.\(^6\) Aggressive periodontal disease usually affects people younger than 30 years old and is characterized by rapid attachment loss and destruction of bone in otherwise healthy individuals.\(^7\) Often the first molar or incisors are the teeth affected by aggressive periodontitis,\(^8\) which are the first permanent teeth to erupt. Prevalence of aggressive periodontitis in those younger than 35 years old ranges from 1-15%.\(^9\)
Unlike periodontal disease which is more uncommon in younger populations, caries is the most common disease of childhood and affects five times more children than asthma.\textsuperscript{10} From the NHANES 2012 survey, the CDC reports that 23\% of children aged 2-5 years have caries on primary teeth, approximately 60\% of adolescence aged 12-19 had experienced caries on their permanent teeth and 15\% had untreated tooth decay.\textsuperscript{11} Caries experience is additive throughout life and 91\% of adults aged 20-64 years in the US have experienced dental caries while 27\% have untreated disease.\textsuperscript{12}

Bacteria in the oral cavity were first described rudimentarily over 300 years ago by Antonie Van Leeuwenhoek, a Dutch scientist who is often referred to as the “father of Microbiology.”\textsuperscript{13} With drawings, he described several different kinds of bacteria, which he called “little animals” in 1683 that were collected from the buildup on teeth. He also discussed the importance of oral hygiene for maintaining healthy teeth and gingiva since he found that more of these “little animals” could be found in the unclean matter on teeth. Interestingly enough, he found that vinegar killed some of this bacteria, but not all, and concluded that it must not penetrate through completely and only destroyed the outermost layer, which today we understand is due to the complex formation of biofilm.\textsuperscript{14}

The association of plaque accumulation and gingival inflammation has been known for many years, and our understanding of the progression of gingivitis and periodontitis has been expanded over time. Epidemiologic studies showed a correlation between debris found intraorally and destruction of the periodontal tissue in the 1950s.\textsuperscript{15,16} In 1965, an experimental study was conducted where participants were instructed not to brush their teeth, and the development of generalized gingivitis was found to occur between 10-21 days. It was noted that interproximal areas had higher gingival scores than buccal and lingual. After the onset of
gingivitis, participants were instructed to resume oral hygiene and the resolution of gingivitis occurred in approximately 7 days.\textsuperscript{17}

After the presence of plaque was shown to cause gingivitis in a healthy periodontium and then its subsequent removal shown to resolve the resulting inflammation, the progression of gingivitis on a bacterial level was explored. Loe \textit{et al.} also described the progression of gingivitis in the following stages. In healthy periodontium, there are few desquamated epithelial cells, few leukocytes, and small groups of bacteria comprising mainly of cocci and short rod bacteria found at the gingival margin. The first stage of gingivitis after tooth cleaning stopped was an increase in cocci bacteria, leukocytes and desquamated epithelial cells. The second phase, beginning 2-4 days after oral hygiene ceased, showed again an increase in leukocytes and the presence of more filamentous forms (mostly \textit{leptotrichia} and \textit{fusobacteria}) and slender rod bacteria. It is interesting that the leukocytes present in this stage of inflammation varies between children and adults, with T-lymphocytes predominating in children\textsuperscript{18} and B-cells predominating in adults.\textsuperscript{4} The onset of the third phase of gingivitis development was more gradual and characterized by the presence of spirochetes and vibrios organisms along with cocci, rods, and filamentous bacteria described in the previous stages. This last phase started six to ten days after brushing had stopped\textsuperscript{17}. Another study described a similar transition from a predominantly gram-positive cocci colonization to a plaque make up of gram negative anaerobic cocci, filaments and spirochetes.\textsuperscript{19}

Additional research revealed that intraoral bacteria colonization has a high degree of order. Supragingival plaque colonizes in a columnar fashion with different species of bacteria growing on the inner surface near the tooth versus on the outer plaque surface.\textsuperscript{20} Subgingival plaque has different colonization patterns characterized by a layer of gram negative species near
the epithelial lining of the periodontal pocket and gram positive rods and cocci adhering to the enamel or root surfaces.\textsuperscript{21,22} Not only are bacterial species organized spatially depending on location, but also certain species of bacteria tend to occur together in complexes. Common complexes include \textit{Porphyromonas gingivalis} and \textit{Tannerella forsythia},\textsuperscript{23} \textit{P. gingivalis} and \textit{Treponema denticola},\textsuperscript{24} and \textit{Fusobacterium nucleatum} and \textit{Prevotella intermedia}.\textsuperscript{25}

The association of various complexes of bacteria with oral disease has been well established. Periodontal disease is a complex process involving an inflammatory host response to oral pathobionts. The bacterial changes observed in periodontal disease development follow a relatively predictable pattern over time, as has been demonstrated in clinical studies of experimental gingivitis\textsuperscript{17} and microbial succession\textsuperscript{26–28}. In 1998, Socransky \textit{et al} described complexes of bacteria associated with subgingival plaque. Two groups of bacteria associated with periodontal disease were described as the orange and red complexes. The core group of bacteria in the orange complex includes \textit{F. nucleatum} subspecies, \textit{Fusobacterium periodonticum}, \textit{P. intermedia}, \textit{Prevotella nigrescens}, and \textit{Parvimonas mica}. The red complex is comprised of three species of bacteria, \textit{T. forsythia}, \textit{P. gingivalis}, and \textit{T. denticola}. This study found an association with the orange complex with deeper periodontal pocket depths while the red complex exhibited a stronger association with deeper pocket depths as well as increased bleeding on probing. Both increased pocket depths and bleeding on probing are clinical indications for disease. The progression of bacterial colonization in periodontal disease is as follows: the orange complex precedes colonization of bacteria in the red complex. The red complex rarely is found in the absence of bacteria from the orange complex\textsuperscript{29} and even within the red complex itself \textit{P. gingivalis} is never found without \textit{T. forsythia}.\textsuperscript{23} These changes indicate how intricately connected bacteria are with one another, both in the formation of mature biofilm and in the
progression of disease. In 2008, Haffajee et al. described supragingival plaque complexes. The yellow complex bacteria is similar to those found in subgingival plaque with the addition of two other streptococcal species, *Streptococcus anginosus* and *Streptococcus constellatus*. The orange and red complexes also showed similarity to their counterparts in subgingival plaque with a few differences in species. *Eubacterium nodatum* is classified as an orange complex bacteria in subgingival plaque but grouped with the red complex for supragingival plaque. The supragingival orange complex did not include *Prevotella micra* or *Eubacterium nodatum* but also included *Fusobacterium periodonticum, Fusobacterium nucleatum vincentii, Fusobacterium nucleatum polymorphum, Leptotrichia buccalis, Campylobacter showae, Campylobacter gracilis, Selenomonas noxia, Gemella morbillorum, and Capnocytophaga ochracea*.30

The tooth surface demineralization that characterizes leads to dental caries occurs in a similar manner with various bacterial species becoming involved in a predictable fashion as the disease progresses. The bacteria associated with dental caries are different species than those involved in periodontal disease. Supragingival plaque is exposed to nutrients in saliva and from the diet. The acid production from carbohydrate metabolism by bacteria causes a drop in pH in the environment surrounding the tooth and leads to demineralization at its surface.31 Mutans streptococci have been established as the principal pathogens involved in dental caries due to their acidogenic (ability to produce acids) and aciduric (ability to live in acidic environments) nature and frequent occurrence in cultures from cavitated dental lesions.32,33 More recently, it has been realized that caries is a dynamic biological process. It has been established that in addition to MS and lactobacilli, members of the genera *Bifidobacterium, Propionibacterium*, and *Scardovia* are associated with caries.34,35 And other bacteria can increase pH as a protective
mechanism by producing ammonia. Besides just the bacteria present, environment plays a role in the disease process.

In 2011, Takahashi and Nyvad described three stages in the progression of caries and the role of bacteria in each. The first stage, the dynamic stability stage, bacteria in dental plaque produce acids but the frequency of exposure to sugars is low so the oral environment is able to recover from these episodes. Bacteria present at this stage are non-mutans streptocci and Actinomyces. In the next stage, the acidogenic stage, exposure to sugars becomes more frequent and bacteria experience low pH for longer periods of time. This more frequent exposure actually increases the acidogenicity of non-mutans strep species such as Streptococcus sanguinis, Streptococcus oralis, Streptococcus gordonii, and Streptococcus mitis and increase their acidurance. Even more frequent and severe acidic environments characterize the third stage, the aciduric stage. In short exposures to severe acid environments, non-mutans strep and Actinomyces have been shown to lose their viability and bacterial growth is slower even after the pH is increased. This allows more aciduric bacteria who have survival advantages, such as MS, lactobacilli and Bifidobacterium, to eliminate many non-mutans strep and Actinomyces species and become much more prevalent in dental plaque. The progression of caries is based on the frequency and severity of a low pH environment which selects for bacterial species with more acidogenic and aciduric properties and doesn’t allow for adequate pH recovery intraorally thus initiating or progressing the caries process.

Fixed Orthodontic Appliances as a Risk Factor for Common Oral Diseases

Fixed orthodontic appliances are a risk factor for gingival inflammation. If orthodontic appliances adversely affect oral hygiene, increased plaque accumulation around brackets is
expected and also puts patients at risk for developing gingival inflammation. Zachrisson noticed that within 1-2 months of orthodontic treatment, a majority of patients had generalized moderate gingivitis, and even patients with good oral hygiene exhibited mild changes in gingival inflammation during treatment. In another study, Kloehn found that before treatment, the percentage of patients with a gingival score of 0 decreased from 20% to 10% in 8 months, meaning there were less patients with healthy gingiva after orthodontic treatment was started. We often think of these gingival changes as temporary while fixed appliances are in place, and that these conditions will resolve after treatment is complete and the teeth are easier to clean. Yáñez found that when orthodontic appliances were removed, in just 10 days, patients showed a significant improvement in gingival inflammation and a decrease in periodontopathogens. Many other studies have reported these inflammatory changes are reversible after appliances are debonded. In the natural course of disease, gingivitis is a precursor for those patients who develop periodontitis. Patients with active periodontal disease have a higher risk of bone loss during orthodontic treatment, making it an unethical intervention for these patients.

Fixed orthodontic appliances and their negative impact on a patient’s oral hygiene also lead to an increased risk of caries as well as enamel decalcification, or white spot lesions. White spot lesions in orthodontic patients have been shown to appear in as little time as four weeks. Studies indicate that the incidence of WSL after orthodontic treatment varies from 12-95%. Mizrahi found that 72% of patients had WSL prior to treatment and 84% had WSL after treatment, with an overall increase in opacity noted. Gorelick found that 24% of patients had WSL prior to orthodontic treatment and 50% had these spots after treatment. In other studies, Richter found that 73% and Lovrov found 95% of patients developed one new WSL during treatment. Therefore, despite interventions such as oral hygiene and fluoride rinses, white spot
lesions often develop in patients with fixed orthodontic appliances. Evaluating bacteria present in orthodontic patients who developed WSL and those who did not revealed higher levels of aciduric flora and *S. mutans* in the WSL group, but findings were not statistically significant.  

Many studies have looked at bacterial involvement and microbial changes in relation to gingivitis and periodontitis, but fewer studies have looked at how the introduction of fixed orthodontic appliances affects the oral microbiome composition. Kim *et al* completed a longitudinal study that collected plaque samples before, during, and after orthodontic treatment and evaluated changes in specific periodontal pathogens using 16s rRNA PCR methods. Of the eight pathogens evaluated (*Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, *Campylobacter rectus*, *Eikenella corrodens*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens* and *Treponema denticola*) they found an increase in *T. forsythia*, *C. rectus* and *P. nigrescens* after fixed appliances were placed. Sandic *et al.* also evaluated microbial changes over time with two groups of patients (those at the beginning of treatment and those towards the end of treatment). Four periopathogens, *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia* and *P. intermedia*, were evaluated using 16s rRNA PCR methods. They found a decrease in *P. gingivalis* upon placement of appliances and decrease in *T. forsythia* upon removal of orthodontic appliances. The only study to look at microbiome changes in orthodontic patients using next-generation sequencing techniques is a study by Koopman et al. They completed a randomized controlled trial to evaluate changes in microbiome of orthodontic patients who used two different fluoride mouthrinses vs a control group. They obtained 2.6 million quality reads and identified bacteria from 15 phyla but found no statistically significant difference in bacteria at any timepoint in either mouthwash groups. In the control group,
*Fusobacterium* decreased significantly between 3 of the earlier timepoints but increased again thereafter.\(^{56}\)

**Oral Microbiome Dysbiosis**

There have been over 700 species identified that live in the oral cavity\(^{57}\) and in health, an individual has between 100-200+ bacterial species present.\(^{58,59}\) Variation in bacterial composition within individuals is due to differences in age, environment, genetics, and lifestyle.\(^{60}\) Oral microbiome is very stable over time in healthy individuals,\(^{61}\) and core species have been shown to be maintained for up to 7 years.\(^{62}\) In health, there is a balance between the oral bacteria that allows a symbiotic relationship with the host and can prevent foreign pathogens from colonizing.\(^{63}\) A change in environment such as a increase in consumption of fermentable carbohydrates and the introduction of fixed oral appliances can perturb the balance of bacteria in the biofilm and contribute to the initiation or progress of periodontal disease and caries.\(^{64,65}\) If healthy individuals have a highly diverse and stable population of bacteria, what do we expect to see with the onset of disease? In patients with Hepatitis B, there was a decrease in the diversity of oral microbiota compared to healthy controls.\(^{66}\) A decrease in the diversity of oral microbiome has been shown to worsen the colonization resistance against pathogenic species, which will eventually lead to dysbiosis.\(^{67}\) In summary, healthy individuals tend to have higher diversity of oral bacterial species which can provide resilience against pathogenic species and it has been shown that in a disease state, the diversity of species is decreased.

**Technology Advances in DNA Sequencing**

The structure of DNA as a double helix was discovered by James Watson and Francis Crick in 1953.\(^{68}\) Several years later, the first nucleic acid to be sequenced was an alanine tRNA
molecule in 1964 by Robert Holley. In 1977, Frederick Sanger and Walter Gilbert independently developed methods to sequence DNA.

Maxam-Gilbert or chemical sequencing was more popular than Sanger sequencing at first since it did not require cloning of DNA and purified DNA could be used directly. DNA was cleaved using a series of four chemical reactions that cut the DNA strand at specific bases. These four reactions cleaved either guanine, guanine and adenine, cytosine, or cytosine and thymine. Using electrophoresis in a gel, the multiple strands that resulted could be separated based on size and reaction type and the sequence of DNA determined. This method fell out of favor because it was slower than Sanger sequencing, used quite a bit of radioactive material, and required use of hydrazine, which is a known neurotoxin.

Sanger sequencing requires cloning of target DNA and formation of a plasmid which was a short strand of plasmid DNA of a known sequence and the remainder being target DNA. Repetitive heating and cooling of the DNA allows the plasmid primer to bond known sequence and then allows for incorporation of other nucleotides along the unknown DNA strand. Radiolabeled nucleotides are randomly incorporated and terminate the sequence. An automated sequencing machine uses electric charge to organize the partial DNA strands by size and then read the terminal radiolabeled nucleotide so that the sequence of the DNA can be determined. Sanger sequencing was widely used for approximately 40 years until Next-Generation Sequencing (NGS) replaced it for large-scale automatic genome analysis.

NGS is a catch-all term to describe techniques that have since widely replaced Sanger sequencing due to their ability to produce an enormous volume of data very quickly, accurately, and inexpensively. The first NGS technologies were launched in 2000 by Lynx Therapeutics
and since then, they have only been improved upon by many different companies. Today, the four modern sequencing technologies used are Illumina® (Solexa) sequencing, Ion PGM sequencing, and SOLiD® sequencing. NGS uses high throughput technology and parallel analysis to increase the volume and speed in which sequencing can be completed.

Analysis of NGS results requires the use of bioinformatics pipelines, such as QIIME™, which can take raw DNA sequencing data and filter them for quality, as well as determine taxonomy of bacterial species present from a known database. Using these methods for determining bacteria present in the oral cavity far outweigh bacterial colonization methods since 35 of oral bacteria have not been able to be cultivated.

It is now easier than ever with next-generation sequencing to evaluate the oral bacterial composition of a plaque sample. Oral health and disease are best understood as dysbiotic shifts in the oral microbiome, yet little is known about specific microbial-ecological events that precede development of disease. Orthodontic treatment with fixed appliances is known to be a risk factor for dental caries and gingivitis which makes individuals undergoing such treatment an interesting population to study. The aim of this longitudinal pilot study is to assess the changes in the oral microbiome of initially healthy patients for whom orthodontic appliances are introduced and maintained, using next-generation sequencing methods.
MICROBIOME SHIFTS IN THE ORAL BIOFILM IN PATIENTS WITH ORAL HEALTH RISK FACTORS: A PILOT STUDY

Introduction

Caries and periodontal diseases are the most common oral diseases. Most adults will have experienced periodontal diseases and/or caries at some point during their lifetime. Gingivitis is reported to affect 37-44% of adults\textsuperscript{1,2} and as high as 60-70% of children\textsuperscript{4,3,5}. Periodontitis is more severe and characterized by destruction of supporting periodontal structures including alveolar bone. Periodontitis is reported to affect 46% of adults\textsuperscript{6} and often affects a younger population (under age 35) in an aggressive form affecting 1-15% of the population\textsuperscript{9}. The relationship between specific bacteria and onset and progression of gingivitis to periodontal disease has been well established.\textsuperscript{16–18,4,19} Gingivitis is the initial indication of disease yet little is known about the events that immediate precede clinically identifiable gingivitis.

Fixed orthodontic appliances often lead to increased plaque accumulation around brackets due to difficult hygiene and lead to an increased risk for gingival inflammation. Several studies have shown an increase in incidence of gingival inflammation after fixed appliances are introduced.\textsuperscript{41,42} These changes have also been shown to improve quickly, in as few as 10 days, after appliances are removed at the end of treatment.\textsuperscript{43} Fewer studies have looked into how fixed orthodontic appliances affect the oral microbiome composition, especially from a global perspective. Most studies to date have used 16s rRNA PCR methods to evaluate changes in a select few species of periodontal pathogens.\textsuperscript{75,76} To our knowledge, only one study has used
next-generation sequencing techniques to identify global changes in oral microbiota in orthodontic patients, but that investigation was a randomized control trial evaluating these changes in a group of participants who used two different fluoride mouthrinses vs. a control group. They found no statistically significant differences in bacteria present at any single timepoint in either mouthwash group but that in the control group, *Fusobacterium* decreased during three of the initial timepoints, but increased again thereafter. Before additional randomized clinical trials, more descriptive information about the microbial changes that naturally occur without intervention are needed.

Studying participants undergoing orthodontic treatment with fixed appliances allows for the study of a unique population who is generally but put at risk for gingivitis and periodontitis through a common, routine treatment modality. Treatment often takes 18-24 to complete which also allows us to follow this at-risk group over an extended period of time. Next-generation sequencing techniques make it easier and faster to determine microbial composite present in a sample by sequencing the highly conserved 16s rRNA gene and comparing against a known database, such as QIIME. Understanding the global oral microbiome changes could provide a better understanding of the disease process that takes patients from a state of health to disease.

In health, there is a balance between the oral bacteria that allows a symbiotic relationship with the host and can prevent foreign pathogens from colonizing. There have been over 700 species identified that live in the oral cavity, and in health, an individual has between 100-200+ bacterial species present. This level of diversity allows for a balance between the oral bacteria and a symbiotic relationship with the host that can prevent foreign pathogens from colonizing. Dysbiosis is an imbalance of the microbiota which can be caused by a change in environment such as plaque accumulation or an increase in consumption of carbohydrates. This
in turn can cause stress on the balance of oral bacteria and lead to progression of disease including periodontal disease and caries.\textsuperscript{64,65}

The specific aims of this study were to longitudinally assess the changes in oral microbiome of initially healthy patients for whom orthodontic appliances are introduced, without any additional intervention, and to establish a protocol in this pilot study for future data collection and analysis.

We hypothesize that with the introduction of fixed appliances leads to an associated dysbiotic shift of the supragingival biofilm

**Materials and Methods**

**Sample**

Patients were recruited from the University of North Carolina School of Dentistry graduate orthodontic clinic during their case presentation appointment, prior to starting any orthodontic treatment. This study was approved by IRB #16-1624 and consent and assent were generated and discussed in person with all participants (and their parents) before any study participation began.

Inclusion and exclusion criteria:

**Inclusion**

1. ASA I or II\textsuperscript{77}

2. Receiving elective orthodontic treatment at the UNC Graduate Orthodontics clinic

**Exclusion**
1. ASA III or above, systemic diseases that may influence oral bacteria e.g. diabetes, ectodermal dysplasia, AIDS, immunocompromised
2. Phase 1, limited orthodontic treatment, or treatment with removable appliances
3. Antibacterial therapy in the past 3 months
4. Smokers

**Data Collection**

This study employed a longitudinal design. Data were collected by a single examiner at four timepoints: before bonding of fixed orthodontic appliances, 1 week after bonding, 6 weeks after bonding, and 12 weeks after bonding. At each visit, intraoral photographs were taken along with a saliva sample, a periodontal evaluation, which included probing depths, bleeding on probing (BOP), plaque index (Quigley Hein)\(^78,79\), gingival score (Silness and Loe)\(^80\), and plaque samples were collected from the buccal surface of four index teeth (UR5, UL1, LL5, LR1). Gingivitis in this study was defined as \(\geq 20\%\) BOP sites with no probing depths \(\geq 5mm\). Participants were also given a short questionnaire asking about changes in their oral hygiene routine and diet.

Plaque samples were collected using a sterile scaler for each site and each plaque sample was placed in 150ug TE buffer, snap frozen, and stored in \(-80^\circ C\). The saliva sample (approximately 3.5mL total) was centrifuged for 10 minutes at 4.1 rpm. The supernatant was removed from the pellet and aliquotted into 4 tubes and stored at \(-80^\circ C\). Prior to DNA isolation, only the plaque samples from UL1 and LL5 were pooled for analysis.

After bonding, all study participants were instructed in oral hygiene and given the standard hygiene kit provided to all patients in the clinic.
DNA isolation

Plaque samples were transferred to sterile 2 ml tubes containing 200 mg of glass beads, \( \leq 106\mu m \) (Sigma, St. Louis, MO) and 0.3 ml of Qiagen ATL buffer (Valencia, CA). Bead beating was carried out for 3 minutes in a Qiagen TissueLyser II at 30Hz. Subsequently Qiagen AL buffer containing Proteinase K (600IU/ \( \mu l \)) was added and samples were incubated at 56°C for 1 hour. DNA was purified using a standard on-column purification method with Qiagen buffers AW1 and AW2 as washing agents and eluted in 10mM Tris (pH 8.0).

16S rRNA amplicon sequencing

12.5 ng of total DNA was amplified using universal primers targeting the V3-V4 region of the bacterial 16S rRNA gene.\(^1\) Primer sequences contained overhang adapters appended to the 5’ end of each primer for compatibility with Illumina sequencing platform. The complete sequences of the primers were:

515F - 5’
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA 3’

806R -
5’GTCTCGTGGGCTCGGAGATGTGTATAAGACAGCAGGACTACHVGGGTWTCTAAT 3’.

Master mixes contained 12.5 ng of total DNA, 0.2 \( \mu M \) of each primer and 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA). The thermal profile for the amplification of each sample had an initial denaturing step at 95°C for 3 minutes, followed by a cycling of denaturing of 95°C for 30 seconds, annealing at 55°C for 30 seconds and a 30 second
extension at 72°C (25 cycles), a 5 minutes extension at 72°C and a final hold at 4°C. Each 16S amplicon was purified using the AMPure XP reagent (Beckman Coulter, Indianapolis, IN). In the next step each sample was amplified using a limited cycle PCR program, adding Illumina sequencing adapters and dual-index barcodes (index 1(i7) and index 2(i5)) (Illumina, San Diego, CA) to the amplicon target. The thermal profile for the amplification of each sample had an initial denaturing step at 95°C for 3 minutes, followed by a denaturing cycle of 95°C for 30 seconds, annealing at 55°C for 30 seconds and a 30 second extension at 72°C (8 cycles), a 5 minutes extension at 72°C and a final hold at 4°C. The final libraries were again purified using the AMPure XP reagent (Beckman Coulter), quantified and normalized prior to pooling. The DNA library pool was then denatured with NaOH, diluted with hybridization buffer and heat denatured before loading on the MiSeq reagent cartridge (Illumina) and on the MiSeq instrument (Illumina). Automated cluster generation and paired–end sequencing with dual reads were performed according to the manufacturer’s instructions.

**Sequencing data analysis**

Multiplexed paired-end fastq files were produced from the sequencing results of the Illumina MiSeq using the Illumina software configureBclToFastq. The paired-end fastqs were joined into a single multiplexed, single-end fastq using the software tool fastq-join. De-multiplexing and quality filtering was performed on the joined results. Quality analysis reports were produced using the FastQC software. Bioinformatics analysis of bacterial 16S amplicon sequencing data was conducted using the Quantitative Insights Into Microbial Ecology (QIIME) software. OTU picking was performed on the quality filtered results using pick_de_novo_otus.py. Chimeric sequences were detected and removed using ChimeraSlayer. Alpha diversity and beta diversity analysis were performed on the data set using the QIIME
routines: alpha_rarefaction.py and beta_diversity_through_plots.py\textsuperscript{82,83}, respectively. Summary reports of taxonomic assignment by sample and all categories were produced using QIIME summarize_taxa_through_plots.py and summarize_otu_by_cat.py.

**Statistical Analysis**

Plaque samples were analyzed using QIIME to determine alpha diversity including PD whole tree and Shannon diversity indices. These were estimated at a rarefaction depth of 20,000 sequences per subsample. Significance for differential abundance by ethnicity was estimated by the Kruskal-Wallis test.

**Results**

The sample size for this study consisted of 10 patients (5 female, 5 male). Mean age was 13.1 years (range 11-15). The self-reported ethnic makeup of our group was 5 Caucasian, 2 Hispanic, and 3 African American. A summary of participant demographics is shown in Table 1.

Three participants had gingivitis at baseline, T0. The remaining participants developed gingivitis throughout the study and all participants had developed the disease by T3, 3 months after bonding. The three participants that started with gingivitis still had an increase in the number of bleeding on probing (BOP) sites from T0 to T3 so that all participants showed a worsening of the periodontal condition during this study. A summary of participant demographics, the initial periodontal condition, and relative changes in periodontal condition from T0-T3 is shown in Table 1.

Data analysis resulted in 3.6 million high quality reads across all time points and identification of 98 genera and 216 species of bacteria.
Alpha diversity of bacteria from plaque samples decreased between T0 and T1, baseline to 1 week after orthodontic appliances were placed for all 10 participants (Figure 2), and the same trend was seen for the 7 participants who started at baseline in a state of health and developed disease during the study (Figure 3). This ecological shift from T0 to T1 for this group was primarily due to a 73% relative decrease in Actinobacteria Corynebacterium matruchotii, 109% relative increase in Proteobacteria Lautropia Mirabilis and 27% relative increase in Fusobacteria genus Veillonella. The two most abundant species of Veillonella detected were Veillonella dispar and Veillonella parvula. Between T1 and T3 (one week to 12 weeks after bonding), alpha diversity returned to baseline levels.

Across all timepoints, the enrichment of two bacterial species varied significantly according to ethnicity, after a Bonferroni correction for multiple testing. Higher levels of Aggregatibacter Actinomycetemcomitans were found in Hispanic patients (p = 0.013), while higher levels of Parvimonas were found in African American patients (p = 0.013).

Two participants developed a hyperplastic gingival response (Figure 3). These two participants were siblings of African American descent. Upon comparing the bacterial composition in plaque between these two participants and the third African American participant in the study, there were no distinct pattern of differences between the three participants and the bacteria present at T0 or T1 (Figure 4).

**Discussion**

From the results of this study, the alpha diversity, or diversity of species that were found within an individual timepoint, decreased most drastically between baseline and 1 week after orthodontic appliances were placed. This drop shows that the intervention of placing orthodontic
appliances is associated with a dysbiotic shift that tends to normalize over time despite the continuing increase in BOP.

The decrease in diversity between T0 and T1 was primarily due to the change in prevalence of a select few bacteria. There was a 73% decrease in Actinobacteria *Corynebacterium matruchotii* which is a non-pathogenic species found in plaque. Fusobacteria, genus *Veillonella* showed a 27% increase from T0 to T1 with the species *Veillonella dispar* and *Veillonella parvula* being the major species present. These species have been associated with chronic periodontal disease. Taken together, these results suggest that there was a significant decrease in a colony of bacteria normally associated with health, along with a significant increase of a pathogenic bacteria. Interestingly, Proteobacteria *Lautropia Mirabilis*, a bacterial species associated with health had an 109% increase despite a shift towards gingivitis and higher BOP levels in all participants. These results are tentative given the small sample size and the exploratory nature of this study, but they suggest a shift in the profile of bacteria present at baseline or health to a dysbiotic profile after orthodontic appliances are introduced.

Another finding was statistically significant differences in specific bacteria present between ethnic groups. *Aggregatibacter actinomycetemcomitans*, which is associated with localized aggressive periodontitis was found at higher levels in Hispanic patients (*p* = 0.013). *Parvimonas* was found in African American patients at higher levels (*p* = 0.013) in this study. *Parvimonas* is a known periodontal pathogen and has been found in higher levels in patients with LAP. Previous studies have found that African Americans have the highest prevalence of localized aggressive periodontitis (LAP) compared to Hispanics and Caucasians. This is suggestive of racial/ethnic differences of the supragingival microbiome composition among this
group of adolescent orthodontic patients. Evidence of such microbiome diversity exists\(^9\); future, larger studies in the oral health domain are warranted to systematically examine these variations.

A hyperplastic gingival response is seen in some orthodontic patients, sometimes despite fairly good oral hygiene. Two participants in our study who were siblings developed this type of response in the first 3 months of treatment. The bacterial composition in their plaque samples compared to the other African American participant did not show any distinct pattern of differences and the non-hyperplastic African American participant actually had a bacterial composite that matched one sibling closer than the two siblings matched each other. These results lead us to believe that it may not be the presence or absence of a specific bacteria that causes this type of gingival reaction, but instead this phenomenon may be genetically-driven.

The main limitation of this pilot study is its small sample size. That limits its power and inferential potential. Nevertheless, this study demonstrates the feasibility of conducting such NGS investigations in the orthodontics domain and provides some initial proof-of-principle evidence regarding the induction of fixed orthodontic appliances-associated dysbiosis. In the future we hope to continue this protocol and enroll a large number of participants as well as have longer follow up time points.

**Conclusions**

These results provide initial proof-of-principle that an elective oral disease risk-increasing intervention, fixed orthodontic appliances, can induce a dysbiotic shift in the supragingival oral biofilm of healthy patients. These initial results also suggest that both ethnic and individual host-centered factors may affect oral dysbiosis, even in the presence of fixed appliances. Future studies should adequately account for such potential influences.
Acknowledgements

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Table 1. Demographic data and periodontal status changes baseline to 3 months after fixed orthodontic treatment

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<th>Participant ID</th>
<th>Age</th>
<th>Gender</th>
<th>Ethnicity</th>
<th>Baseline % BOP</th>
<th>Absolute Change in % BOP*</th>
<th>Baseline Plaque Score</th>
<th>Relative Change in Plaque Score (%)**</th>
<th>Baseline Gingival Score</th>
<th>Relative Change in Gingival Score (%)**</th>
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* Absolute change: Final - Baseline
** Relative Change: (Final - Baseline)/Baseline * 100
AA: African American, C: Caucasian, H: Hispanic
BOP: Bleeding on Probing
SD: Standard Deviation
Figure 1. Alpha Diversity T0 through T3, n=10

Figure 1 illustrates a decrease in alpha diversity between T0 and T1, baseline to 1 week after orthodontic bonding. Between T1 and T3, alpha diversity increases back to baseline.

Figure 2. Alpha Diversity T0 through T3, n=7

Figure 2 illustrates a similar trend in alpha diversity changes with the group of 7 patients who started at baseline in a state of health and developed gingivitis throughout the study.
Figure 3. Hyperplastic gingival response

Example of hyperplastic gingival response seen in two participants who were African American and also siblings.
Figure 4 shows the relative abundance of bacteria for African American patients at T0 and T1. Participants HP1 and HP2 developed gingival hyperplasia within three months of starting orthodontic treatment while participant NH did not develop gingival hyperplasia.
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