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AAO Foundation Final Report Form
(a/o 6/30/2019)

In an attempt to make things a little easier for the reviewer who will read this report, please consider these two questions before this is sent for review:

- *Is this an example of your very best work, in that it provides sufficient explanation and justification, and is something otherwise worthy of publication? (We do publish the Final Report on our website, so this does need to be complete and polished.)*
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Please prepare a report that addresses the following:

Type of Award, e.g., Orthodontic Faculty Development Fellowship Award, Postdoctoral Fellowship Award, Biomedical Research Award, Center Award, Educational Innovation Award, Program Award, Research Aid Award Biomedical Research Award

Name(s) of Principal Investigator(s) Flavio Uribe

Institution: University of Connecticut

Title of Project Dental microbiota composition of adolescent patients in fixed orthodontic treatment with and without white spot lesions: a cross-sectional study

Period of AAOF Support (e.g. 07-01-19 to 06-30-20): 7-01-2019 to 12-31-2020

Amount of Funding: $30,000

Summary/Abstract

White spot lesion formation is a common sequela of orthodontic treatment driven by cariogenic bacteria. We hypothesized that there is a different oral microbial community composition between patients with and without white spot lesions with fixed orthodontic appliances. A prospective clinical study aimed to correlate the oral microbiome composition to white spot lesion formation in the orthodontic patients while considering various secondary clinical factors was conducted.

A total of 92 subjects (orthodontic patients), including 46 (23 male/23 female) with white spot lesions (WSL group) and 46 (23 male/23 female) without white spot lesions (WSL- group) were recruited for this study with an average age of 14 years. Primary outcome included the measurement of the supragingival microbiota in the maxillary lateral incisors using 16S rRNA gene sequencing (all samples) and

metagenomic whole genome sequencing (mWGS, 10 subjects per group). Secondary outcomes included clinical parameters such as salivary pH, salivary buffer capacity, Bleeding on Probing (BOP), stimulated salivary flow, supragingival plaque score, diet, fluoride exposure, Decayed/Missing/ Filled Teeth (DMFT) index, and time in fixed appliances.

As determined by 16S rRNA gene sequencing, 790 Operational Taxonomic Units (OTUs) were identified from all 92 subject samples. A total of 142 OTUs were unique to the WSL- group and 173 OTUs were unique to the WSL group. The WSL had a higher microbiome diversity. When comparing specific OTUs between groups, OTU20 *Eikenella*, OTU55 *Prevotella nanceiensis* and OTU149 *Leprotrichia* were significantly more abundant in the WSL- group, while OTU 157, 243 *Prevotella*, OTU94 *Selenomonas,* OTU130 *Streptococcus mutans*, OTU232 *Dialister*, OTU131 *Actinomyces*, OTUs 234 and 289 *Fusobacterium* and OTU304 *Bifidobacterium* were significantly more abundant in the WSL group. Among the *streptococcus* species, *S. sanguinis* was more abundant in the WSL- group while *S. mutans, S. anginosus* and *S. parasanguinis* were significantly more abundant in the WSL group. On streptococcus specific network analysis, *S. mutans* had a negative correlation with *S. sanguinis* and had a high correlation with *Selenomonas noxia* and also a high correlation with Bifidobacterium in the WSL group. Additionally, *S. sanguinis* correlated highly with *Rothia* and *Haemophilus* in the WSL- group but not in the WSL group.

MWGS identified 194 total species. Abundance differences revealed significant differences between both groups with *Gemella morbillorum, Lautropia mirabillis, Neisseria sicca Porphyromonas* sp. oral taxon, and *Haemophilus parainfluenzae* more abundant in the WSL-. *S. mutans, Megasphaera micronuciformis, Dialister invisus, Cryprtobacterium curtum,* unclassified *Gemella* species, *Shuttleworthia satelles*, and *Alloprevotella rava* were more abundant in the WSL group. When analyzing just streptococcus species, *S. mutans, S. anginosus* and *S. parasanguinis* were more abundant in the WSL group. Among the fungal species, *Malassezia restricta* was significantly more abundant in the WSL- group, while *Candida Albicans* was significantly more abundant in the WSL group. The viruses identified were significantly greater in the WSL-, while no viruses were found in greater abundance in the WSL group.

Among the clinical parameters, increased BOP and plaque score, decreased buffer capacity, and increased time in fixed appliances were variables associated with the presence of white spot lesions. The clinical factors that explain the variation of microbiome composition between the groups were white spot presence, salivary pH, salivary buffer capacity, BOP, and race of the subject.

Summing up, we found that the microbiome composition of supragingival plaque is different between the WSL and WSL- groups, with an increased diversity associated with white spot formation.
Specific species of bacteria and fungi are associated with healthy plaque and with cariogenic plaque. There are *streptococcus* bacteriophages and fungi that correlate with the absence of white spot lesions.

Detailed results and inferences:

1. If the work has been published please attach a pdf of manuscript OR **(See thesis attached)**
2. Describe in detail the results of your study. The intent is to share the knowledge you have generated with the AAOF and orthodontic community specifically and other who may benefit from your study. Table, Figures, Statistical Analysis and interpretation of results should be included.

Respond to the following questions:

1. Were the original, specific aims of the proposal realized? Yes, all the aims were achieved
2. Were the results published?
    a. If so, cite reference/s for publication/s including titles, dates, author or co-authors, journal, issue and page numbers
    b. Was AAOF support acknowledged?
    c. If not, are there plans to publish? If not, why not?
    The results have not been published in a manuscript but are being submitted as the requirements for the Master's in Dental Science of a resident that graduated in June 2020. A manuscript is being worked on to submit to the Journal of Dental Research where proper credit will be given to the AAOF research support.
3. Have the results of this proposal been presented?
    a. If so, list titles, author or co-authors of these presentation/s, year and locations
    b. Was AAOF support acknowledged?
    c. If not, are there plans to do so? If not, why not?
    The results will be presented formally in a thesis defense for the Master's of Dental Science of a graduate student (Dr. Nyle Blanck). The date for the defense has been scheduled for January 15, 2021.
4. To what extent have you used, or how do you intend to use, AAOF funding to further your career?
    This funding gave me the ability to embark in a new line of research. A multidisciplinary team with significant expertise on the microbiome was established for future projects. These results will serve as a pilot for further studies and submission of future grant proposals to the NIH.

Accounting for Project; i.e., any leftover funds, etc.

The leftover funds amounted to $11,633.12

Dental Microbiota Composition of Adolescent Patients in Fixed Orthodontic Treatment With and Without White Spot Lesions: A Cross-Sectional Study

Master's in Dental Science
University of Connecticut
Nyle Blanck DMD

Major Advisor: Flavio Uribe
DDS, MDentSc

Research supported by the American Association of Orthodontists Foundation (AAOF) Biomedical Research Award 2019

Introduction:

Background

Oral Health with Orthodontic Treatment

Orthodontic therapy is a unique approach to improving discrepancies in the dentofacial complex utilizing fixed or removable appliances. While the goal of treatment is a better functional and esthetic outcome, there are multiple risks involved in the patient's temporary and long-term oral health. One major concern is the patient's compromised ability to maintain optimal oral hygiene throughout treatment.[1] Less than ideal oral hygiene results in an increased risk for plaque-induced gingivitis and subsequent periodontitis.[2] Another increased risk is the development of white spot lesions and subsequent cavitation of enamel. The extent of these orthodontic "scars" becomes most apparent after the removal of appliances at the end of treatment. The etiology of white spot lesions is an early demineralization of enamel due to byproducts of metabolic activity of cariogenic bacteria. Formation of white spot lesions is the most common iatrogenic side effect of orthodontic treatment, with an occurrence reported in the literature ranging from 2-96%.[3-8] One study reported that patients receiving fixed orthodontic treatment are more prone to white spot lesion formation than those without fixed appliances.[9]

White Spot Formation

Fixed orthodontic appliances include bands, brackets, archwires, ligature wires, and elastomeric modules. All of these components, though necessary for control of tooth movement, are plaque-retentive factors fostering establishment and growth of plaque biofilm.[10,11] As the patient consumes sucrose, *Streptococcus mutans (s. mutans)* and other cariogenic bacteria compete with beneficial bacteria for growth. As the biome evolves, with poor oral hygiene and a frequent

supply of sucrose, there is a proportional and absolute increase in *S. mutans*. This leads to a decrease in the pH of the oral environment. *S. mutans* ferments sucrose via the enzymes invertase and glucosyltransferase. Invertase divides sucrose into glucose and fructose components to be metabolized into lactic acid via fermentation. Glucosyltransferase converts the glucose moiety into glucans to allow *S. mutans* to adhere to plaque-retentive factors. When the pH drops below 5.0-5.2, the salivary buffers become overwhelmed and the hydroxyapatite, which makes up enamel, dissociates into its calcium and phosphate ion components.[12] According to the literature, a measurement of 10^6 colony forming units per milliliter of saliva is associated with future decay.[13]

Fluoride provides a protective quality to enamel. The presence of fluoride leads to the formation of hydroxyfluorapatite on demineralized enamel. Hydroxyfluorapatite has a lower critical pH for demineralization, allowing this newly formed enamel substitute to be more caries resistant than its initial purely hydroxyapatite structure. This altered enamel structure is more resistant to an acidic challenge and subsequent demineralization because it has a lower critical pH of about 4.5. Thus, an increased fluoride exposure would indicate a lower caries risk in an acidic environment because more fluoride is present for remineralization.[14]

It is well-known and frequently published in the literature that *S. mutans* is a cariogenic bacterium found at the site of white spot lesions.[15] Though *S. mutans* is the bacterium most commonly associated with carious lesions, there is an entire microbiome of inter-microbial interactions to consider.[16] Some orthodontic patients develop white spot lesions, while others do not. This indicates that there might be alternative microbial consideration involved beyond the presence of *S. mutans* affecting white spot lesion development.[17] For example, one study found that *Scardovia wiggsiae* is associated with white spot lesion formation in the presence and absence of *S. mutans*. This study also demonstrated that, along with *S. mutans*, there could be an association

between *Granulicatella elegans, Veillonellaceae* sp., and *Bifidobacteriaceae* sp. and white spot formation. The same study found that the bacterium *Cardiobacterium hominis* is more common in the absence of white spot lesions, which could indicate a protective quality.[18] An increase in certain bacteria ultimately could increase or decrease the risk of white spot formation in the orthodontic patient. Knowledge of the microbiome composition could give insight into which patients may be at a higher risk. For these patients, management options include either continuous oral hygiene instruction or potentially targeted drug therapy. A better understanding of supragingival microbiome composition and the inter-microbial interactions could aid in identifying orthodontic patients more prone to developing white spot lesions, which could advance the pharmaceutical field of treating these higher risk patients.

Caries Risk Factors

There are many factors contributing to the risk level of a patient.[19] The American Dental Association (ADA) created a caries risk assessment that separates the risks into three categories: high, moderate, and low. Contributing conditions include: total fluoride exposure, diet including frequency of sugar exposure, caries experienced by caregiver, and maintaining a dental home (i.e. being an established patient of record in a dental office and receiving regular dental care). General health conditions to be considered include: special health care needs (developmental, physical, medical or mental), eating disorders, and pH and flow of saliva (affected by genetics, drugs, or other therapies that could reduce salivary flow). Clinical conditions include: presence of carious lesions, visible plaque, unusual tooth morphology, previous restorations, exposed root surfaces, and dental or orthodontic appliances. To distinguish if a certain bacterium is the cause, all components of caries risk must be considered. The DMFT (decayed, missing, filled teeth) score

gives insight on history and current status of carious lesions. Plaque score and measure of bleeding on probing (BOP) can be affected by the patient's oral hygiene. Information on diet can be gathered directly from conversation with the patient. Salivary pH and flow can be measured with quick and inexpensive analysis at the chairside. Fluoride intake can be deduced from information provided by the patient, such as utilization of fluoridated toothpaste and degree of fluoridation of the patient's household drinking water.

To quantify white spot lesion formation, the Gorelick index has been established as a form of identification of disease progression.[19] This index divides the severities of white spot lesion formation into four categories: no lesion, slight lesion formation, severe lesion formation, and cavitation. Slight lesion formation is typically observed at the height of contour on the labial surface of anterior teeth, whereas severe is more generalized across the labial surface. A modified version of this index combines the "slight" and "severe" categories. The modified index would then comprise of "No WSL", "WSL", and "Cavitation". Furthermore, there has been a strong association observed between white spot lesion formation and gingivitis in the orthodontic patient.[18] The periodontium can be compromised throughout orthodontic treatment due to excessive plaque retention caused by fixed orthodontic appliances. This negative effect can be monitored by measuring BOP and plaque score for each patient.

Microbiome Analysis Technique

Historically, bacteria could only be detected via stains targeting specific physiologic characteristics at the taxonomic level. This requires the organism to be grown *in situ* for identification based on colony morphologic features, colony growth on particular media, or colony metabolic byproducts.[20] This time consuming, non-specific process led to DNA extraction-based

methods of species identification such as fluorescent *in situ* hybridization (FISH). This low-throughput metagenomic assay is inefficient and cannot be used to investigate entire microbial communities.[21] More recently, next generation high-throughput sequencing has been developed to examine samples, such as the oral microbiome, with large numbers of species. This is based on utilization of the ubiquitous bacterial 16S rRNA marker gene, unique to each bacterium, to match against databases to give the relative abundance of operational taxonomic units (OTU) in a microbial sample.[22] State of the art technology includes whole-metagenome shotgun sequencing (wMGS) to assay uncultured microbes from a human host. This technique uses gene fragments as taxonomic markers to be compared against databases to provide relative abundance in the microbial sample at the species level.[23,24] Recent advances in metagenomic sequencing have facilitated the ability to examine the relative abundance of species in the microbiome of fixed orthodontic patients with white spot lesions.

Oral Microbiome

With metagenomic sequencing technology continuously advancing, it has become much more affordable to examine entire microbiome populations.[25] Recent studies have reported findings drawn from the human host microbiome, particularly from the oral cavity. By comparing 3-month old infants to 3-year old children, next generation methods have demonstrated that the diversity of the microflora increases with age and that several taxa within the oral biofilm could be associated with the presence or absence of caries.[26] However, another study using 454-pyrosequencing determined that children have a more complex biodiversity compared to adults, suggesting a decrease in diversity into adulthood.[27] Beyond age, the environment and genetics also have an impact on a person's oral microbiome. There is a greater difference in oral microbiome

composition of non-related subjects compared to twins inhabiting the same environment.[28] Within the same oral cavity, the microbiome varies depending on geographic location, such as tongue, saliva, soft tissue, teeth, and subgingival and supragingival tooth surface. A more disputed subject is diversity with progressive periodontal disease. Some studies demonstrate decreased bacterial diversity and others indicate increased diversity with disease.[29,30]

Within the orthodontic literature, metagenomic sequencing is a relatively new and scarcely utilized technique. One study comparing microbial profile of metallic and ceramic brackets concluded that there is no significant difference in the abundance of caries-inducing bacteria between the bracket materials.[31] As for type of ligation, it was determined there is no difference in the composition of microorganisms between conventional ligation, self-ligating brackets, and steel ligatures. However, it was determined that the presence of fixed orthodontic appliances increases the presence of *S. mutans, Streptococcus sobrinus, Lactobacillus casei,* and *Lactobacillus acidophilu*s with the greatest increase in *S. mutans*.[32] Another study profiled the subgingival plaque biofilm of patients wearing clear aligners. It was determined that the diversity of subgingival plaque decreases during clear aligner therapy, but the relative abundance of periodontal pathogens remains stable.[33]

With such a high prevalence of white spot lesion formation in fixed orthodontic therapy, it would be beneficial to have a better understanding of biofilm composition during treatment. There have been a few published studies utilizing metagenomic sequencing to determine bacterial diversity in the orthodontic patient. One study found an increase in bacteria associated with white spot lesion formation and periodontitis; however, the results were not significant enough to conclude that orthodontic treatment shifts the microbiota to a more pathogenic composition.[34] Only one study has attempted to draw conclusions about the association between the microbiota and

white spot lesion formation in the orthodontic patient. They used DNA microarray to determine a difference in the microbiome between orthodontic patients with and without white spots.[18] No studies have quantified white spot lesion formation in conjunction with profiling the microbiota using 16S rRNA gene sequencing and metagenomic whole genome shotgun sequencing compared to a control group. Furthermore, there have been no studies with an adequate power analysis for determining a sufficient sample size.

White Spot Formation in Orthodontic Treatment

In the literature, there is published research examining enamel demineralization with fixed orthodontic appliances. In one study, a specially designed orthodontic band for plaque accumulation was placed on premolars that were treatment-planned to be extracted to relieve severe crowding. This extreme environment demonstrated that white spot lesions can develop within 4 weeks of placement of an ill-fitting band. Upon microradiographic and scanning electron microscope examination, the continuous cariogenic challenge caused softening of the enamel surface. The study concluded that caries formation in fixed orthodontic therapy can be a rapid process without proper oral hygiene and fluoride supplementation.[35] In the typical oral environment of a patient with fixed orthodontic appliances, 40% of patients develop white spot lesions within the first six months. This is not significantly different from the 43% of patients developing white spot lesions after twelve months of treatment. This indicates that the majority of white spot lesions develop within the first six months of treatment. The same study concluded that the mandibular first molars and maxillary lateral incisors are the teeth most susceptible to white spot lesion formation in the orthodontic patient. The maxillary lateral incisor is significant because it is in the esthetic zone of a patient's smile. Only considering the maxillary anterior teeth, the

order of highest to lowest risk of white spot formation is: lateral incisor, canine, then central incisor.[36]

The goal of orthodontics is to improve a patient's malocclusion, while maintaining good oral health throughout the duration of treatment. A better understanding of the supragingival microbiome could advance the field by ultimately preventing white spot formation.

Rationale and Objectives

Conclusions may be drawn from correlating white spot lesion formation to biofilm composition in the fixed orthodontic patient, particularly in areas in which white spot lesions are commonly observed. A better understanding of the supragingival microbiome composition could identify patients considered to be at higher risk of white spot formation during treatment. This could also lead to the development of preventive therapies to avoid the iatrogenic growth of cariogenic bacteria during orthodontic treatment. However, development of white spot lesions is a multifactorial process in which all risk factors must be considered. This prospective clinical study aims to correlate the oral microbiome composition to white spot formation in the orthodontic patient while also considering various secondary clinical factors.

Hypothesis

Null Hypothesis

There is no difference in supragingival microbiome composition in patients undergoing fixed orthodontic appliances with and without white spot lesions.

Alternate Hypothesis

Patients who develop white spot lesions in fixed orthodontic treatment have a different supragingival microbiome composition than those who do not.

Specific Aims (SA)

SA#1: To determine the supragingival microbiota composition after at least 6 months of fixed orthodontic appliance treatment in patients with and without white spot lesions

SA#2: To correlate microbiota composition and caries risk factors to white spot lesion formation with placement of fixed orthodontic appliances

Materials and Methods

Study design

This was a prospective, cross-sectional study evaluating a single timepoint plaque sample in correlation with caries risk factors of adolescent orthodontic patients that do and do not develop white spot lesions over the duration of treatment. It was approved by the Institutional Review Board at the University of Connecticut. (IRB#: 19-112-2)

Inclusion Criteria:

- Patients with fixed orthodontic appliances
- In treatment at least 6 months
- Male and Female
- With and without white spot lesions on the labial surface of the maxillary lateral incisors
- Age 10-19

Exclusion Criteria

- History of white spot lesions prior to orthodontic treatment
- Syndromic patients
- Cleft lip/palate patients
- Prescribed antibiotics in preceding 3 months of sample collection
- Fixed appliances bonded with fluoride-releasing adhesive
- History of previous orthodontic treatment

Patient records were screened from the clinic at the University of Connecticut School of Dental Medicine, Division of Orthodontics, Farmington, Connecticut from June 2019 to January 2020. Patients in fixed orthodontic treatment for at least 6 months between the ages of 10-19 were identified. They were sequentially approached for consent and examined for the presence or absence of white spot lesions on the maxillary lateral incisors with loupes (2.5x magnification). If the patient and/or guardian consented and fulfilled inclusion/exclusion criteria, they were included in the study. Patients in the study were instructed to refrain from brushing their teeth at least three hours prior to the next adjustment appointment to ensure there was sufficient plaque present to obtain a sample. Plaque sample and all secondary measurements were collected at that following appointment. The samples and measurements were deidentified by the study coordinator (N.B).

Outcome Variables

The primary outcome measurement was the microbiota composition of supragingival plaque in patients that develop and do not develop white spots in fixed orthodontic treatment. The secondary outcome measurements included other factors that contribute to caries risk:

- Salivary pH: The saliva is the environment that encompasses the enamel of teeth. When the pH of saliva is more acidic, the hydroxyapatite is driven towards demineralization. A more acidic salivary pH would indicate higher caries risk.[19]
- Stimulated salivary flow: When a patient begins mastication, the salivary flow increases as part of the digestion process. The saliva has protective qualities for enamel to prevent caries by washing away sugar and buffering acidic challenges. Increased stimulated salivary flow would indicate decreased caries risk.[19]
- Salivary buffering capacity: When an acidic challenge is introduced to the enamel from either diet or acid byproducts of microbial metabolism, buffers in the saliva prevent the oral pH from decreasing significantly. An increased buffer capacity allows the saliva to maintain a relatively higher pH when presented with an acidic challenge. A decreased buffer capacity would indicate higher caries risk.[19]
- Supragingival plaque score: The presence of supragingival plaque on the teeth gives insight into the oral hygiene and caries risk of the patient. An increased amount of supragingival plaque denotes worse oral hygiene and would indicate higher caries risk.[19]
- Bleeding on probing (BOP): This is a widely used technique to diagnose gingival inflammation. The presence of gingivitis gives insight into the patient's oral hygiene and caries risk. Teeth with an increased number of sites with BOP would indicate worse oral hygiene, thus higher caries risk.[19]
- Diet: A frequency of three or more sugary snacks per day, excluding meals, would increase the amount of opportunities for supragingival bacteria to consume and metabolize sucrose and other carbohydrates, thus increasing the time the enamel is exposed to an acidic

challenge from fermentation. A higher frequency of sugary snacks would indicate higher caries risk.[19]

- Fluoride exposure: Fluoride provides a protective quality for enamel. Fluoride allows for formation of hydroxyfluorapatite from demineralized enamel. Hydroxyfluorapatite has a lower critical pH for demineralization, allowing this alternate enamel form to be more caries resistant than the initial, purely hydroxyapatite structure. An increased fluoride exposure would indicate lower caries risk.[19]
- Decayed, missing, filled teeth (DMFT) index: The DMFT index gives insight on both historical and current status of caries in the patient. One point is given to each tooth that has decay, is missing due to decay, or was filled due to decay. A higher DMFT score indicates higher caries risk in the patient.[19]

Preliminary Studies

Dr. Yanjiao Zhou, M.D., Ph.D. from the University of Connecticut, School of Medicine conducted a study that investigated the subgingival microbiome changes in patients (aged 12-29) receiving fixed orthodontic treatment. She and her team conducted 16S rRNA gene sequencing on collected subgingival plaque and found that the microbiome changed significantly after 6 and 12 weeks of bracket placement, compared to healthy controls without treatment. The changes were exemplified by increased *Fusobacterium* and decreased *Streptococcus Mitis* (the closest taxa to the reference database) (Figure 1). The microbiome controls also changed during the study period, but with less magnitude. They also found that bacterial diversity, including Richness and Shannon Diversity, demonstrated a significant increase after bracket placement (Figure 2), suggesting a potential overgrowth of different types of bacteria due to treatment.

Sample Size Determination

Sample size: 92 subjects

Sample size justification: Power calculation was performed based on a previous study showing a significant difference of *S. mutans* between patients with and without white spot lesion by qPCR.[18] A total of 92 subjects, including 46 (23 male/23 female) with white spot lesions and 46 (23 male/23 female) without white spot lesions, allowed an 80% power to detect a mean difference of 0.78, and give a standard deviation of 0.78 by two sample t-test, at a significance level (α) of 0.001. This conservative α value was utilized to justify multiple comparisons in the high dimensional microbiome data analysis ($\alpha = 0.001$ allowing up to 50 multiple comparisons with an FDR-adjusted P value of 0.05). This sample size also took into consideration a 10% failure rate during sample preparations and sequencing, and upscale by another 15% to account for the use of non-parametric statistical tests required for microbiome analysis.

Data Collection

Patients of record at the orthodontic clinic at the University of Connecticut School of Dental Medicine were identified, sequentially approached, informed of the study, and included in the study if the patient and/or guardian consented and if all of the inclusion and exclusion criteria were fulfilled. The initial diagnostic photographs were examined to confirm the absence of white spot lesions at the beginning of treatment. The maxillary anterior teeth were examined by the study coordinator (N.B.) under 2.5x magnification to determine if white spots were present on the labial surface. The initial procedure was to use a priority of lateral incisor, then canine, then central incisor to find teeth with white spots. However, every subject that had white spots on the canines or central incisors also had white spots on one or both of the lateral incisors. As such, samples

were only taken from lateral incisors, for both the white spot and no white spot groups. The tooth examined and the presence/absence of white spots were recorded for plaque collection at the following appointment. The subject was instructed to refrain from brushing three hours prior to their next routine orthodontic appointment, typically one month after recruitment and examination, for sample and data collection.

At the following appointment, plaque and data were collected after confirming that the patient had refrained from brushing for at least three hours. The tooth was examined again by the study coordinator (N.B.) to confirm the presence or absence of white spots. The tooth was isolated using a cotton roll and a supragingival plaque sample was taken from the buccal surface with a sterilized dental scaler and placed into a PowerBead tube from the DNeasy PowerSoil® kit (QIAGEN, Venlo, Netherlands). The PowerBead tube was placed on ice and transported to storage at -80°C until DNA extraction could be performed using the same kit at a later date. Intraoral photographs, including frontal and buccal left and right views were taken.

For the secondary outcomes, the Saliva-Check Buffer Kit® (GC Corporation, Tokyo, Japan) was used, following the kit's included protocol. The subject was instructed to expectorate any pooled saliva into a collection cup at this second visit. A pH strip was placed into the sample and held for 10 seconds. The color of the strip was then compared to the chart of known pH colors provided in the kit to determine the salivary pH of the subject (Figure 3). Next, the subject was instructed to chew on a piece of paraffin wax to stimulate salivary flow and to allow the saliva to pool for 30 seconds. The subject was then instructed to expectorate the pooled saliva into a volumetric collection cup and continue chewing/collecting saliva in the cup at regular intervals for a total of 5 minutes and 30 seconds. The amount of stimulated salivary flow was recorded at the end of the stated time. Following this, a sufficient amount of saliva was drawn from the collection

cup using a sterile pipette and placed on each of the three pads on the buffer test strip. The strip was immediately turned 90° on a tissue to allow the excess to be absorbed. After two minutes, the color on each pad was observed and compared to a conversion table to determine the buffer capacity of the saliva (Figure 4). The DMFT score was determined via intraoral examination and previous radiographs. Each tooth with decay was counted as 1 point. Each missing tooth was counted as 1 point. Teeth congenitally missing, unerupted, missing due to orthodontic therapy or due to trauma/fracture were not counted. Each tooth with a filling due to decay was counted as 1 point. No tooth counted for two categories, such as a tooth with both decay and a filling, in which case this tooth was counted as 1 point. The score was summed and recorded. Using a periodontal probe, the subject was probed in the gingival sulcus at six locations (distobuccal, buccal, mesiobuccal, distolingual, lingual, mesiolingual) on every tooth, excluding third molars. The presence or absence of BOP was recorded for each site. The subject was instructed to chew GUM® Red-Cote (Sunstar Americas, Inc., Illinois, USA) disclosing plaque tablets and subsequently swish the saliva for 30 seconds to allow staining and visualization of the supraginigival plaque. The presence or absence of plaque on the buccal and lingual surface of each tooth was recorded, excluding the third molars. The total number of teeth for each subject was recorded so that the DMFT, BOP, and plaque scores could be calculated as ratios for a relative score. The subject was surveyed to obtain information about diet, fluoride exposure, and oral hygiene. Subject demographic information such as age, gender, race, and ethnicity was collected. The time in fixed orthodontic treatment at the time of sample collection was also recorded.

Data Analysis

Once plaque samples were collected for all subjects, the DNA extraction was performed following the protocol and using the solutions provided in the DNeasy PowerSoil® (QIAGEN, Venlo, Netherlands) kit. The isolated metagenomic DNA was then used for downstream sequencing. All 92 samples were subjected to 16S rRNA gene sequencing. 16S V4 regions from the metagenomic DNA were amplified and sequenced. Sequencing reads were processed by removing the sequences with low quality (average qual<35) and ambiguous nucleotides (N's). Chimeric amplicons were removed using UChime software. The processed reads were used for operational taxonomic unit (OTU) generation by an automated pipeline. Each OTU was classified from phylum to genus level using the most updated Ribosomal Database Project (RDP) classifier and training set. A taxonomic abundance table was 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 was presented as relative abundance (the read counts in a given taxon divided by total reads in the sample).

A subset of samples (10 subjects per group, 20 total) were subjected to metagenomic whole genome shotgun sequencing (mWGS) to identify bacteria to species or strain level, fungi, viruses, genes, and metabolic pathways in the microbial community. The 10 white spot and 10 no white spot samples were picked based on highest abundance of *Streptococcus* genera. The subsample demographics were checked prior to proceeding with mWGS sequencing. Libraries were constructed with an average insert of 500 bases and then sequenced on the HiSeq2500 (Illumina), producing 150 base read pairs from each fragment and yielding ~15 million read pairs per sample. The raw data was processed through: (1) removal of duplicate reads utilizing a custom script; (2) screening host contamination with BMTagger; (3) trimming of Illumina adaptors with Flexbar; (4)

removal of low-complexity sequences; and (5) filtering out all reads that are shorter than 60 bases or reads with less than 60 high quality bases. The processed reads were aligned to the reference genome database consisting of over 5000 bacterial genomes, over 5000 viral genomes, and 64,450 fungal sequences using the Real Time Genomics (RTG) aligner. The taxonomic table was presented in a fashion similar to the 16S rRNA approach. Microbial genes were identified using a number of programs such as MetaGUN, a metagenomic gene finder based on a Support Vector Machine (SVM) algorithm. Metabolic pathways were reconstructed from metagenomic samples using HUMAnN2 software. The resulting abundance tables of gene families and metabolic pathways were generated and presented in a format similar to the taxonomic table.

Statistical Analysis

Exploratory multivariate analyses and formal statistical testing were performed to discover microbiome distribution patterns and to determine the microbiome difference between patients with and without white spot lesions, using all of the 16S rRNA data. (1) For exploratory multivariate analysis, non-metric multidimensional scaling (NMDS) was applied to evaluate the microbiome similarity among all of the samples. Clinical variables including salivary pH and flow, diet, fluoride intake, plaque score, measure of BOP and DMFT were mapped to the NMDS plot to identify sample clustering patterns. (2) To determine whether the microbiome was statistically significant between groups, permutational multivariate analysis of variance (PERMANOVA) was performed for each clinical variable individually. In the final PERMANOVA model, the microbiome difference in patients with and without white spot lesions was compared after adjusting confounding clinical variables. (3) For microbiome diversity index, the Richness and Shannon Diversity gave indicators of microbiome complexity. Non-parametric Wilcoxon rank

testing was performed to determine whether the microbial diversity differed between the two groups. (4) To identify specific microbes that are associated with white spot lesion, DESeq was used after adjusting for other clinical variables. (5) Spearman or Pearson correlation was performed to identify the relationships between microbe-microbe as well as microbe-clinical variables. A network was constructed based on the correlation. This approach identified microbial clusters composed of multiple organisms. The statistical analysis of the microbiome data from mWGS was performed in a similar fashion as the 16S rRNA data analysis.

Wilcoxon test was used to obtain p-values for all secondary outcomes to determine if these variables were statistically significant between the white spot and no white spot groups. All of the p-values were corrected by false discovery rate approach for multiple sample comparisons. A p-value ≤ 0.05 was considered as statistically significant. All analyses were performed in R environment.

Results

For each demographic variable measured, there were no statistically significant ($p > 0.05$) results observed between the white spot and no white spot groups (Table 1). The average age of subjects in the white spot group was 14.17 ± 1.55 years and the average age of subjects in the no white spot group was 14.28 ± 1.83 years ($p = 0.908$). The number of subjects of each gender was predetermined as a factor to be equal in each group. There were 23 males and 23 females in both white spot and no white spot groups, to reach 46 total males and 46 total females ($p = 1.00$). With regard to race, there were 6 African American subjects in the white spot group and 5 African American subjects in the no white spot group, 36 Caucasian subjects in the white spot group and 38 Caucasian subjects in the no white spot group, and 4 Asian subjects in the white spot group and

3 Asian subjects in the no white spot group ($p = 0.866$). Regarding ethnicity, there were 9 Hispanic/Latino subjects in the white spot group and 12 Hispanic/Latino subjects in the no white spot group and 37 non-Hispanic/Latino subjects in the white spot group and 34 non-Hispanic/Latino subjects in the no white spot group ($p = 0.619$).

Based on answers from the questionnaire, the usage of fluoridated toothpaste and frequency of sugary snacks between meals were both statistically insignificant ($p > 0.05$) (Table 2). All 46 subjects in the white spot group and 45 subjects in the no white spot group used fluoridated toothpaste, while 1 did not ($p = 1.00$). In the white spot group, 32 subjects consumed less than 3 and 14 subjects consumed 3 or more sugary snacks between meals, while in the no white spot group 38 subjects consumed less than 3 and 8 subjects consumed 3 or more sugary snacks between meals daily ($p = 0.222$).

The average DMFT score and total possible DMFT score were statistically insignificant between the two groups ($p > 0.05$). The average DMFT score was 1.04 ± 1.63 points for the white spot group and 0.59 ± 1.02 points for the no white spot group ($p = 0.214$). The total possible DMFT score was 26.91 ± 1.75 points for the white spot group and 26.83 ± 2.00 points for the no white spot group ($p = 0.732$). The average salivary pH was statistically insignificant between the groups ($p > 0.05$). The average salivary pH was 6.85 ± 0.37 in the white spot group and 6.93 ± 0.40 in the no white spot group ($p = 0.392$). The average salivary buffer capacity was statistically significant between the groups ($p < 0.01$). The average salivary buffer capacity was 8.70 ± 1.62 points in the white spot group and 9.54 ± 1.90 points in the no white spot group ($p = 0.00715$). The average stimulated salivary flow was statistically insignificant between the groups ($p > 0.05$). The average stimulated salivary flow was 7.48 ± 3.24 mL in the white spot group and 7.91 ± 3.37 mL in the no white spot group ($p = 0.736$). The average BOP score was statistically significant between the

groups ($p < 0.001$), while the total possible BOP score was statistically insignificant ($p > 0.05$). The average BOP was 29.9 ± 28.8 points in the white spot group and 11.5 ± 12.3 points in the no white spot group ($p = 0.0000608$). The total possible BOP was 162 ± 10.2 points in the white spot group and 160 ± 12.2 in the no white spot group ($p = 0.966$). The average plaque score was statistically significant between the groups ($p < 0.05$), while the total possible plaque score was statistically insignificant ($p > 0.05$). The average plaque score was 33.7 ± 13.9 points in the white spot group and 26.9 ± 16.0 points in the no white spot group ($p = 0.0202$). The total possible plaque score was 53.9 ± 3.51points in the white spot group and 53.5 ± 4.08 points in the no white spot group ($p = 0.966$). Because the total possible BOP and plaque score was statistically insignificant between the groups, it was not necessary to calculate the relative scores, as this would redundant. The average time in braces at the timepoint that the sample was taken was statistically significant between the groups ($p < 0.05$). The average time in braces was 21.7 ± 9.99 months in the white spot group and 18.1 ± 9.17 months in the no white spot group ($p = 0.0431$).

As determined by 16S rRNA gene sequencing, 790 Operational Taxonomic Units (OTUs) were identified from all 92 subject samples. Of the 790 total, 475 OTUs were shared by both white spot and no white spot groups. These 475 accounted for 98.0% of the total abundance of OTUs present. A total of 142 OTUs were unique to the no white spot group and 173 OTUs were unique to the white spot group (Figure 5). The mean relative abundance of the top 25 OTUs (Figure 6) between the no white spot and white spot groups were significantly different at the phylum and genus taxonomy levels (Figures 7 and 8). There were bacteria more prevalent in the absence of white spots and bacteria more prevalent in the presence of white spots (Figure 9). With the Shannon diversity index, the white spot group had a higher microbiome diversity than the no white spot group ($p = 0.0029$) (Figure 10). The alpha diversity between the groups was statistically

significant at the genus level ($p = 0.0074$) (Figure 11), but not at the OTU level ($p = 0.15$). The beta diversity was statistically significant between the groups at the OTU level ($p = 0.0023$) (Figure 12).

The statistically significant clinical factors compared to OTU as determined by Principal Component Analysis (PCA) were: presence of white spot ($p = 0.001$), salivary pH ($p = 0.001$), race ($p = 0.006$), salivary buffer capacity ($p = 0.011$), and BOP ($p = 0.014$) (Table 3) (Figures 13 and 14). The same statistically significant clinical factors of presence of white spot ($p = 0.001$), race ($p = 0.002$), salivary pH ($p = 0.002$), salivary buffer capacity ($p = 0.004$), and BOP ($p = 0.018$) were determined by Distance-based Redundancy Analysis (dbRDA) as the PERMANOVA (Figure 15). Using Pearson correlation, the clinical factors and the 25 most abundant OTUs had very weak correlations ($-0.34 < r < 0.36$) (Figure 16).

The race of the subjects was statistically insignificant ($p = 0.866$) between the no white spot and white spot groups. There was a significant difference in OTU composition (Figure 17) between the three races (African American, Asian, Caucasian) with Shannon diversity ($p = 0.035$) (Figure 18) and alpha diversity ($p = 0.0096$) (Figure 19). The Asian group was missing 10% of the OTUs in the top 25 most abundant (Figures 20 and 21), but had a higher OTU diversity than the African American and Caucasian cohorts. The most significant OTUs in the Asian cohort, not represented by the other groups were *Neisseria, Moxarella, Aggregatibacter, Eikenella,* and *Capnocytophaga*.

When comparing specific OTUs in the no white spot and white spot groups, there were significant differences observed (Table 4). Using DESeq to analyze abundance differences at padj < 0.01, OTU20 *Eikenella*, OTU55 *Prevotella nanceiensis,* and OTU149 *Leptotrichia* were significantly more abundant in the no white spot group and OTUs 157 and 243 *Prevotella,* OTU94

Selenomonas, OTU130 *Streptococcus mutans,* OTU232 *Dialister,* OTU131 *Actinomyces,* OTUs 234 and 289 *Fusobacterium*, and OTU304 *Bifidobacterium* were significantly more abundant in the white spot group (Figure 22). For abundance differences at padj < 0.05, OTU83 *Lachnoanaerobaculum,* OTU1 *Corynebacterium*, and OTU23 *Streptococcus* were significantly more abundant in the no white spot group and OTU212 *Prevotella,* OTU191 *Veillonella*, OTU230 *Streptococcus*, and OTU222 *Schwartzia* were significantly more abundant in the white spot group (Figure 23). More differences were noted at padj < 0.15, but this was not considered statistically significant (Figure 24). All *Lactobacillus* OTUs were not significantly different between the groups. *Lactobacillus, Granulicatella, Scardovia,* and *Gemella* were previously reported cariogenic bacteria that were not significantly different between the groups. Many *Actinomyces* OTUs were significantly more abundant in the white spot group. *Cardiobacterium* was a previously reported caries protective bacteria in which no significant difference was observed between the groups.

On closer examination of *Streptococcus* species specifically (Figure 25), significant differences in relative abundance between the no white spot and white spot groups were observed (Figure 26). Using DESeq to analyze abundance difference, OTU23 *Streptococcus sanguinis* was significantly more abundant in the no white spot group ($p = 0.000052$). In the white spot group, OTU130 *Streptoccocus mutans* ($p = 0.0000035$), OTUs 230 and 453 *Streptococcus anginosus* ($p = 0.00047$ and $p = 0.043$, respectively), and OTU225 *Streptococcus parasanguinis* ($p = 0.012$) were significantly more abundant. The most abundant OTUs present, but not statistically significant between the groups were OTU2 *Streptococcus mitis* ($p = 0.26$) and OTU29 *Streptococcus gordonii* ($p = 0.6$) (Figures 27 and 28). On *Streptococcus* specific network analysis (Figure 29), *S. mutans* had a negative correlation with *S. sanguinis* ($r = -0.2$). There were no strong

correlations on the SparCC of *Streptococcus* OTUs ($r < -0.4$ or $r > 0.4$). Network analysis of *Streptococcus* against the top 25 most abundant OTUs was performed (Figure 30). Scatterplots of important correlations gave p-values and correlation coefficients (Figure 31). *S. mutans* highly correlated with *Selenomonas noxia* ($r = 0.4$, $p = 0.0058$). *S. mutans* correlated with *Bifidobacterium* in the white spot group ($r = 0.42$, $p = 0.000029$), but not in the no white spot group ($r = -0.062$, $p = 0.0058$). In the no white spot group, *S. sanguinis* highly correlated with *Rothia* ($r = 0.48$, $p = 0.00065$) and *Haemophilus* ($r = 0.71$, $p = 0.000000044$), but not in the white spot group ($r = -0.039$, $p = 0.8$ and $r = -0.068$, $p = 0.65$, respectively).

A subset of samples of 10 no white spot and 10 white spot was selected for mWGS based on the highest abundance of *Streptococcus* OTUs (Figure 32). The demographics of the two groups were even with the only statistically significant difference of BOP being greater in the white spot group (Table 5). For the initial group of 92 subjects, BOP was significantly higher in the white spot group as well. The top 25 OTUs of the subsample for mWGS was consistent with the full subject relative abundance bar plot (Figure 33). For the mWGS subsample, OTU7 *Haemophilus*, OTU 131 *Actinomyces*, and OTU94 *Selenomonas* were slightly different from the larger sample. For *Streptococcus* species specifically, the relative abundance of species was consistent with the initial sample (Table 6). For the subsample, *S. sangunis* was significantly greater in the no white spot group ($p = 0.039$) and *S. mutans* ($p = 0.008$), *S. parasangunis* ($p = 0.012$), and *S. anginosus* ($p = 0.043$) were significantly greater in the white spot group.

With mWGS, the top 25 most abundant OTUs were reliably identified to the species level (Figure 34). There were some discrepancies between OTU and species because the basic local alignment search tool (BLAST) was used to narrow down the OTUs. In the subsample, 194 total species were identified. Using DESeq to analyze abundance differences at $p < 0.02$, *Gemella*

morbillorum (p = 0.0056), *Lautropia mirabillis* (p = 0.0056), *Neisseria sicca* (p = 0.014), *Poryphyromonas* sp. oral taxon (p = 0.014), and *Haemophilus parainfluenzae* (p = 0.019) were significantly more abundant in the no white spot group. At p < 0.02, *S. mutans* (p = 0.0048), *Megasphaera micronuciformis* (p = 0.012), *Dialister invisus* (p = 0.017), *Cryptobacterium curtum* (p = 0.0063), unclassified *Gemella* species (p = 0.015), *Shuttleworthia satelles* (p = 0.015) and *Alloprevotella rava* (p = 0.015) were significantly more abundant in the white spot group (Figure 35). For the abundance differences at p < 0.05, *Capnocytophaga gingivalis* (p = 0.031) and *Capnocytophaga* sp. oral taxon (p = 0.043) were significantly more abundant in the no white spot group. At p < 0.05 *S. parasanguinis* (p = 0.037), *S. anginosus* (p = 0.035), *Selenomonas flueggei* (p = 0.031), unclassified *Veillonella* species (p = 0.029), *Actinomyces* sp. oral taxon (0.037), *Atopobium rimae* (p = 0.024), *Atopobium parvulum* (p = 0.026), *Peptostreptococcus stomatis* (p = 0.035), *Oribacterium* sp. oral taxon (p = 0.043), and unclassified *Olsenella* species (p = 0.05) were significantly more abundant in the white spot group, with *Veillonella* and *Actinomyces* having very high relative abundance (Figure 36).

When just considering *Streptococcus* species, three additional species were identified by mWGS, not initially observed in 16S rRNA sequencing (Figure 37), but these did not show a significant difference in abundance between the groups. *S. mutans, S. parasanguinis,* and *S. anginosus* remained more prevalent in the white spot group. Using DESeq to analyze abundance differences, no species were significantly more abundant in the no white spot group. *S. sanguinis* (p = 0.088) and *S. infantis* (p = 0.075) were marginally significantly increased in the no white spot group. *S. mutans* (p = 0.0048), *S. anginosus* (p = 0.035), and *S. parasanguinis* (p = 0.037) were significantly more abundant in the white spot group (Figure 38).

Of the metabolic pathways with a statistically significant difference in abundance using DESeq, several were determined to be more abundant in both the no white spot and white spot groups (Figure 39) (Table 7). The relative abundances of pathways were low. Pyruvate fermentation to acetate, aerobic respiration, and NAD NADH phosphorylation dephosphorylation were some of the pathways to show a significantly higher abundance in the no white spot group. The three pathways with a statistically significant higher abundance in the white spot group were peptidoglycan biosynthesis from *Enterococcus faecium*, L rhamnose degradation, and methyl ketone biosynthesis. The unmapped pathways were 18.5% on average in the samples. The unintegrated pathways were 68.8% on average in the samples. The pathways with the highest correlation to specific bacteria ($r < -0.35$ or $r > 0.35$, $p < 0.5$) were identified. *Rothia denocariosa* had a positive correlation with the superpathway of threonine biosynthesis, glycolysis from glucose, aminoimidazole-ribonucleotide biosynthesis I, aminoimidazole-ribonucleotide biosynthesis II, and the superpathway of 5-aminoimidazole-ribonucleotide biosynthesis. *Corynebacterium matruchoti* had a negative correlation with urate biosynthesis inosine-5-phosphate degradation. *Campylobacter gracilis* had a negative correlation with isoleucine biosynthesis I from threonine and valine biosynthesis (Figure 40).

Only 16 fungal species were identified in all of the samples (Figure 41). *Malassezia restricta* and *Candida albicans* were the most abundant species of all of the fungal species. *M. restricta* was significantly more abundant in the no white spot group ($p = 0.009$) and *C. albicans* was significantly more abundant in the white spot group ($p = 0.05$) (Figure 42). The mean abundance of *C. albicans* relative to all fungal species present was 7.14% in the no white spot group and 99.85% in the white spot group.

There were 49 virus types identified in all of the samples. Of the 49 identified, 35 were *Streptococcus*-specific phages (Figure 43). The viruses identified to be significantly greater in the no white spot group were *Streptococcus* phage phiAR10462 ($p = 0.035$) and *Streptococcus* phage phiAR10746 ($p = 0.052$) (Figure 44). No viruses were identified to be significantly greater in the white spot group.

Discussion

In orthodontic treatment, the most common iatrogenic side effect is the formation of supragingival white spot lesions, particularly adjacent to fixed appliances.[3] Unlike gingivitis, which is another frequently observed iatrogenic side effect, white spot lesions are irreversible.[2] These early carious lesions, typically on the buccal surface in the esthetic zone, require restoration by a dentist to achieve the same healthy appearance as the unaffected, healthy surface prior to beginning treatment.[36] One of the main goals of orthodontics is to achieve a beautiful smile. When white spots are present at the time of fixed appliance removal, that goal has not been achieved, even though the teeth have been straightened. Thus, it is paramount for the patient to maintain good oral health throughout the duration of treatment. Furthermore, it becomes extremely valuable for the orthodontist to know the exact causes of white spot formation to ultimately be able to prevent them and achieve ideal esthetic outcomes.

The first aim of this study was to determine the supragingival microbiome composition of patients, with and without white spot lesions, who had been in fixed orthodontic treatment for at least 6 months. White spot formation is a multifactorial process involving many factors that contribute to the microbiome composition. Hence, the second aim of this study was to correlate the microbiome composition to risk factors associated with caries formation. *S. mutans* is the most

well-known cariogenic bacteria with an established mechanism of action. It was determined from a previous study, examining *S. mutans* in white spot formation, that inclusion of 92 subjects would achieve the power level necessary to be deemed statistically significant.[18] Because the subjects were obtained by sequentially approaching patients that fulfilled the inclusion criteria, a wide range of demographics were represented. For all of the demographic variables considered (age, gender, race, and ethnicity), there were no statistically significant differences between the no white spot and white spot groups, indicating that both groups had similar representation of these demographic variables.

The American Dental Association (ADA) has identified several factors associated with caries formation. Because white spot lesions are defined as an early demineralization of enamel, these caries risk factors were used as white spot lesion risk factors for the purpose of this study. Contrary to what was hypothesized, the use of fluoridated toothpaste, frequency of sugary snacks between meals (≥ 3), DMFT score, salivary pH, and salivary flow were statistically insignificant between the no white spot and white spot groups. These factors did not contribute to the risk of white spot formation in patients with fixed orthodontic treatment. All of the subjects lived in areas with a fluoridated water supply, through which they received adequate fluoride supplementation in addition to using fluoridated toothpaste (except 1 in the no white spot group). The assumption that the white spot group would have a significantly higher snacking frequency and DMFT score was not observed. The average salivary pH in both groups was well above the pH of 5.0-5.2 necessary for demineralization of enamel.[12] According to the literature, a stimulated salivary flow of ≤ 3.5 mL/min is considered a high caries risk. The vast majority of subjects in this study were well above this threshold for high caries risk.[37]

The caries risk factors with a statistically significant difference between the no white spot and white spot groups were buffer capacity, BOP, plaque score, and time spent in fixed appliances. The no white spot group had a statistically significant higher buffer capacity than the white spot group. Though the average buffer capacity was overall higher in the no white spot group, both groups were considered to have a high, healthy buffer capacity, according to the literature.[38] An increased BOP and plaque score is indicative of poor oral hygiene, and hence, higher caries risk.[19] These variables were observed to be higher in the white spot group, as expected. Poor oral hygiene leads to excessive growth of the supragingival biofilm, and subsequent enamel demineralization and gingivitis. Lucchese et. al.[36] reported that the majority of white spot lesions form in the first six months of orthodontic treatment. This disagrees with our finding of increased time in fixed orthodontic appliances as a risk factor for white spot lesion formation. This could be explained by the fact that the fixed, plaque-retentive factors remain on teeth for an extended period of time. Additionally, maintaining proper oral hygiene when fixed orthodontic appliances are present is more time consuming on a daily basis; thus, oral hygiene fatigue could settle in as treatment continues up to two years and beyond.

For microbiome analysis with 16S rRNA gene sequencing, the no white spot and white spot groups had different OTU compositions. Of the total 790 OTUs found between the groups, there was an overlap of 475 OTUs. These 475 OTUs accounted for 98.0% of the OTU abundance in the samples. This indicates that the majority of bacterial species that exist in the supragingival plaque do not contribute to the caries risk. There were many OTUs unique to both the no white spot and white spot groups. The white spot group had a significantly higher microbiome alpha and beta diversity than the no white spot group. This could indicate that supragingival microbiome diversity increases as white spot formation and subsequent caries progress.

The presence of white spots, salivary pH, salivary buffer capacity, BOP, and race are clinical factors that could explain the OTU variation in the samples. However, the clinical factors and most abundant OTUs had very weak correlations. Differences in OTU abundances were observed between the races. Yang et. al.[39] found a difference in oral microbiome composition between races as determined by 16S rRNA gene sequencing. Our results agreed with this finding as the compositions differed across the three races included. The African American and Caucasian groups had similar top 25 OTUs, but the Asian group was missing 10% of these top 25. The Asian group had a higher diversity and included *Neisseria, Aggregatibacter, Eikenella,* and *Capnocytophaga* OTUs.

S. mutans is the most acknowledged cariogenic bacteria. Because of this, the subset of samples from the no white spot and white spot groups selected for mWGS were based on the highest relative abundance of *Strepotococcus* genera. As demonstrated by 16S rRNA gene sequencing, various clinical factors can affect OTU abundances. The demographics and clinical variables of the subset matched that of the full sample set, indicating that this smaller group of 20 samples was a good representation of all 92 samples. The mWGS species abundances were overall consistent with the top 25 OTUs, further demonstrating a good representation. There were slight differences in OTUs, such as *Haemophilus, Actinomyces,* and *Selenomonas*. Discrepancies could be explained by the use of BLAST to narrow down OTUs. From mWGS, samples at the species level were reliably identified. This was compared to 16S rRNA sequencing that identifies at the genus level for most OTUs, with some at the species level. Similar to the results from 16S rRNA sequencing, mWGS determined that the white spot group had a higher diversity than the no white spot group.

Whether in the no white spot group or white spot group, many bacteria were observed to have a statistically significant difference. However very few bacteria had a significant abundance across the samples. This is an important consideration before drawing any conclusions such as the discovery of a novel cariogenic or caries antagonist species. If a bacterium is determined to be significantly higher in abundance in one of the groups, the overall relative abundance must also be considered.

The mechanism of action of caries progression caused by *S. mutans* is well-established, making it the most known and most commonly studied cariogenic bacteria.[12] Our study agrees with all existing literature that *S. mutans* is more abundant in the white spot group. The difference in relative abundance of *S. mutans* between the two groups was the most statistically significant of all bacteria identified. *S. mutans* metabolizes sucrose to form glucans to allow the bacteria to adhere to hydroxyapatite and produces lactic acid as a byproduct. This allows for prolific growth and a decrease in the pH of the oral environment, leading to enamel demineralization. Though *S. mutans* is considered to be the ubiquitous cariogenic bacteria, the abundance is relatively low (<5%) compared to other bacteria observed, which indicates that there are other bacteria that play a significant role. Other common *Streptococcus* species associated with caries development are *Streptococcus anginosus* and *Streptococcus parasanguinis*. The results from 16S rRNA sequencing and mWGS in our study show an increased abundance of these bacteria in the white spot group in accordance with published literature. Drucker and Green[40] first identified *S. anginosus* as cariogenic. Nonong et. al.[41] determined that *S. anginosus* has the glucosyltransferase enzyme than converts glucose to glucans, which aids in adherence to plaque retentive factors. *S. parasanguinis* has been reported to be present in caries in the absence of *S. mutans*.[42] This bacterium can ferment multiple carbohydrates into lactic acid and can bind to tooth structure to act

as a cornerstone to biofilm formation. All of these bacteria have acid tolerance and can survive at a pH of 5.0 and lower, allowing them to exist in an environment that provokes enamel demineralization.

Contrary to cariogenic *Streptococcus* species, *Streptococcus sanguinis* is associated with a healthy plaque biofilm. Zhu et. al.[43] reported that *S. sanguinis* has an inverse relationship with cariogenic species. There is an antagonistic relationship that exists between *S. mutans* and *S. sanguinis*: *S. sanguinis* produces hydrogen peroxide that represses the growth of *S. mutans* while *S. mutans* produces mutacins I and IV that suppress *S. sanguinis*. This mutual inhibition is driven by environmental conditions. In a nutrient rich environment, *S. mutans* thrives due to the increased availability of carbon sources to metabolize, leading to increased mutacin production, and subsequent decrease in *S. sanguinis* and hydrogen peroxide production. This further decreases the inhibitory ability of *S. sanguinis* on *S. mutans*. Our results are comparable with the literature in that a higher abundance of *S. sanguinis* can be found in the no white spot group. This species was significantly more abundant with the 16S rRNA sequencing, but only marginally significant with mWGS.

According to 16S rRNA sequencing, bacteria found in statistically significant higher abundance in the no white spot group were *Eikenella, Leptotrichia, Lachnoanaerobaculum, Rothia, Strepotococcus* species, and *Corynebacterium*. According to mWGS, the bacteria in statistically significant higher abundance in the no white spot group were *Gemella morbillorum, Lautropia mirabilis, Neisseria sicca, Poryphyromonas* sp. oral taxon, *Haemophilus parainfluenzae,* and *Capnocytophaga gingivalis*.

Jiang et. al.[44] reported that *Eikenella*, *Poryphoromonas*, and *Capnocytophaga* have all been found in higher abundance in the absence of caries. Aas et. al.[13] found that *Leptotrichia* and

Capnocytophaga are found at higher abundances in the absence of white spot lesions. The same study found *G. morbillorum* at higher levels in healthy subjects and significantly reduced on the healthy enamel of subjects with caries. Johannson et. al.[45] reported that *Lachnoanaerobaculum* is part of the core plaque microbiome, but was not in higher abundance in the presence or absence of caries. Qudeimat et. al.[46] reported that *L. mirabilis* was relatively more abundant in caries-free subjects. Kressirer et. al.[47] found *Neisseria sicca* in higher abundance in caries-free children. Alcohol dehydrogenase has been mapped mainly to *N. sicca*, which is confirmed to produce this enzyme. Alcohol dehydrogenase neutralizes bacterial acid production by reducing nicotinamide adenine dinucleotide, buffering a potentially acidic environment. This could indicate a potential protective quality of *N. sicca*. All of these findings are comparable to the results of our study, in which these bacteria having a higher abundance in healthy plaque.

Schoilew et. al.[48] identified *Haemophilus* and *Rothia* as bacteria that are important for early biofilm formation, but not necessarily more prominent in plaque that is conducive to caries progression. Aas et. al.[13] found *Rothia dentocariosa* as a bacterium in higher abundance in caries. This indicates that certain species of the *Rothia* genus could be associated with caries formation while other species could be associated with health. Because *Rothia* was only significant in 16S rRNA sequencing in our study, only the *Rothia* OTU could be identified and not the particular species. Qudeimat et. al.[46] also found that *Corynebacterium* species were more prevalent in caries-free subjects. This disagrees with the findings of Jiang et. al.[44] which reported higher abundances of *Corynebacterium* in subjects with caries and, more specifically, white spot lesions. Johansson et al.[45] reported that *Corynebacterium* is another core bacterium that can be considered a cornerstone in plaque development. The majority of bacteria found in the no white spot group were comparable to what is published in the literature, with the only significant discrepancy being

Corynebacterium. Since this was also only identified at the OTU level, some species could be associated with health, while others could be associated with caries progression.

In the white spot group, bacteria found in statistically significant higher abundance according to 16S rRNA sequencing were *Fusobacterium, Prevotella, Selenomonas, Dialister, Bifidobacterium, Actinomyces, Schwartzia,* and *Veillonella*. The bacteria in higher abundance in the white spot group according to mWGS were *Cryptobacterium curtum, Megasphaera micronuciformis, Alloprevotella rava,* unclassified *Gemella* species, *Shuttleworthia satelles, Dialister invisus, Atopobium rimae, Atopobium parvulum,* unclassified *Veillonella* species, *Selenomonas flueggei, Peptostreptococcus stomatis, Actinomyces* sp. oral taxon, *Oribacterium* sp. oral taxon, and unclassified *Olsenella* species.

Actinomyces is commonly associated with cariogenic bacteria. Jiang et. al.[44] reported a higher abundance in subjects with white spot lesions, but a lower abundance in cavitated enamel. They concluded that *Actinomyces* plays a significant role in the initial formation of caries. Aas et. al.[13] also reported that *Actinomyces* was associated with caries. This agrees with both of our 16S rRNA and mWGS results of an increased abundance in the white spot group. *Actinomyces* also has a strong coaggregation with *Veillonella, Prevotella, Streptococcus*, and *Gemella* species. *Actinomyces* has been shown to be suppressed by an acidic environment, which is required for enamel demineralization.[44] This supports the idea that *Actinomyces* plays a role in initial caries formation, but shows a decreased abundance in later caries progression.

As determined by both Aas et. al.[13] and Jiang et. al.[44], *Veillonella, Prevotella,* and *Olsenella* species are associated with carious lesions. Tanner et. al.[18] also determined that *Veilonella* is more abundant specifically in white spot lesion biofilm. As determined by both Aas et. al.[13] and Tanner et. al.[18], *Bifidobacterium* is more abundant in the presence of caries and, more specifically, white

spot lesions. Aas et. al.[13] reported an increase in *Selenomonas, Dialister,* and *Peptostreptococcus* species in caries. Jiang et. al.[44] reported an increase in *Cryptobacterium, Megasphaera,* and *Shuttleworthia* in caries. Kressirer et. al.[47] reported an increase in *Atopobium rimae* and *Atopobium parvulum* abundance in children with caries. These results are comparable to our results, which demonstrated an increased abundance of these bacteria in the white spot group. Our study identified *Schwartzia* and *Oribacterium* species in higher abundances in the white spot group. There is no published literature correlating these bacteria with caries progression, suggesting that these could be novel cariogenic bacteria.

In our study, *Fusobacterium* was observed to be in higher abundance in the white spot group. This agrees with the results of Aas et. al.[13], but disagrees with the results of Jiang et. al.[44], which reported *Fusobacterium* as being more prevalent in health. *Fusobacterium* was only found to be significantly more abundant as determined by 16S rRNA sequencing and not by mWGS in our study. This could indicate that the *Fusobacterium* genus has some species more abundant in health and others more abundant in caries. This cannot be distinguished at the OTU level. Schoilew et. al.[48] reported that *Fusobacterium* forms bridges in healthy and diseased plaque that allow for the biofilm to mature. *Alloprevotella* was determined to be in higher abundance in the white spot group. Aas et. al.[13] reported that bacteria in this genus are considered part of the core bacteria that form the dental biofilm. Though this bacterium is important in biofilm formation, there is no difference in its abundance between healthy and cariogenic plaque. In the same study, specific *Gemella* species were identified as an important factor in root caries. In our study, an unclassified *Gemella* species was more abundant in the white spot group.

Many studies identify various *Lactobacillus* species as cariogenic.[13] In our study, there was no difference in the abundance between the no white spot and white spot groups. *Lactobacillus*

species have been observed to have an increased abundance in dentin caries and enamel cavitation. Because white spot lesions are early demineralization of enamel, rather than dentin caries or cavitation, a higher abundance of this genera of bacteria is not expected. Tanner et. al.[18] reported *Granulicatella* and *Scardovia* as having increased abundance in the presence of white spot lesions. Our study disagrees with these findings, as there was no difference in abundance of these bacteria between the no white spot and white spot groups. Tanner et. al.[18] also identified *Cardiobacterium* as potentially protective against enamel demineralization. In our study, there was no statistically significant difference in abundance observed between the no white spot and white spot groups.

Several metabolic pathways of bacteria were discovered to be in higher abundance in both the no white spot and white spot groups. The relative abundance of each pathway is very low, but there could still be clinical significance. Most of these pathways have not been reported in the literature to have a positive or negative correlation to caries progression. Only assumptions and hypotheses can be made about the clinical significance of each pathway. Conclusions may be drawn from further investigation of each pathway found to be in statistically significant abundance in each group.

Korithoski et. al.[49] reported that when *S. mutans* is grown in excess of glucose, lactate is the major fermentation product. This highly acidic end product of glycolysis can contribute to a decrease in the pH of the dental environment, leading to enamel demineralization. Under conditions of limited glucose, acetate is produced instead of lactate. Our study found the metabolic pathway of pyruvate fermentation to acetate to be more abundant in the no white spot group. This could indicate that the no white spot group has less pyruvate fermentation to lactate, allowing the oral environment to maintain a higher pH, and thus reducing the process of enamel demineralization.

Another pathway found to be in higher abundance in the no white spot group was aerobic respiration. The main product of glycolysis, which is a common mechanism of glucose metabolism in bacteria, is pyruvate. The metabolism of pyruvate can occur in an aerobic (oxygen present) or anaerobic (oxygen absent) environment. When oxygen is absent, fermentation occurs, leading to an end product of lactic acid. When oxygen is present, aerobic respiration occurs through the Krebs cycle and oxidative phosphorylation, and lactic acid is not produced as a byproduct.[50] Because aerobic respiration was determined to be in higher abundance in the no white spot group, it can be hypothesized that less fermentation occurred in this group, and that subsequently, less lactic acid was produced. With less lactic acid present in the environment, pH can be maintained at a normal level, preventing enamel demineralization. The NAD NADH phosphorylation dephosphorylation pathway, which is part of oxidative phosphorylation in aerobic respiration, was also in higher abundance in the no white spot group. This pathway does not have a lactic acid byproduct, and thus, would not contribute to a decrease in pH in the oral environment.

The pathway of taxadiene biosynthesis was observed to have a higher abundance in the no white spot group. Taxadiene is formed from the metabolism of acetyl-CoA, which is formed from the metabolism of pyruvate.[51] If pyruvate is being used for the taxadiene biosynthesis, less pyruvate is available for fermentation to lactic acid. The pathways of palmitate[52], polyisoprenoid[53], and farsenol[54] biosynthesis were also observed to have a higher abundance in the no white spot group. Like taxadiene biosytnethesis, acetyl-CoA, and hence pyruvate, is necessary for these pathways. It can be hypothesized that these pathways can therefore be caries resistant because less pyruvate would be available for fermentation to lactic acid. The pentose phosphate pathway (PPP) was also observed to have a higher abundance in the no white spot group. The PPP is a shunt off of glycolysis to form NADPH and ribose 5-phosphate. Because this is a deviation from glycolysis,

pyruvate is not formed and cannot be metabolized to lactic acid.[55] Of the 36 pathways identified to have a statistically significant difference in abundance between the no white spot and white spot groups, 33 were more abundant in the no white spot group. Many of these were found to use starting compounds that could otherwise be fermented into lactic acid. It can then be theorized that increased prevalence of these alternate pathways that do not produce lactic acid could decrease the abundance of starting compounds available for fermentation into lactic acid, which would protect from enamel demineralization.

One pathway with a significantly higher abundance in the white spot group was the L-rhamnose degradation pathway. This pathway metabolizes the deoxy sugar L-rhamnose to produce NADH with lactic acid as a byproduct.[56] With lactic acid as a byproduct, a decrease in the environmental pH is expected. Our study has only brushed the surface of microbial pathway analysis in white spot formation by identifying several pathways with a statistically significant difference in abundance between the groups. Future studies can more closely examine these pathways to solidify theories on how they contribute to the cessation or progression of caries. *S. mutans* is considered cariogenic due to its production of lactic acid via glucose metabolism; hence, the involvement of lactic acid in these pathways can be an evidence-based foundation to future studies.

The oral microbiome does not only consist of bacteria. Other microorganisms could contribute to or protect against the progression of caries. Along with the ability of mWGS to identify metabolic pathways and bacteria at the species level, the sequencing can identify fungi as well. In our study, only 16 species of fungi were identified with mWGS, with the greatest abundance being *Candida albicans* and *Malassezia restricta*. Both of these fungi had a statistically significant difference in abundance between the two groups with *M. restricta* being increased in

the no white spot group and *C. albicans* being increased in the white spot group. Baraniya et. al.[57] reported similar findings with *Malassezia* genus as the most commonly detected, followed by *Candida*. *Candida albicans* is the most commonly investigated oral fungus because of its ability to cause oral mucosal infections, particularly in an immunocompromised patient. Beyond *Candida,* there is little known about fungal contribution to caries. *M. restricta* has been reported as a core fungi in the oral microbiome, and has been positively correlated with health-associated microorganisms and negatively correlated with cariogenic bacteria, such as *Actinomyces*. This caries antagonistic trait agrees with our study with a statistically significant higher abundance in the no white spot group. In the white spot group, the abundance was relatively nonexistent with an abundance close to 0%.

Fechney et. al.[58] found no association between the presence of *C. albicans* in children with caries. Baraniya et. al.[57] had contrasting results with a higher abundance in children with caries. They also found that *C. albicans* correlated with caries-associated bacteria, such as *S. mutans*. Our study found an increased abundance of *C. albicans* in subjects with white spot lesions. The no white spot group only had a mean abundance of 7.14%, indicating that other fungal species accounted for more than 92% of the fungal microbiome. Contrary to the no white spot group, *Candida albicans* accounted for 99.85% of the fungal species present in the white spot group.

Malassezia globosa has been shown to be more abundant in caries-free and early caries individuals, such as white spot lesions.[57] This would explain the lack of a significant difference in abundance between the groups. Our study was examining a difference between no white spot and white spot groups, which would be similar to caries free and early caries individuals.

Beyond bacteria and fungi, acellular viruses can also be identified with mWGS. In all of the samples, 49 virus types were observed, with 35 of them being *Streptococcus*-specific phages.

Only two of the viruses were identified to have a statistically significant difference in abundance between the no white spot and no white spot group, with a higher abundance in the no white spot group. Both of these viruses, *Streptococcus* phage phiAR10462 and *Streptococcus* phage phiAR10746, are *Streptococcus*-specific bacteriophages, indicating that they only parasitize the *Streptococcus* genera. There have been almost 50 bacteriophages known to infect *S. mitis, S. mutans, S. oralis, S. salivarius,* and *S. sobrinus*.[59] The life cycle of a bacteriophage includes: (1) attachment to host receptor, (2) phage DNA injection, (3) replication of bacteriophage DNA, (4) biosynthesis of bacteriophage proteins, (5) maturation by forming and assembling new bacteriophage particles, and (6) bacterial cell lysis to release the newly formed virus particles. The key step in the bacteriophage life cycle is lysis of the host cell. This ultimately leads to death of host cell, which decreases the abundance of the bacteria. This is beneficial if the bacteriophage is specific to cariogenic bacteria, such as *S. mutans*. It can be hypothesized that an increase in *S. mutans*-specific bacteriophages could lead to protection from caries progression. The most commonly studied *S. mutans*-specific bacteriophage is M102. Our study did not identify this bacteriophage as significantly more abundant in the no white spot group. Because the two phages identified to be more abundant in the no white spot group are specific to *Streptococcus* genera, parasitism and eventual lysis of cariogenic *Streptococcus* species could have played a role in decreased white spot formation.

A few weaknesses can be identified in this study. When determining if subjects have white spots, only a qualitative, binary yes or no answer was given, rather than a quantitative value of white spot progression. Several studies identified a shift in microbiome composition as caries progressed from initial demineralization to cavitation. Also, some of the white spot subjects just had white spots adjacent to the bracket, while others had generalized white spots across the buccal

surface. This can alter the results of microbe abundance. Future studies can use the full Gorelick index to quantify the white spot instead of our modified version. Another weakness is that this was a one time-point, cross-sectional sample collection of subjects who have and have not developed white spots. A future study could take a longitudinal approach, by starting with a sample of subjects without white spots at the start of orthodontic treatment and take plaque samples periodically. At the last timepoint, white spot formation can be quantified and the microbiome composition can be compared between the multiple timepoints. Another consideration is the significant difference in number of subjects in each race. There were 74 subjects in the Caucasian group, 11 subjects in the African American group, and 7 subjects in the Asian group. Race was identified as a demographic variable in which the microbiome composition differed. There is a vast difference in number of subjects in the Caucasian group compared to the African American and Asian groups. A future study could have groups with a closer number of subjects of each race to allow for adequate power analysis of this variable. Finally, the utilization of diagnostic intraoral photos to confirm the absence of white spots at the beginning of treatment could be a limitation. The quality of photos differed significantly, depending on the provider, camera, and camera settings. Future studies can consider standardizing these variables across all subjects.

As supported by the literature and the results of this study, white spot lesion formation is a multifactorial process with contributions from demographic, genetic, clinical, and microbial factors. Demographic and genetic factors cannot be altered. Clinical factors such as proper oral hygiene instruction can be beneficial in preventing white spot formation. The microbiome composition has become of interest recently with high throughput sequencing, such as 16S rRNA and mWGS. By identifying cariogenic or protective microorganisms, patients can be identified as having high or low caries risk prior to beginning orthodontic treatment. The field of

pharmaceuticals has not yet considered antibacterial, antifungal, probiotic, or proviral approaches to caries prevention. By knowing the exact bacteria, fungi, and viruses with a causation or correlation to caries progression, targeted therapy could be developed to decrease the relative abundance. If certain bacteria or fungi have protective qualities to prevent caries, probiotic oral supplements could be utilized. Finally, cariogenic bacteriophages could be introduced into the supragingival plaque to lyse harmful cells. This study lays the foundation for many approaches to prevent white spot lesions.

Many differences were noted between the groups, particularly in microbiome composition, metabolic pathways, and bacteriophages. Future studies are needed to examine specific mechanisms of action and how they relate to caries progression, particularly in the orthodontic patient.

Conclusions

1. Poor oral hygiene (increased BOP and plaque score), decreased buffer capacity, and increased time in braces are variables associated with the presence of white spot lesions.
2. The microbiome composition of supragingival plaque is different between the no white spot and white spot groups, with an increased diversity associated with white spot formation.
3. The clinical factors that explain the variation of microbiome composition between the groups are white spot presence, salivary pH, salivary buffer capacity, BOP, and race of the subject.
4. Specific species of bacteria and fungi are associated with healthy plaque and with cariogenic plaque.

5. Specific metabolic pathways are associated with no white spots and with white spots.
6. There are *Streptococcus* bacteriophages that correlate with the absence of white spot lesions.

Tables and Figures

Figure 1: Microbial profile of subgingivae in patients with and without bracket treatment by 16S rRNA gene sequencing. Samples were sequenced at baseline before bracket treatment and after 6 and 12 weeks of treatment.[13]

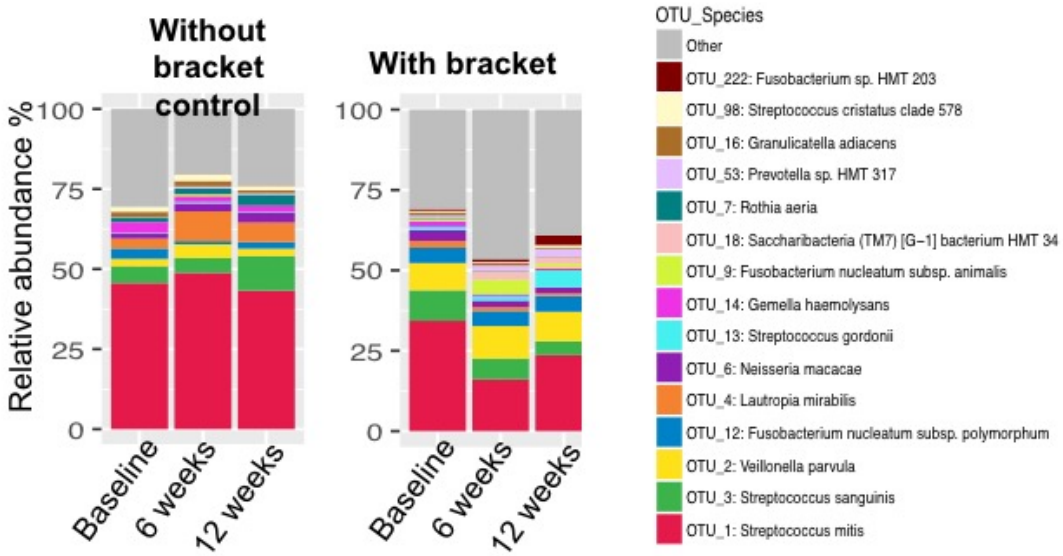


Figure 2: Increased bacterial diversity (Richness and Shannon Diversity) in patients with brackets compared to controls after 6 and 12 weeks of bracket treatment.

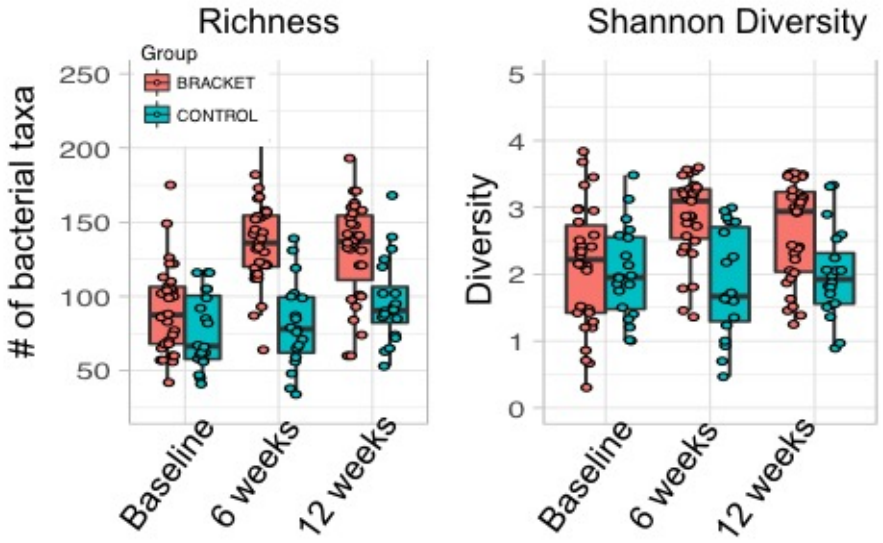


Figure 3: Dental saliva pH indicator chart included in the GC Saliva-Check Buffer Kit®.

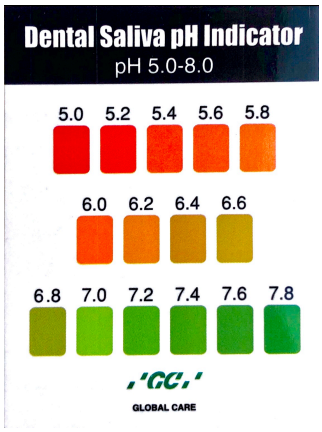


Figure 4: GC buffer capacity color to point conversion table included in the GC Saliva-Check Buffer Kit®.

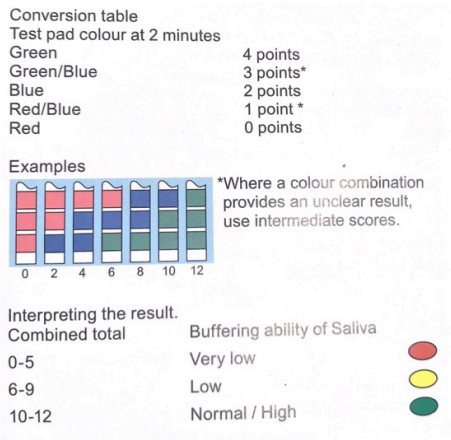


Figure 5: Of 790 OTUs identified by 16S rRNA gene sequencing, 475 were shared by both groups, 142 were unique to no white spot group, and 173 were unique to white spot group.

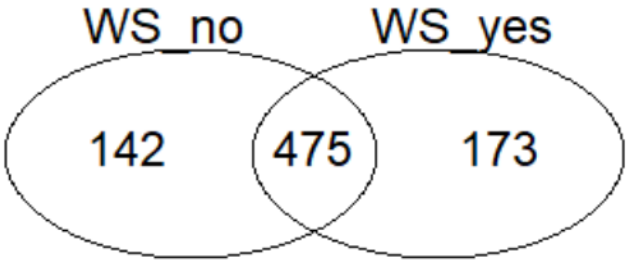


Figure 6: Top 25 most abundant OTUs in all samples.

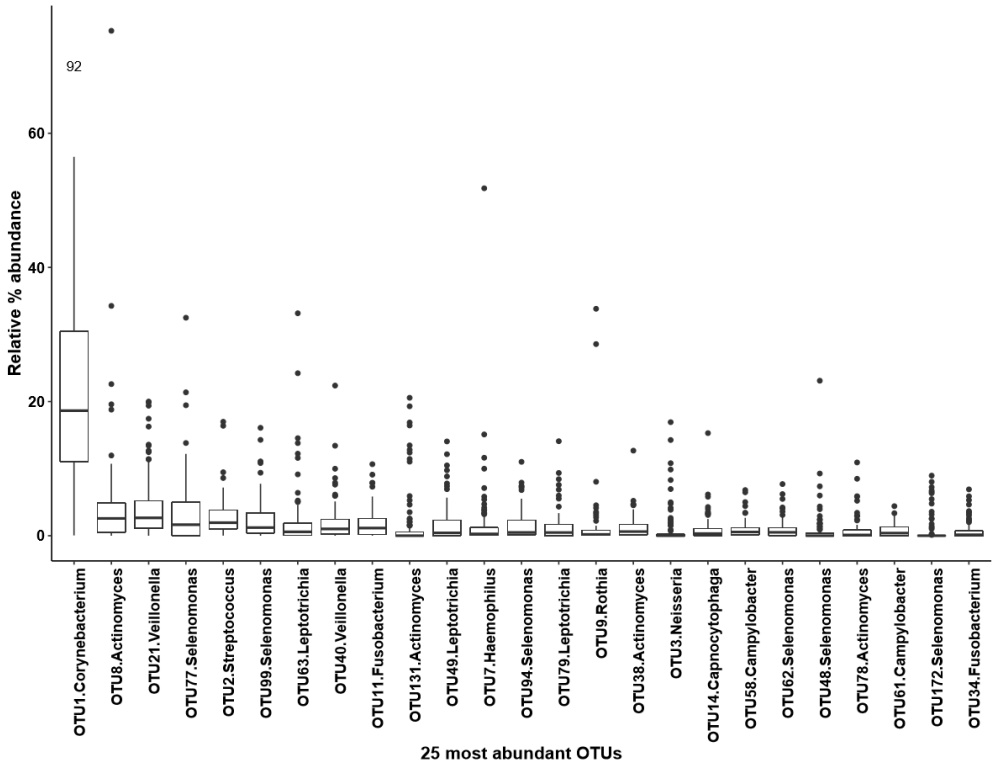


Figure 7: Phylum taxonomy of top 25 OTUs between no WS and WS groups.

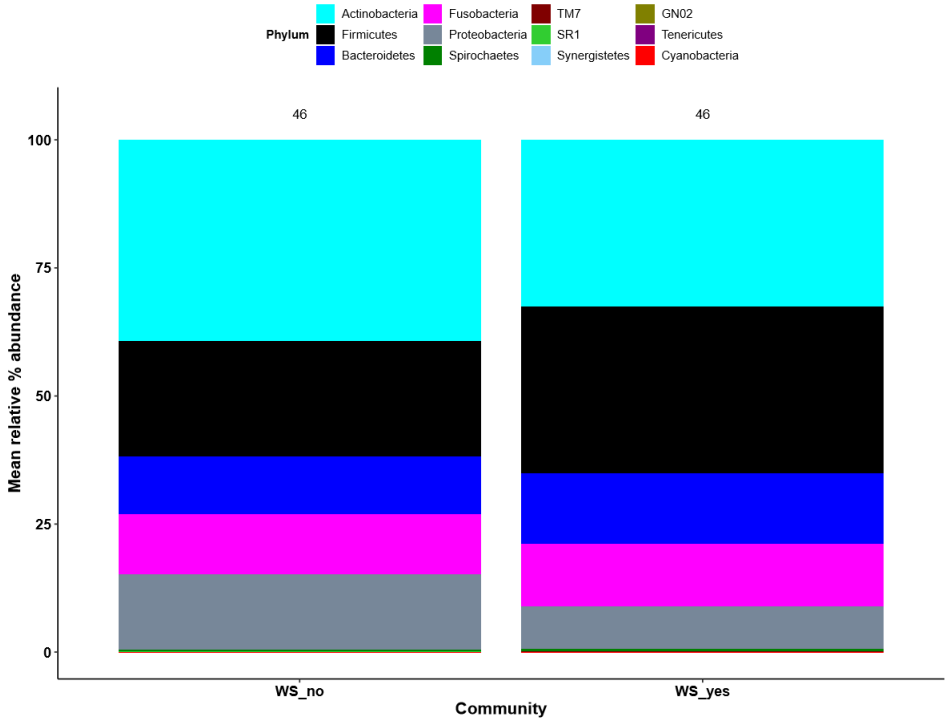


Figure 8: Genus taxonomy of top 25 OTUs between no WS and WS groups.

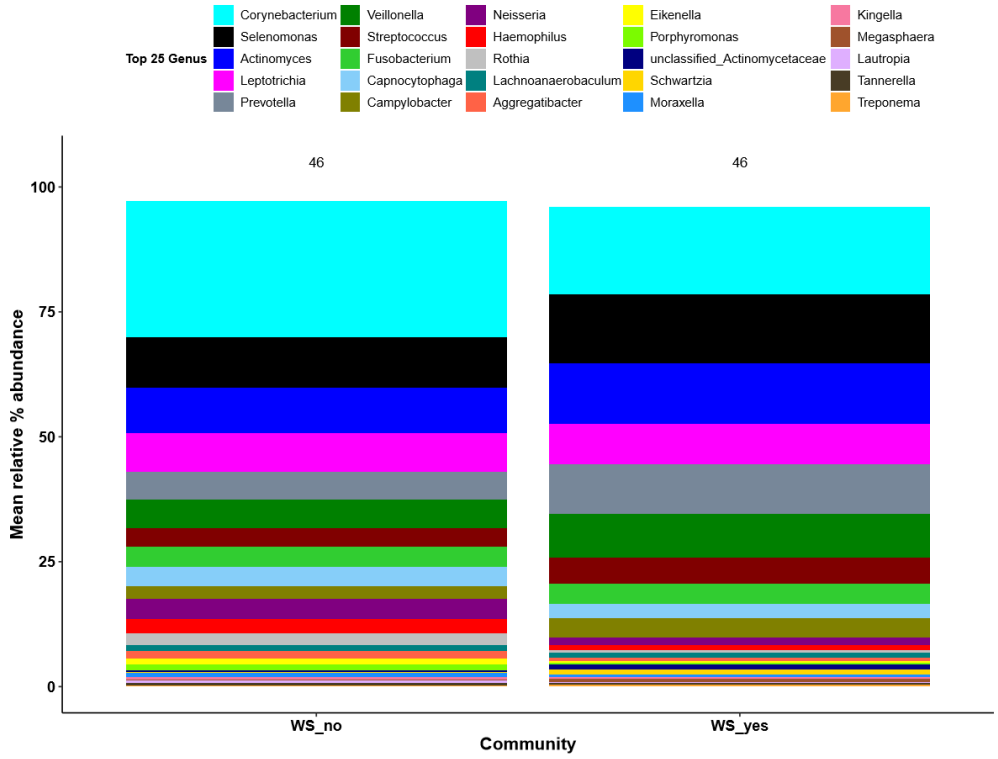


Figure 9: Prevalence of top 25 OTUs between no WS and WS groups, ordered by greatest difference between no WS and WS; top: no WS > WS, bottom: no WS < WS.

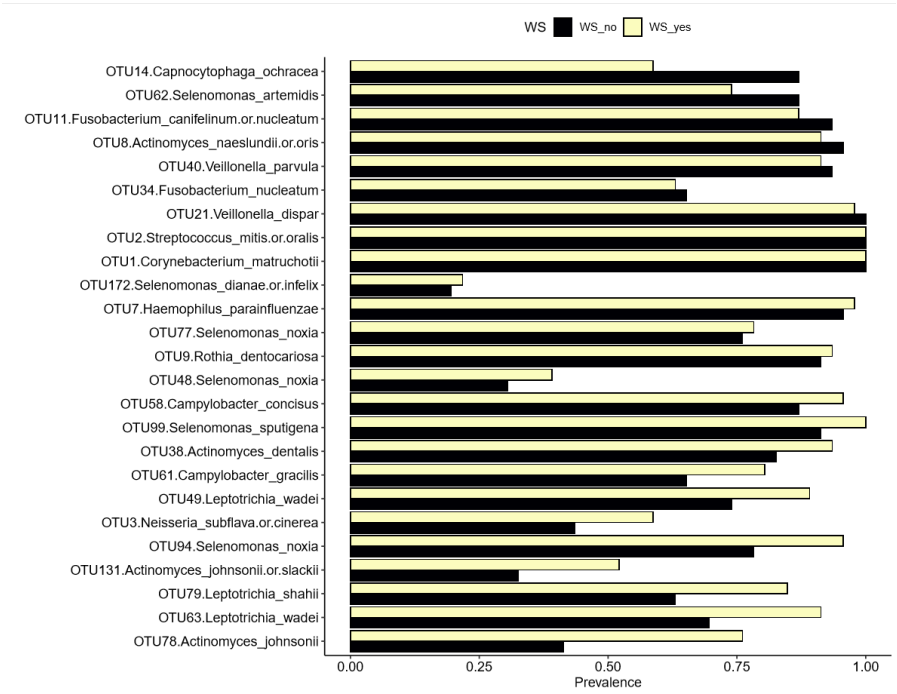


Figure 10: Shannon diversity index demonstrating white spot group has a higher microbiome diversity than the no white spot group, as determined by 16S rRNA gene sequencing.

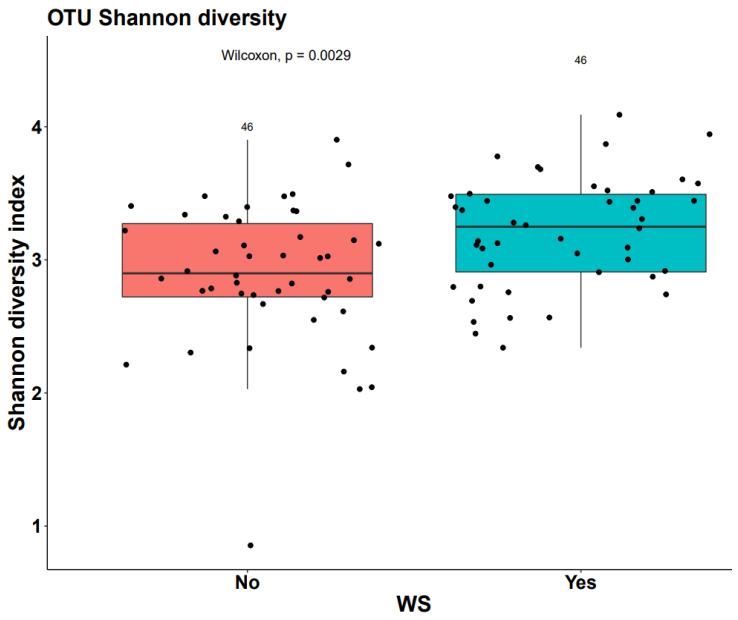


Figure 11: Alpha diversity is statistically significant at the genus level as determined by 16S rRNA gene sequencing.

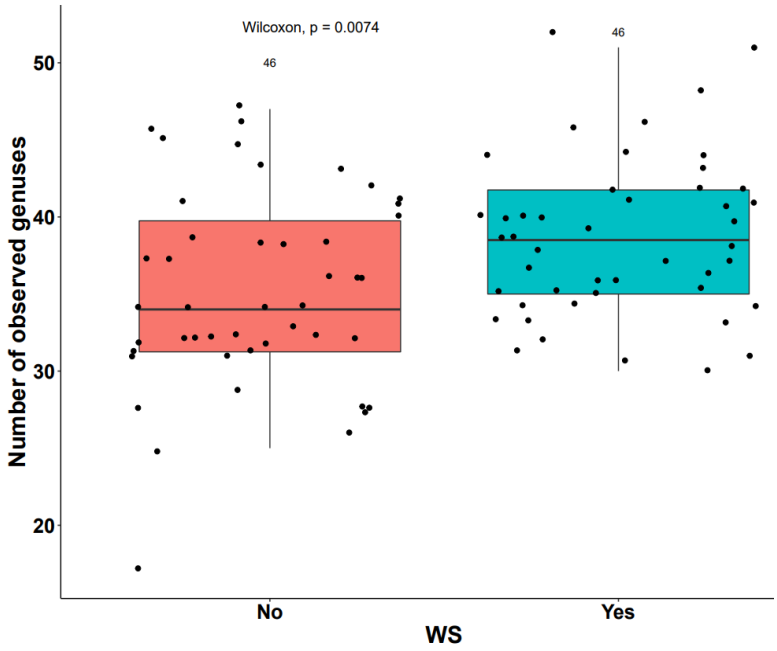


Figure 12: Beta diversity is statistically significant at the OTU level as determined by 16S rRNA gene sequencing.

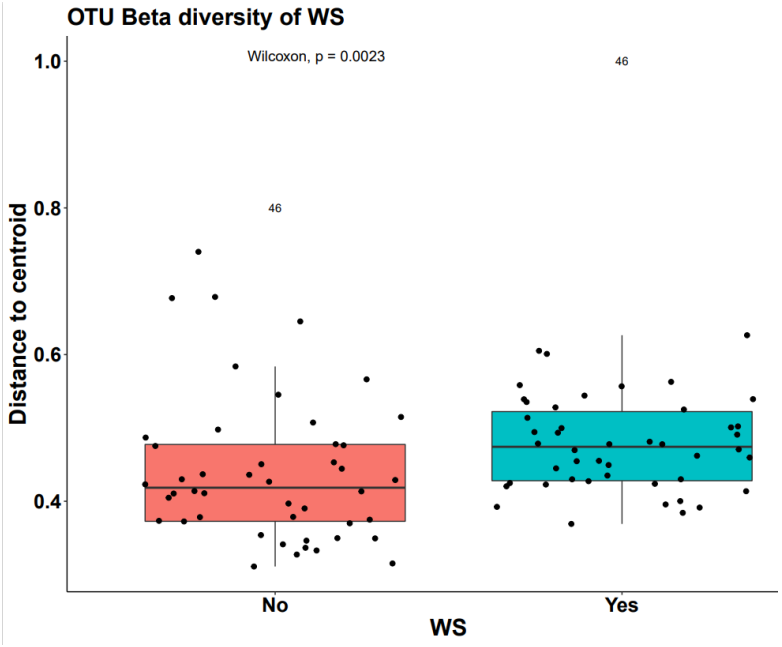


Figure 13: Principal Component Analysis (PCA) of WS to OTU composition demonstrating a significantly different composition between the groups.

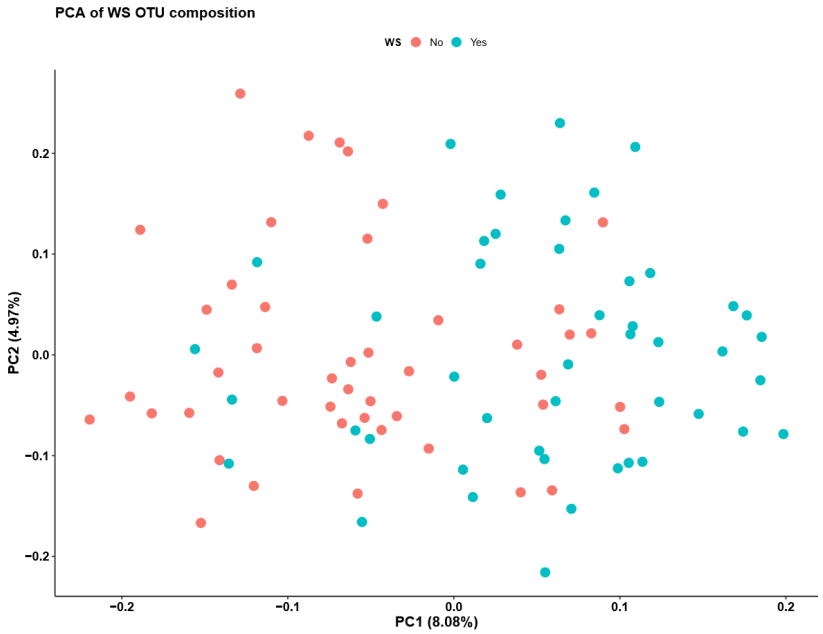


Figure 14: PCA of Race, BOP, Saliva pH, and Saliva buffer capacity to OTU composition.

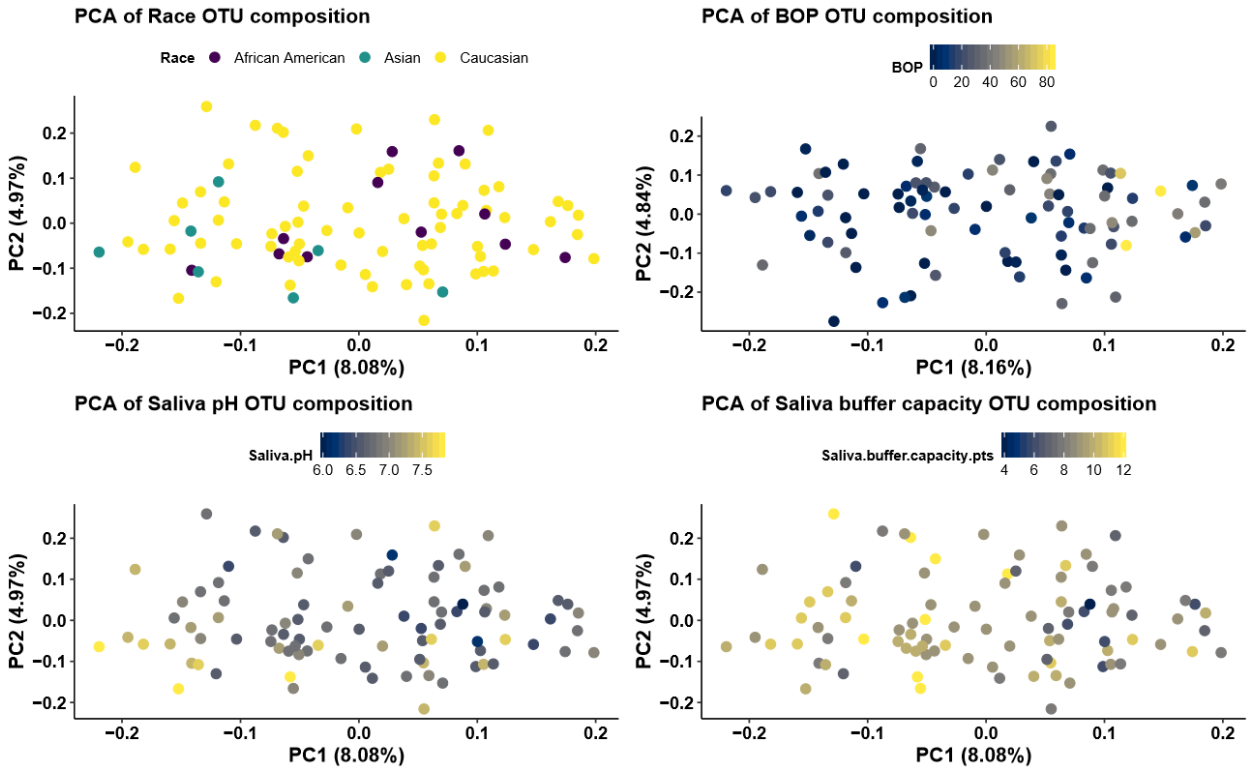


Figure 15: Distance-based redundancy analysis of clinical factors compared to OTU composition.

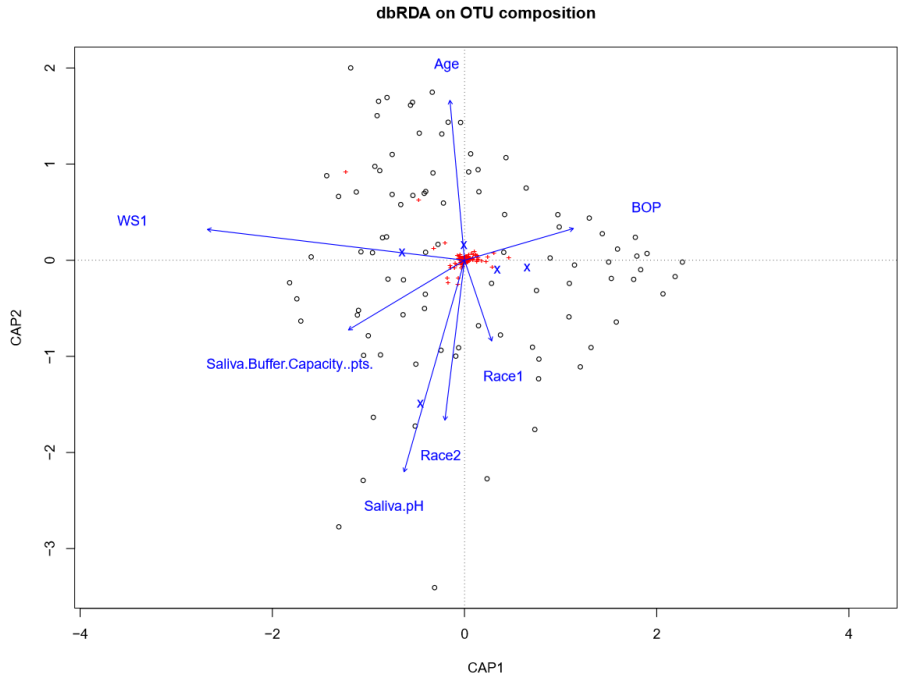


Figure 16: Pearson correlation of clinical factors and 25 most abundant OTUs.

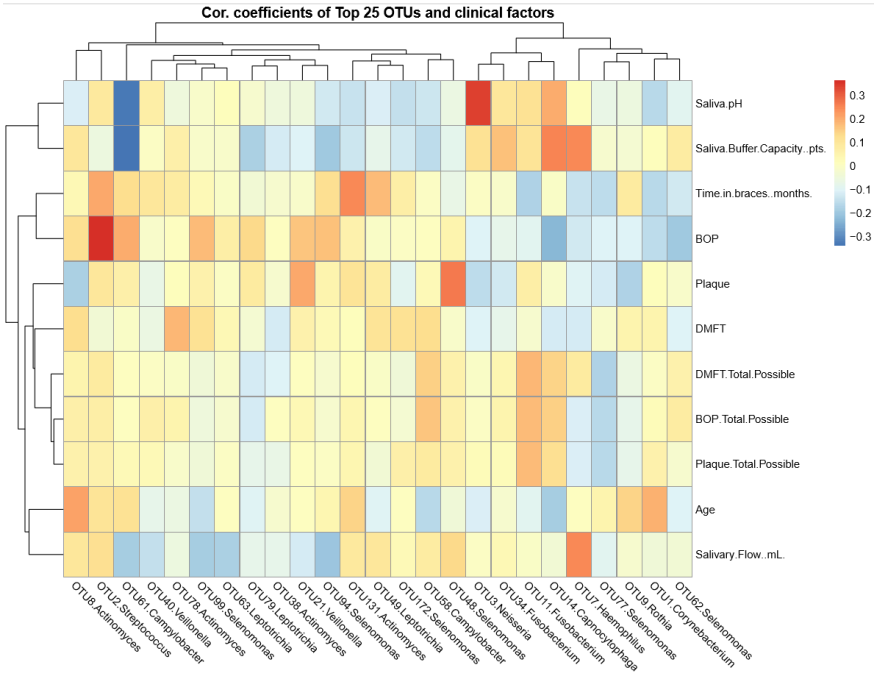


Figure 17: Race OTU prevalence ordered by greatest difference between no WS and WS groups; top: African American > Asian, bottom: Asian > African American.

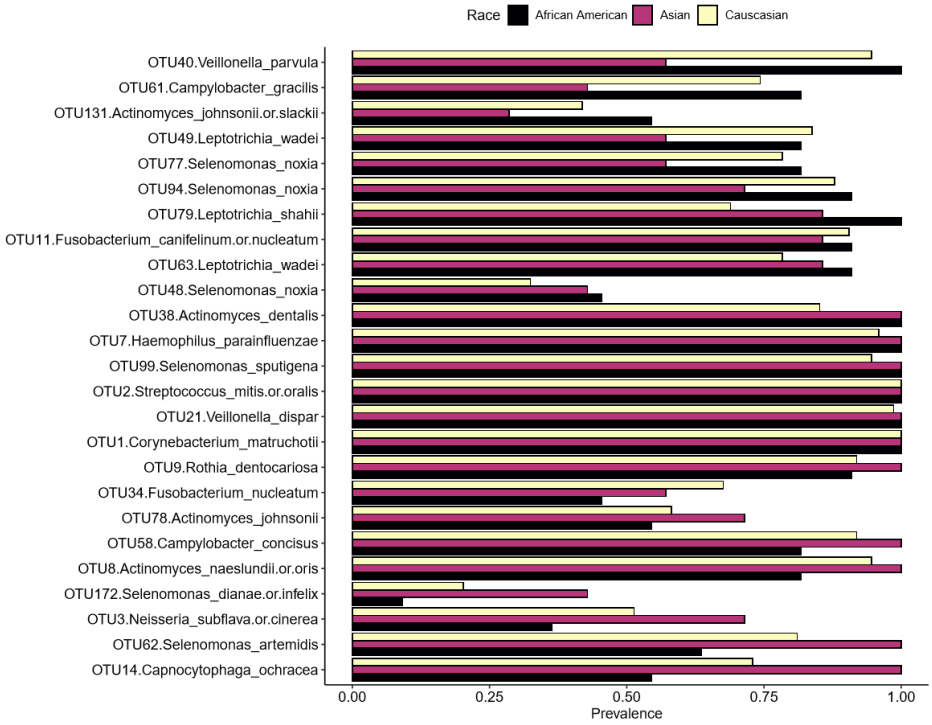


Figure 18: Shannon diversity of three races represented by the subjects demonstrating statistically significant OTU composition between them.

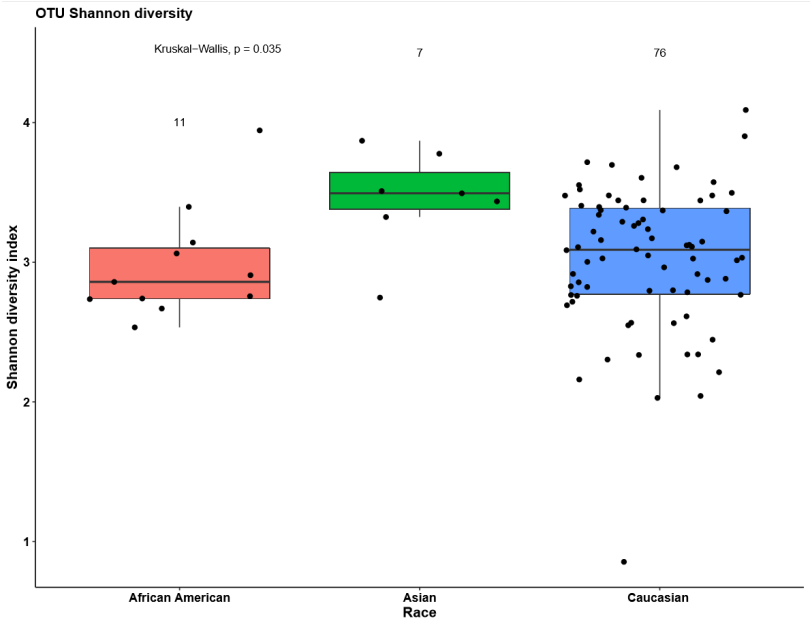


Figure 19: Alpha diversity of three races represented by the subjects demonstrating statistically significant OTU composition between them.

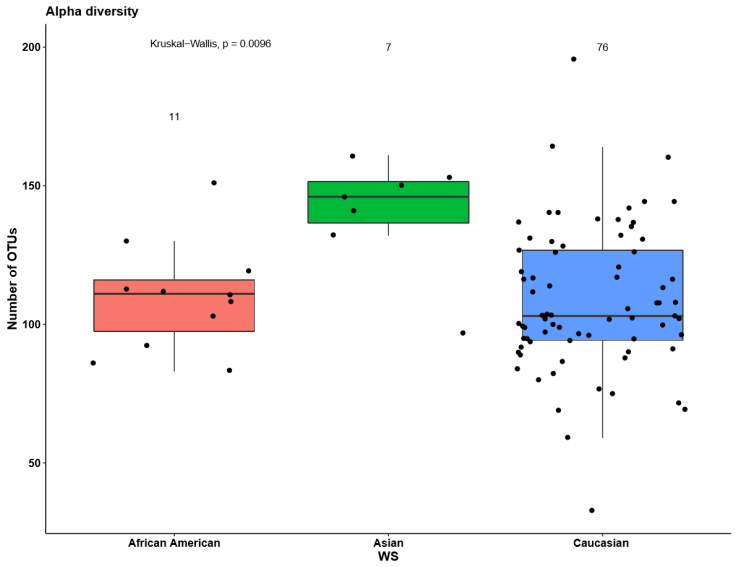


Figure 20: Asian cohort was missing 10% of top 25 most abundant OTUs.

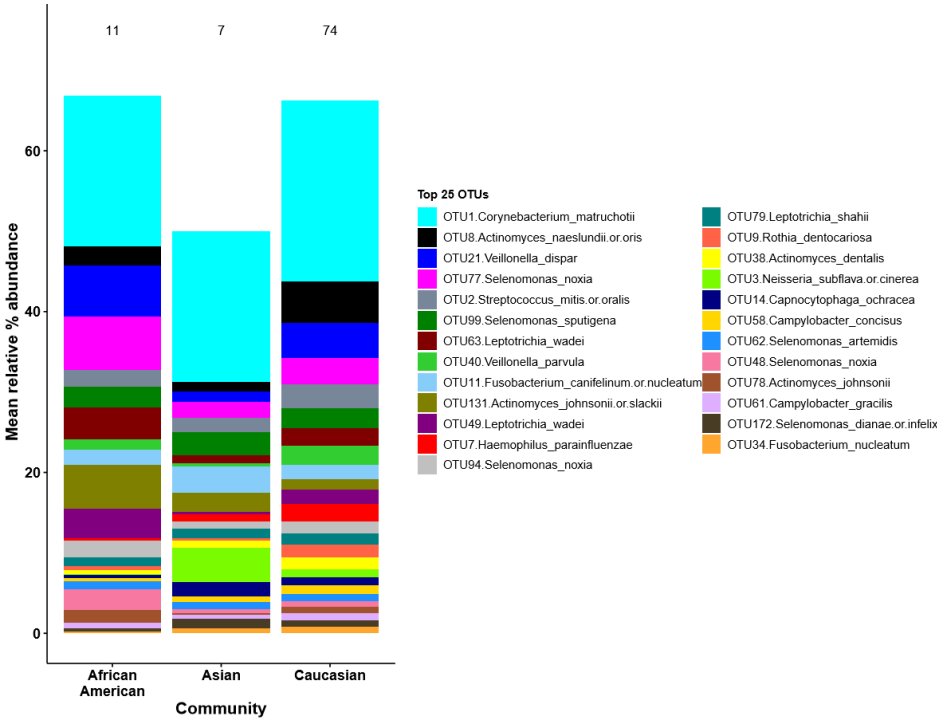


Figure 21: Top 25 OTUs of the Asian cohort compared to other races.

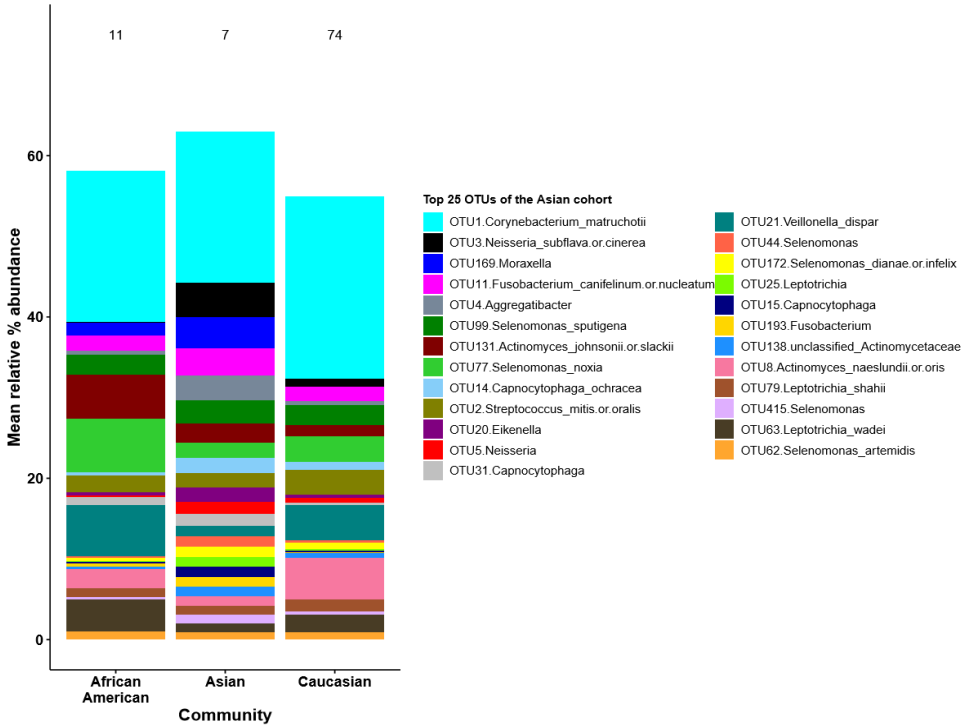


Figure 22: DESeq padj < 0.01 difference in % abundance between no WS and WS groups.

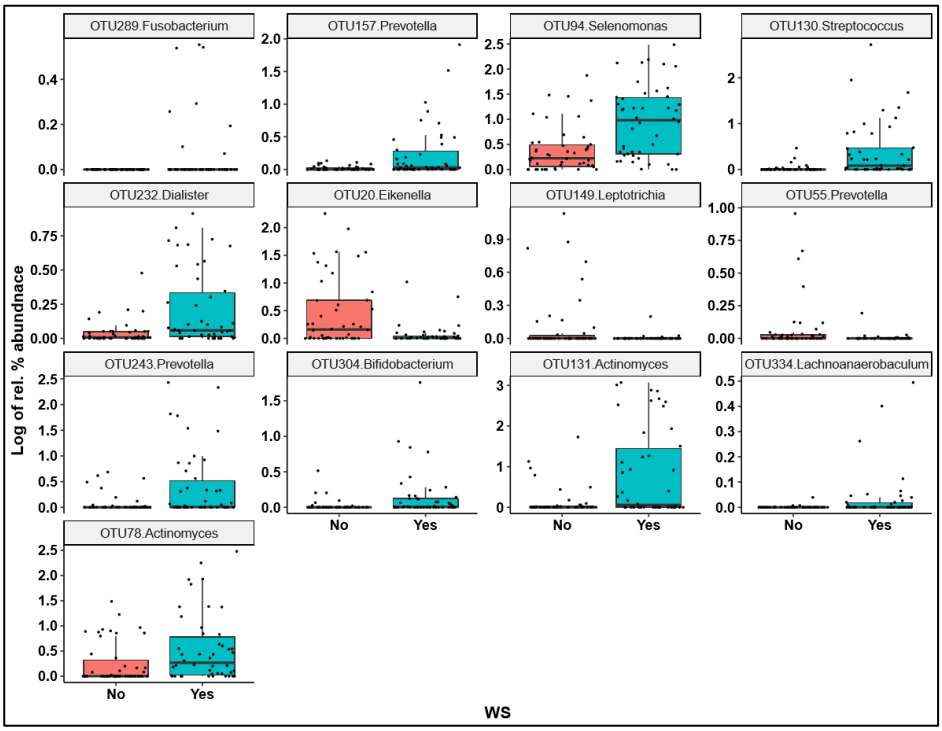


Figure 23: DESeq padj < 0.05 difference in % abundance between no WS and WS groups.

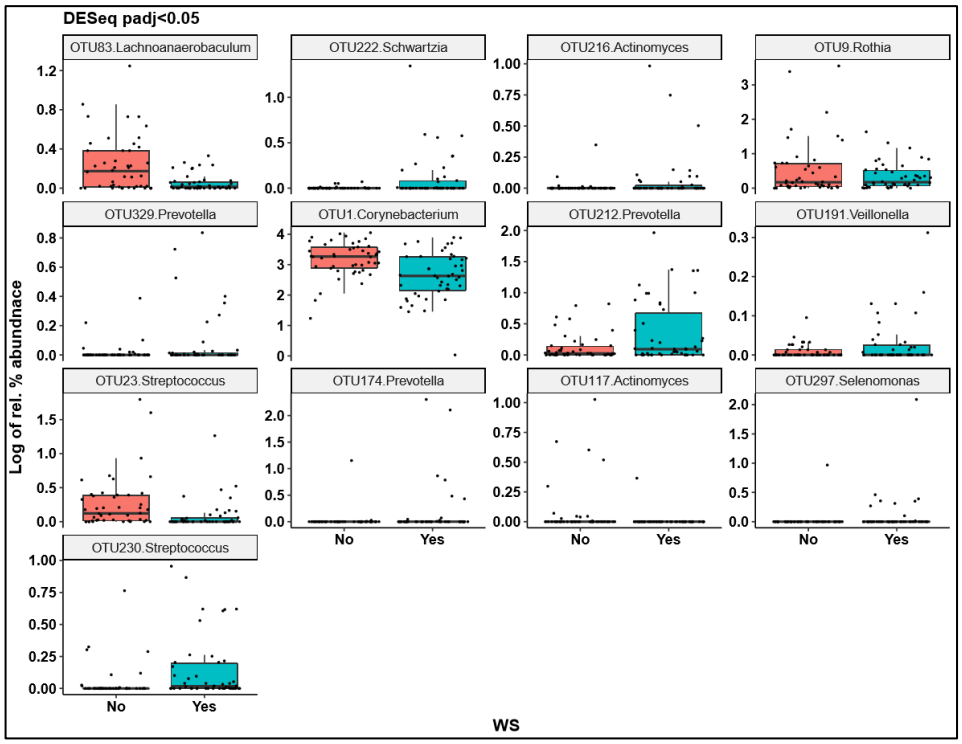


Figure 24: DESeq padj < 0.15 difference in % abundance between no WS and WS groups.

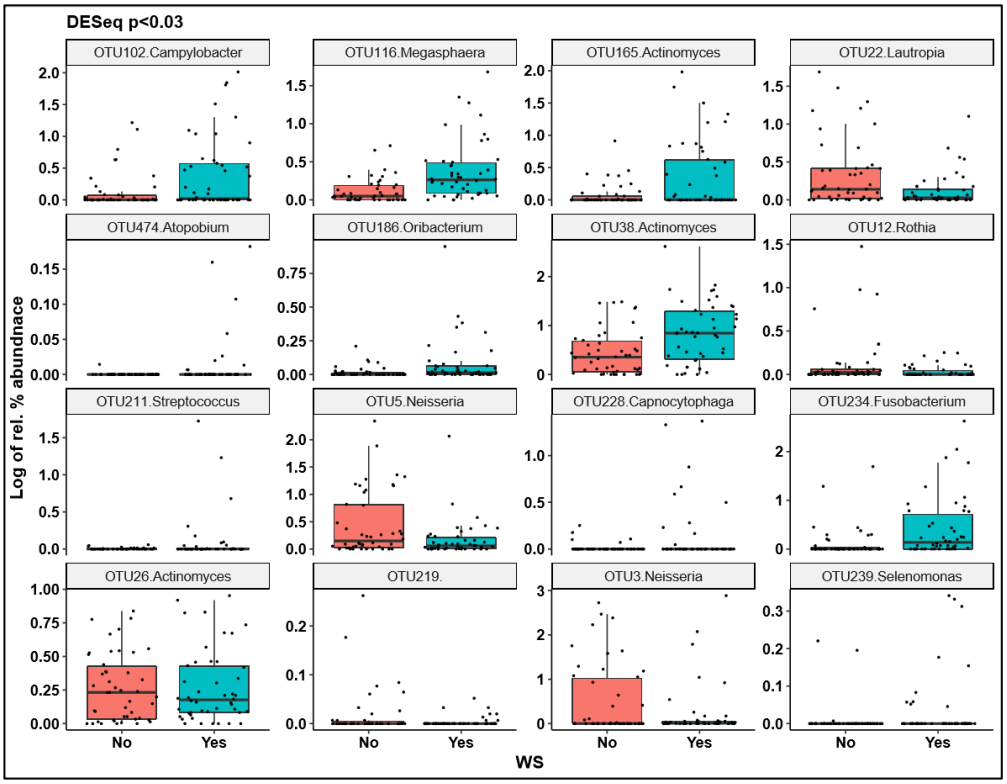


Figure 25: Relative abundance of *Streptococcus* OTUs in all subjects.

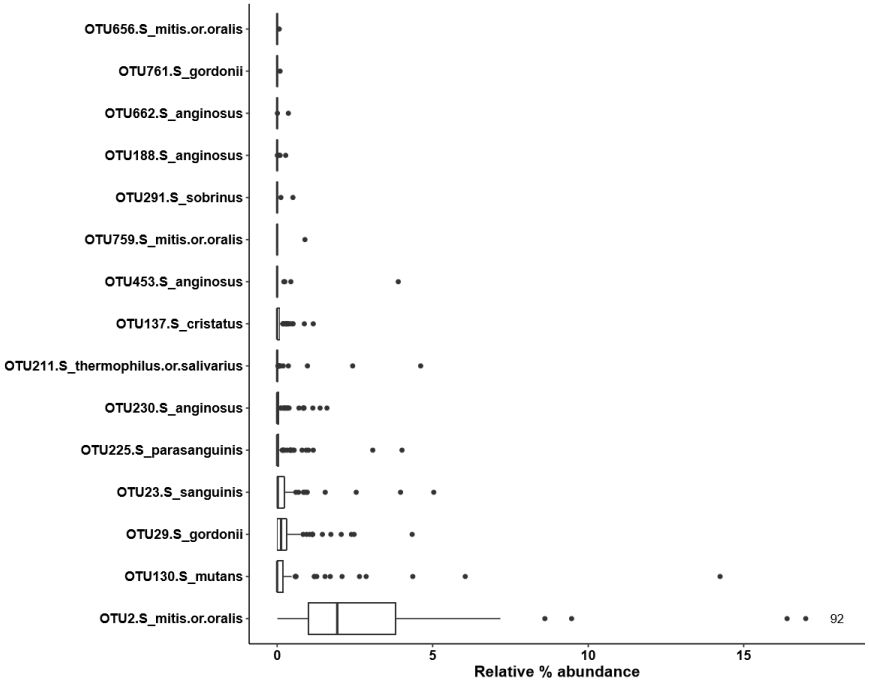


Figure 26: Relative abundance of *Streptococcus* OTUs in no WS and WS groups.

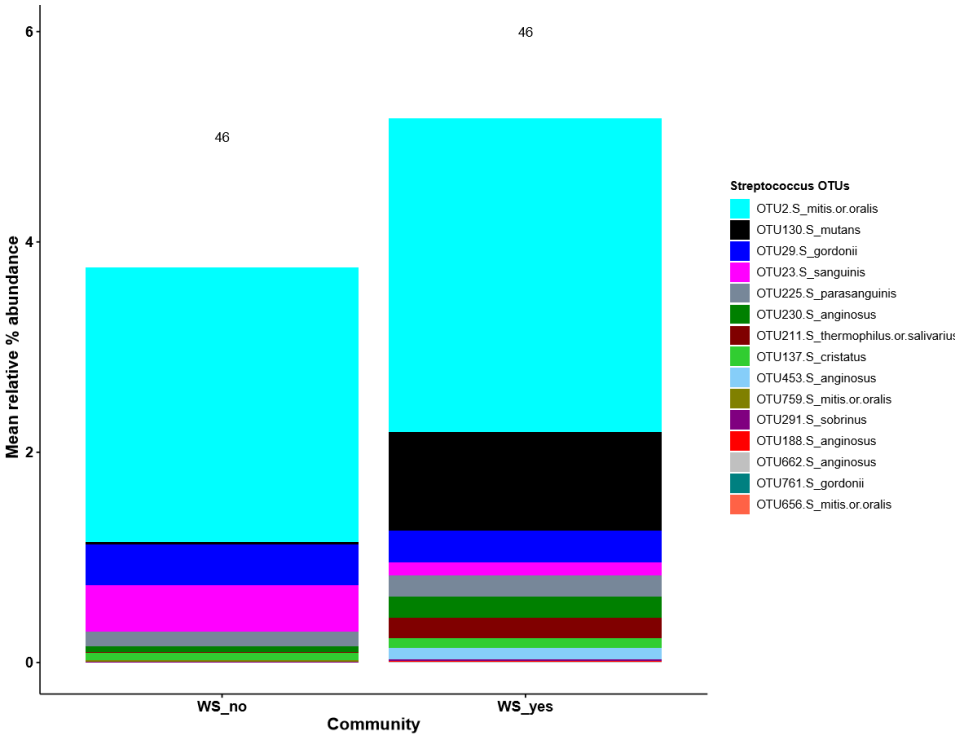


Figure 27: DESeq difference in relative abundance between no WS and WS groups for *Streptococcus* OTUs.

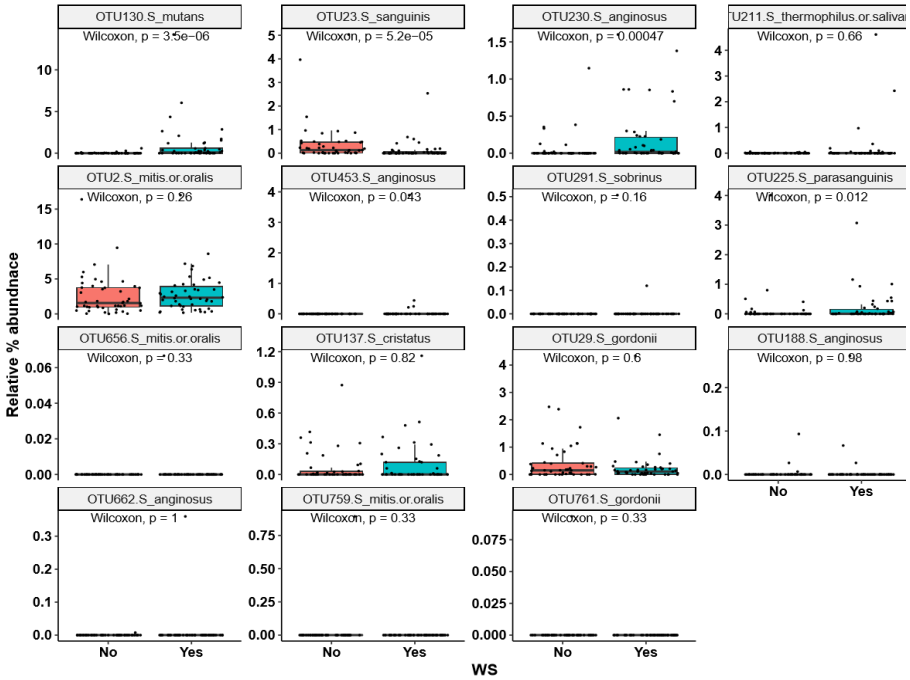


Figure 28: Prevalence of *Streptococcus* OTUs ordered by greatest difference between no WS and WS; top: no WS > WS, bottom: no WS < WS.

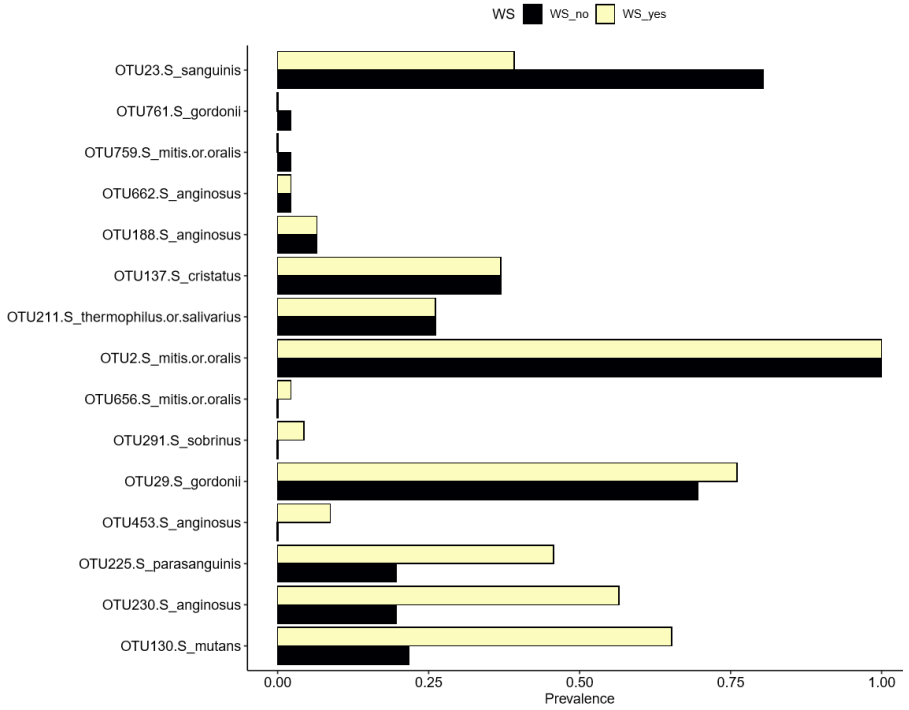


Figure 29: *Streptococcus* species network analysis.

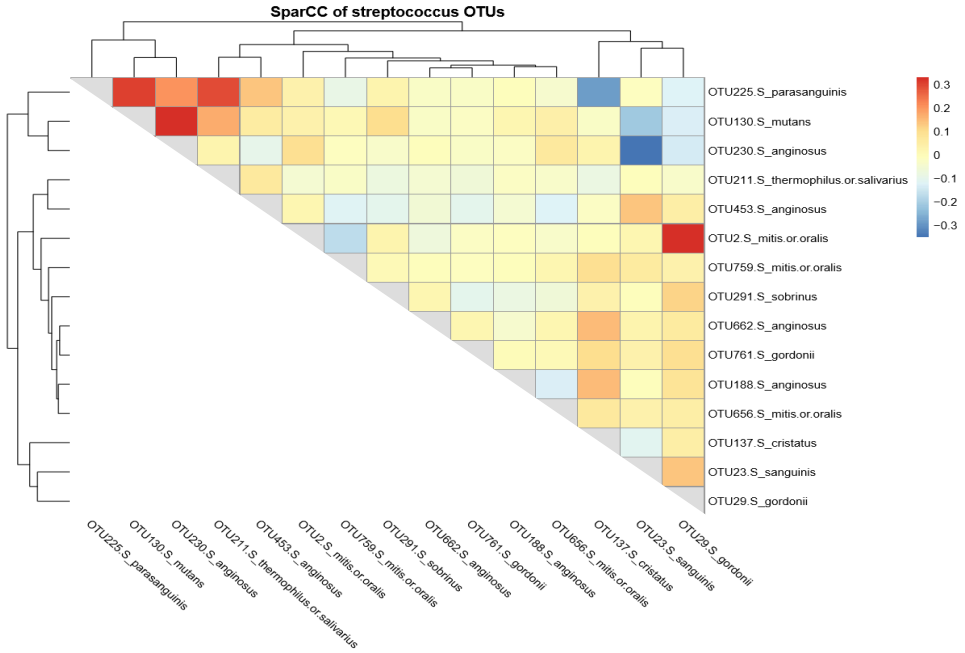


Figure 30: Network analysis of *Streptococcus* species correlated with top 25 most abundant OTUs.

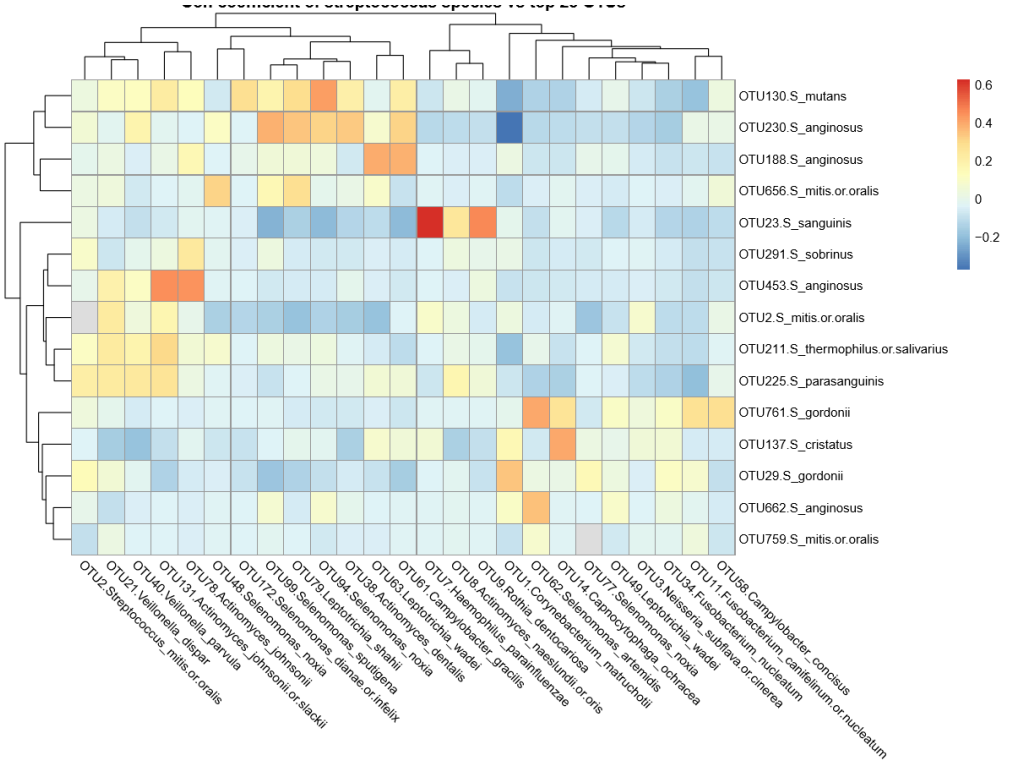


Figure 31: Scatterplots of highest correlation from network analysis of *Streptococcus* against top 25 most abundant OTUs.

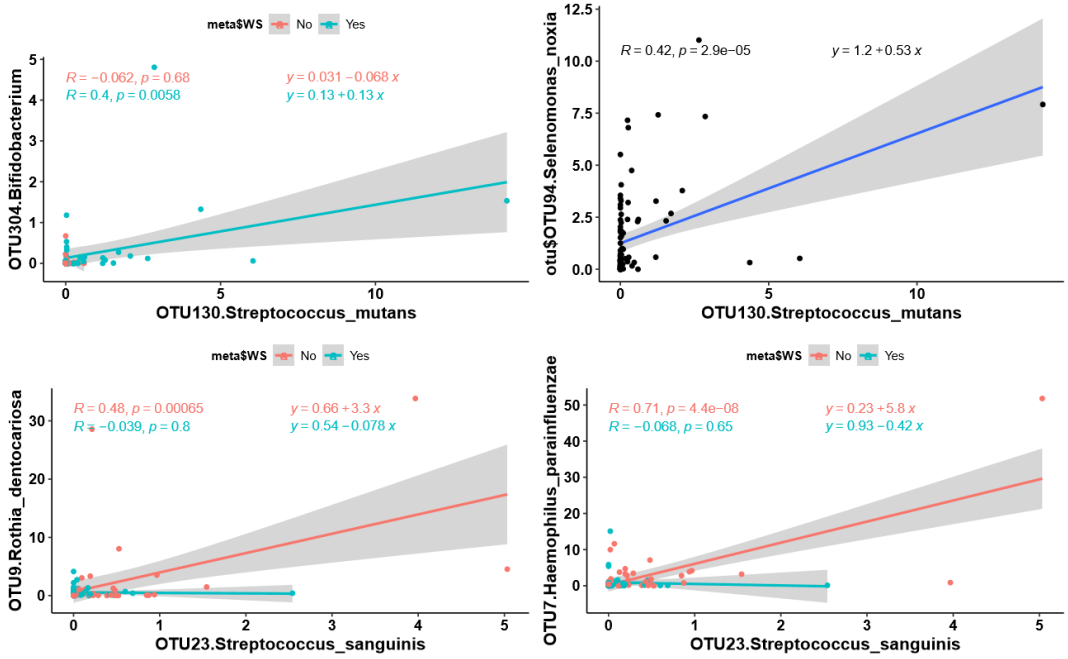


Figure 32: The subset of 10 samples from each group for mWGS sequencing were determined by picking the highest abundance of *Streptococcus* OTUs.

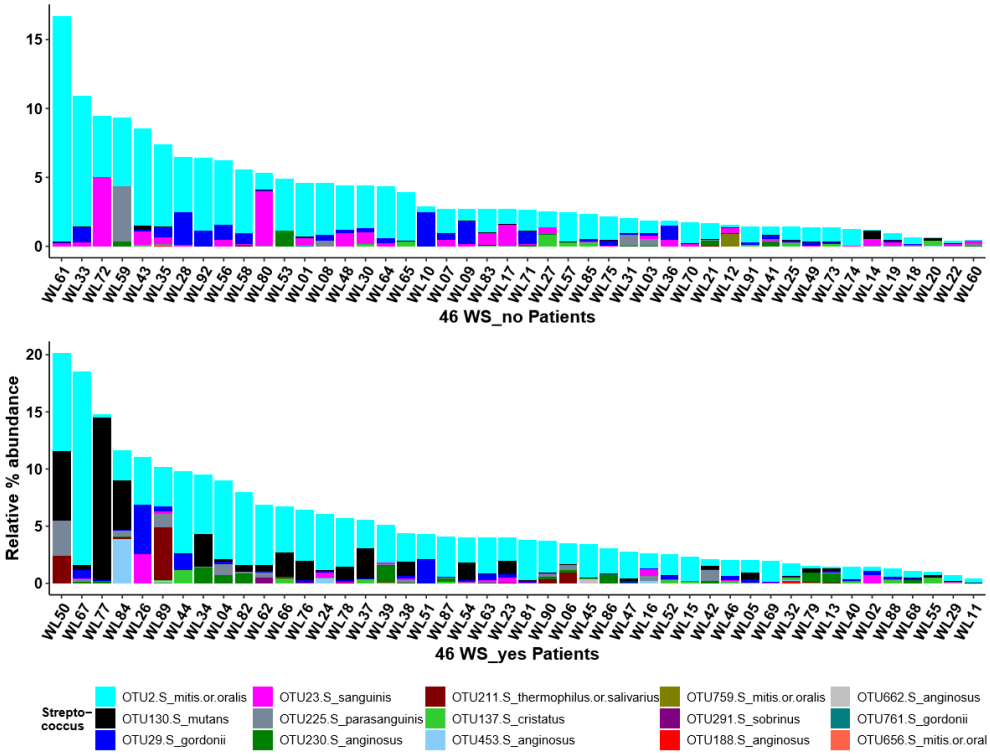


Figure 33: Relative abundance of each of OTUs of n=92 (left) and n=20 (right) subset for mWGS sequencing.

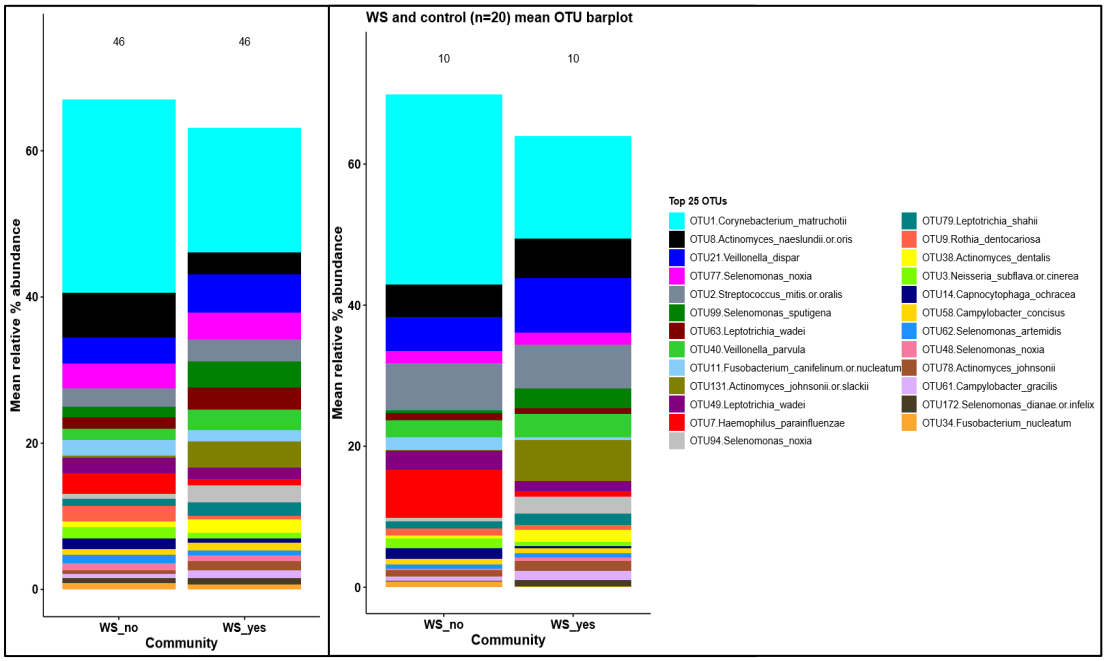


Figure 34: mWGS reliably identifies top 25 most abundant species.

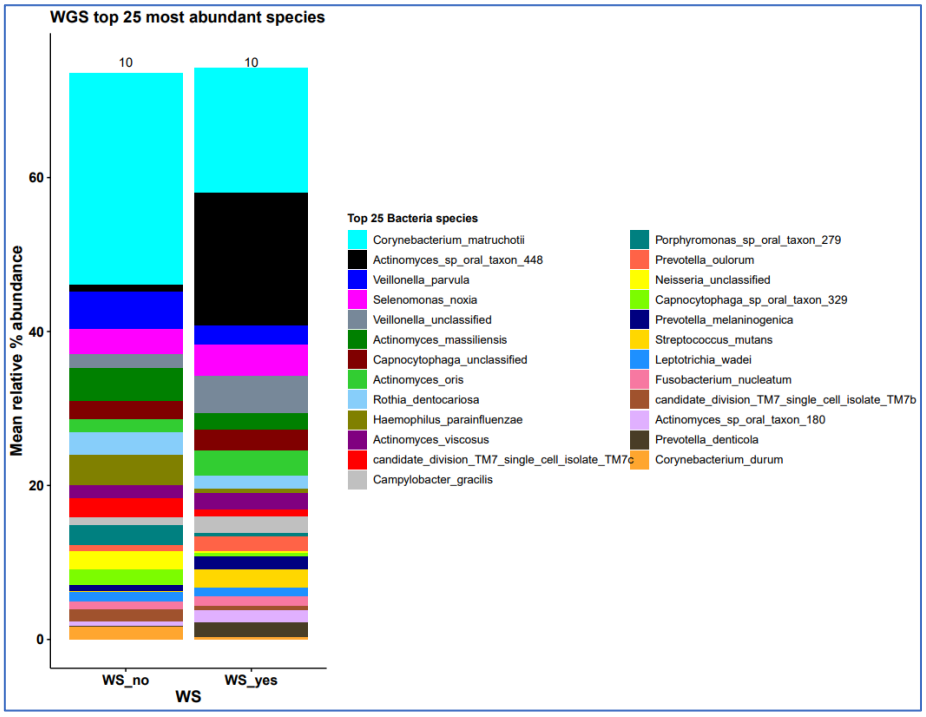


Figure 35: DESeq $p < 0.02$ for mWGS between white spot and no white spot groups.

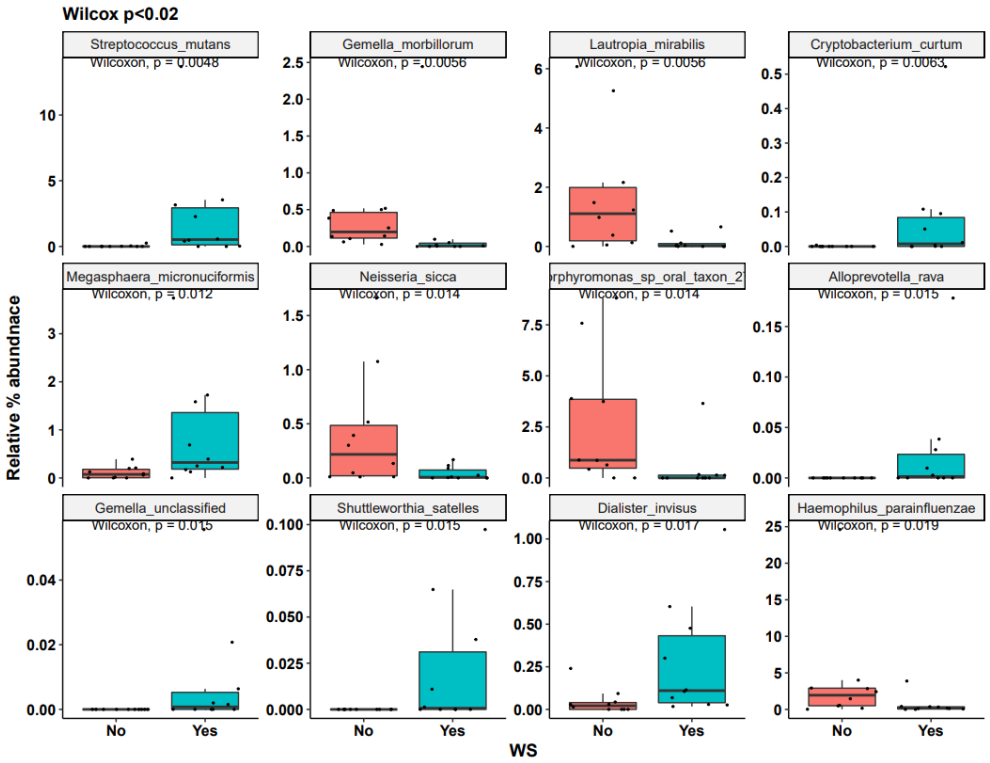


Figure 36: DESeq $p < 0.05$ for mWGS between white spot and no white spot groups.

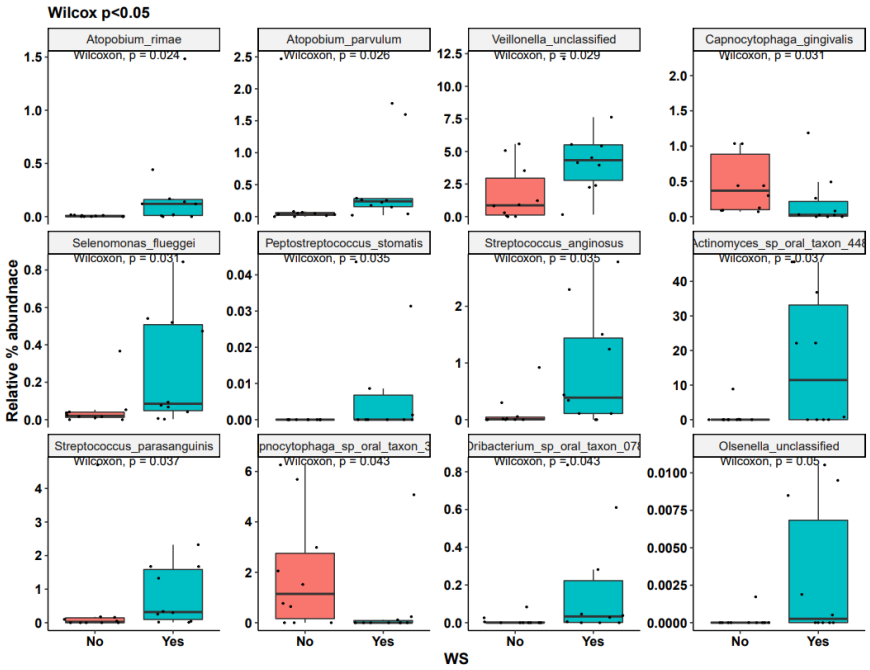


Figure 37: Prevalence of *Streptococcus* species ordered by greatest difference between no WS and WS; top: no WS > WS, bottom: no WS < WS.

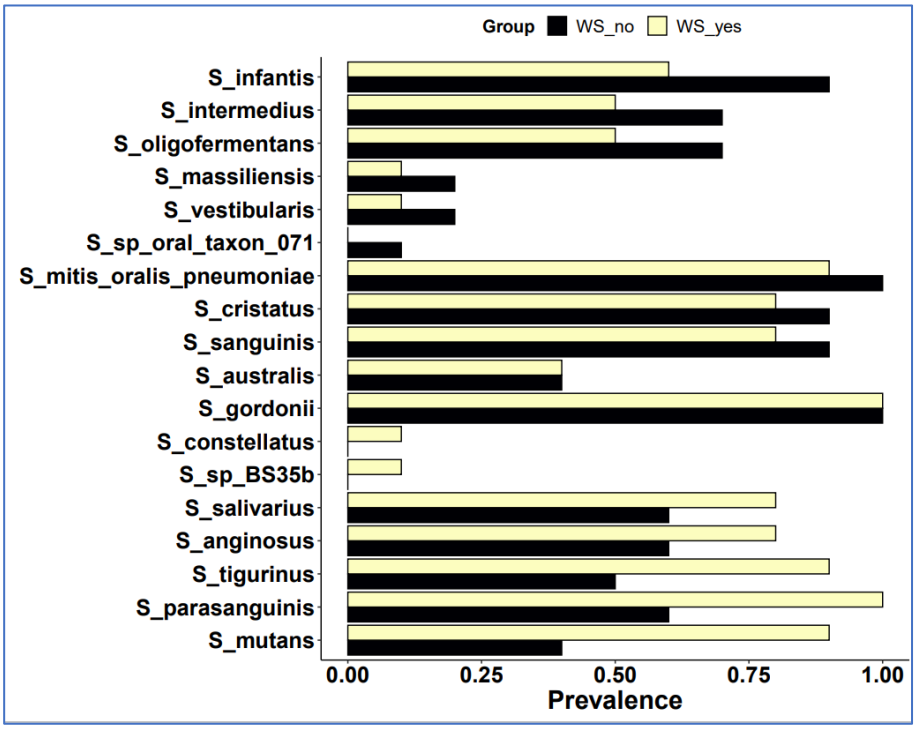


Figure 38: DESeq for mWGS of specifically *Streptococcus* species between no white spot and white spot groups.

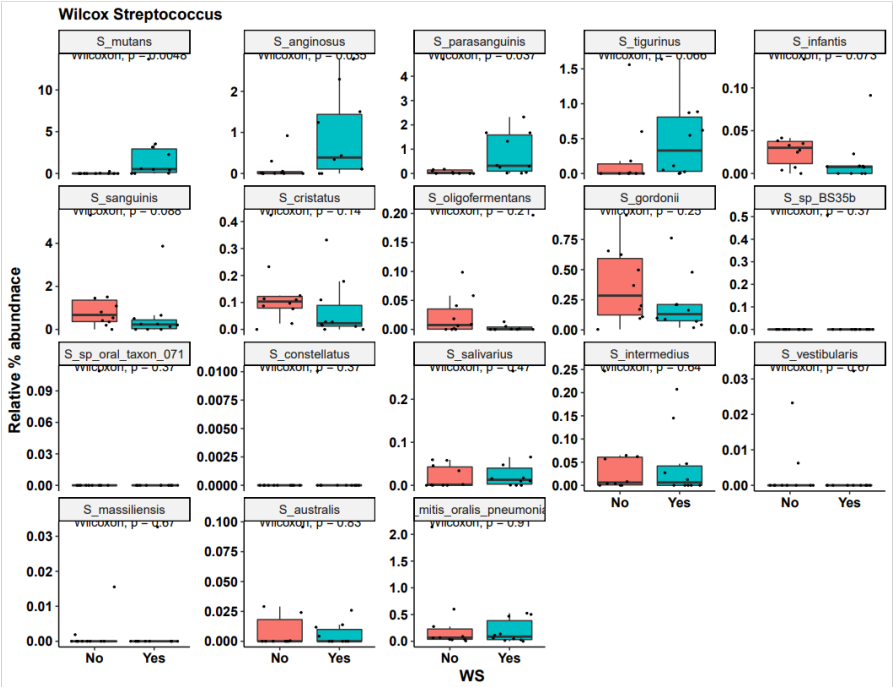
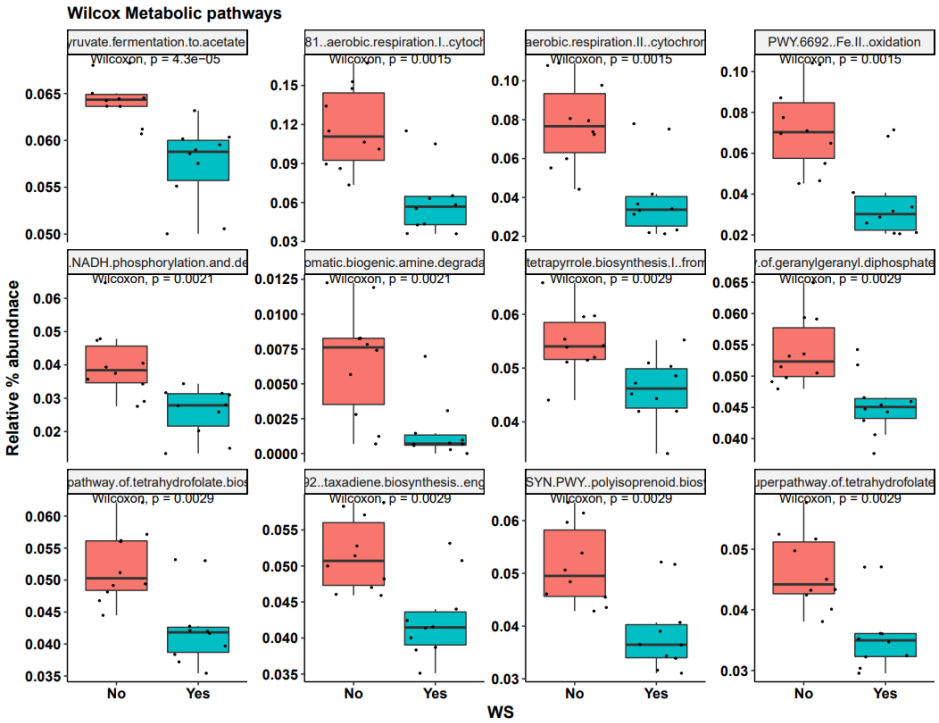
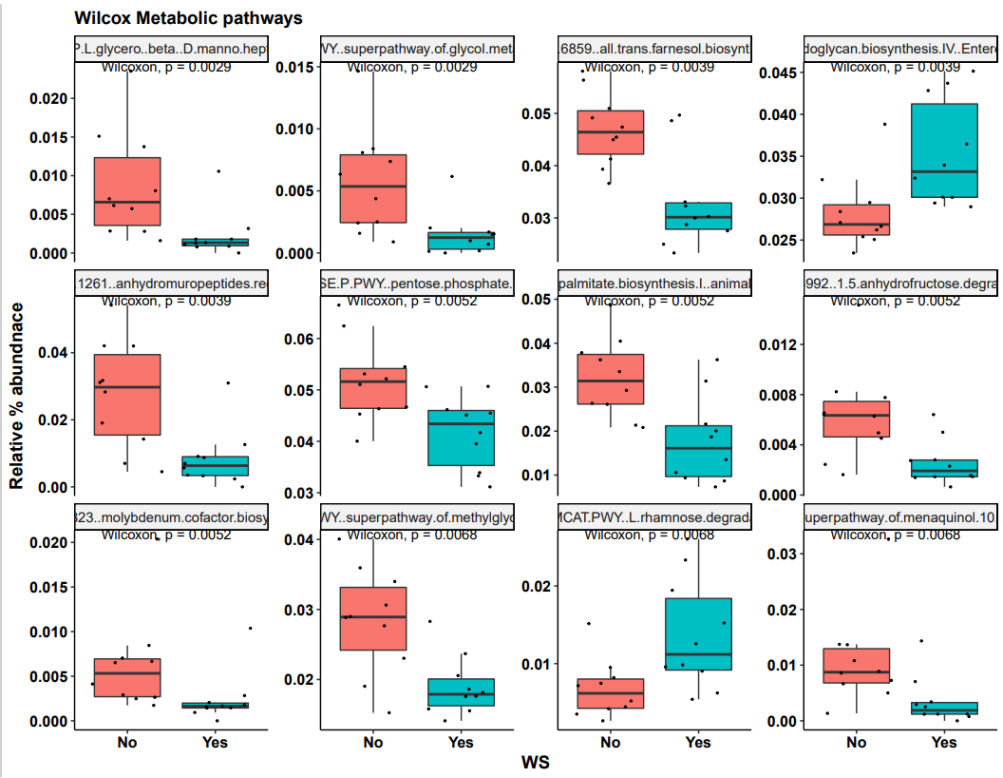


Figure 39: DESeq of metabolic pathway abundances between the no white spot and white spot groups.




Wilcox Metabolic pathways
Wilcoxon, p = 0.0029
Wilcoxon, p = 0.0039
Wilcoxon, p = 0.0052
Wilcoxon, p = 0.0068
Relative % abundnace
No
Yes
WS

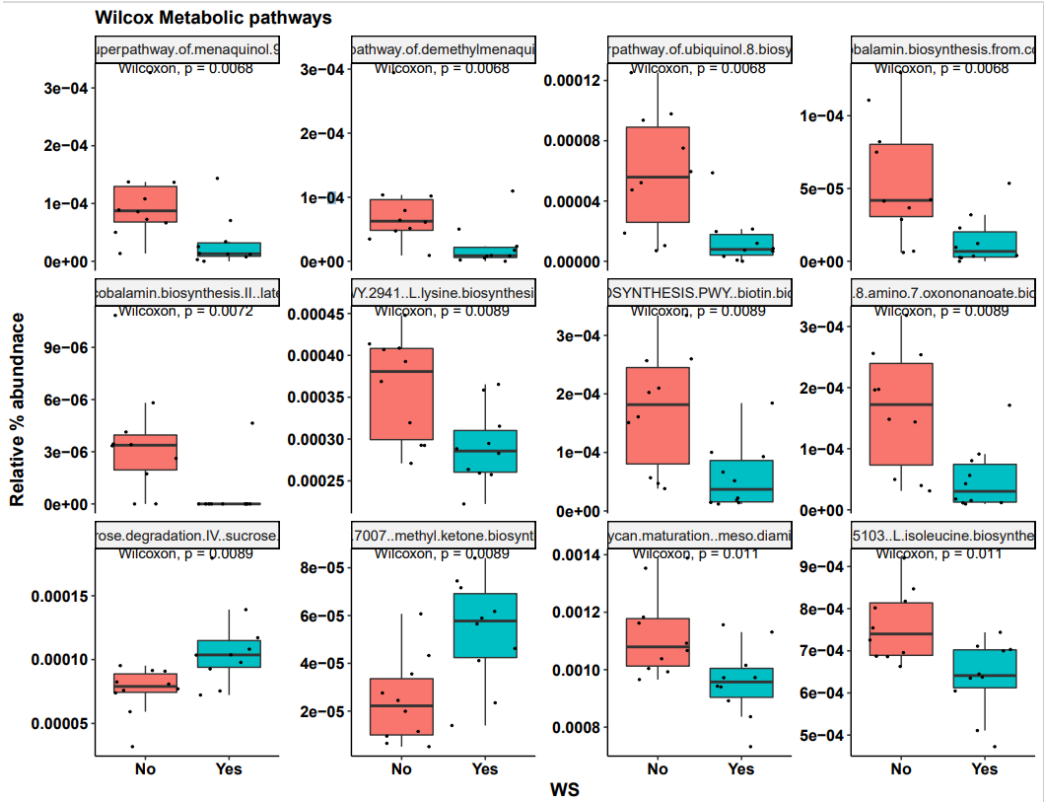

Wilcox Metabolic pathways
Wilcoxon, p = 0.0068
Wilcoxon, p = 0.0072
Wilcoxon, p = 0.0089
Wilcoxon, p = 0.011
Relative % abundance
No
Yes
WS

Figure 40: Metabolic pathway and bacteria correlations; top is unfiltered correlations and bottom is filtered to r < -0.35 or r > 0.35 and p < 0.05.

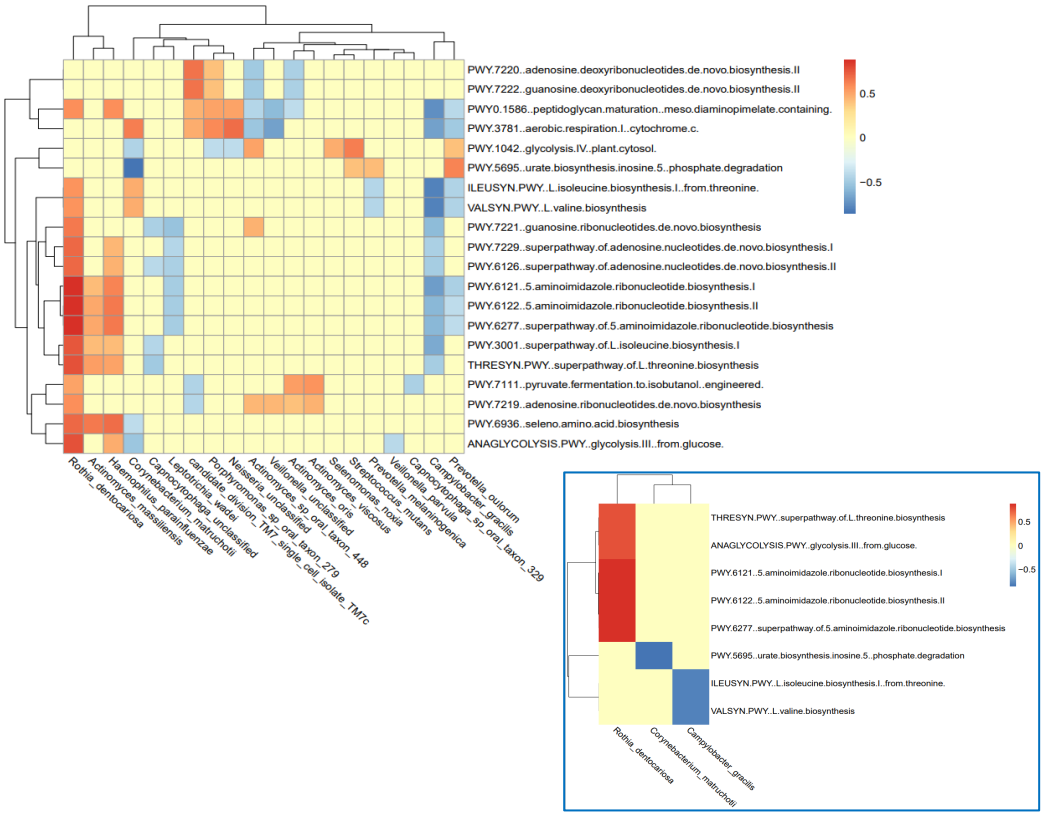


Figure 41: Prevalence of fungal species ordered by greatest difference between no WS and WS; top: no WS > WS, bottom: no WS < WS.

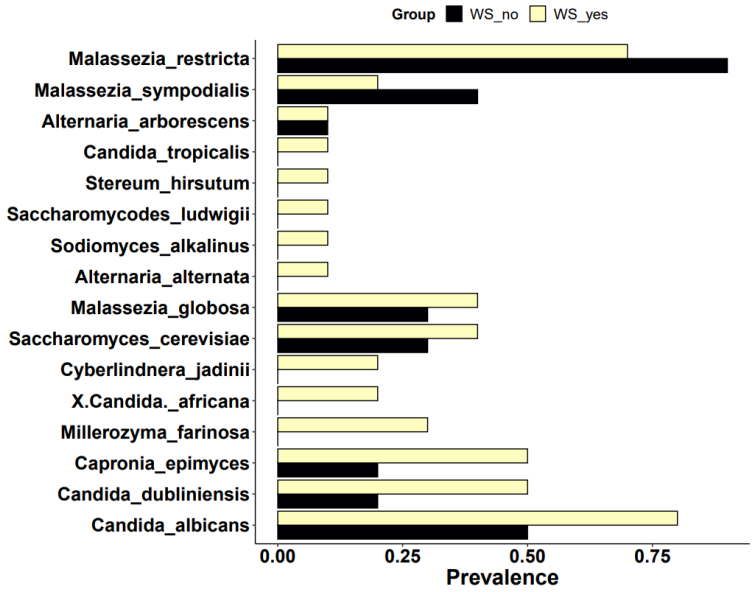


Figure 42: DESeq difference in relative abundance of fungus species at $p < 0.05$ between no white spot and white spot groups.

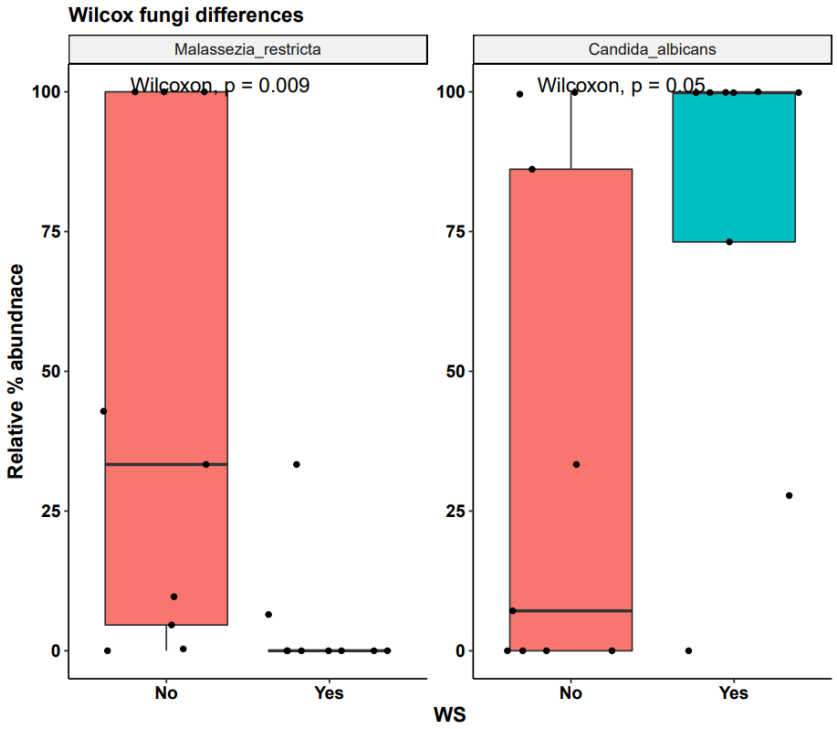


Figure 43: Prevalence of viruses ordered by greatest difference between no WS and WS; top: no WS > WS, bottom: no WS < WS.

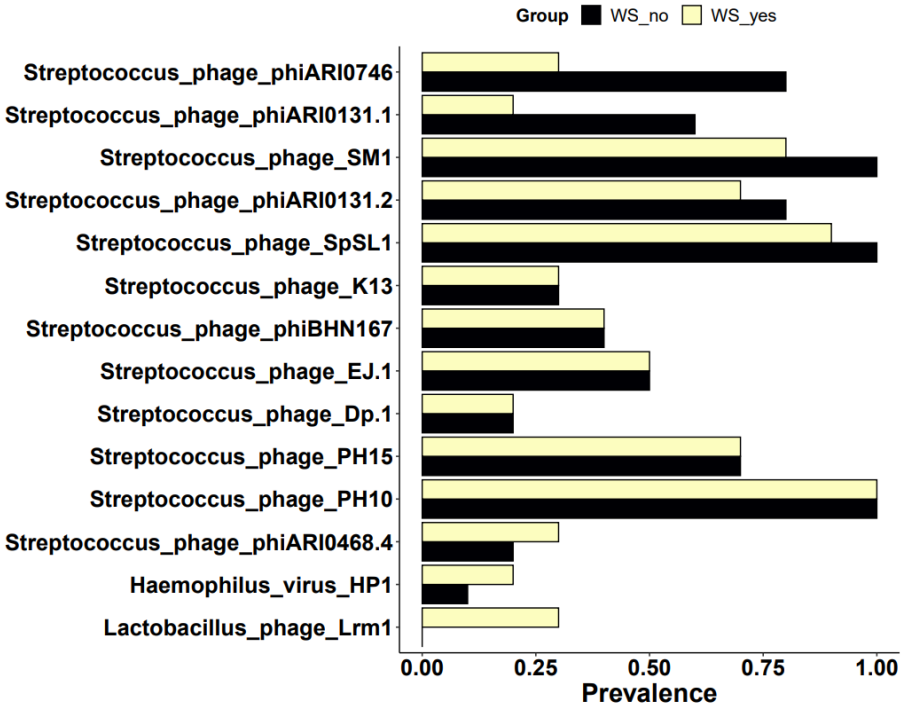


Figure 44: DESeq difference in relative abundance of virus between no white spot and white spot groups for statistically significant viruses.

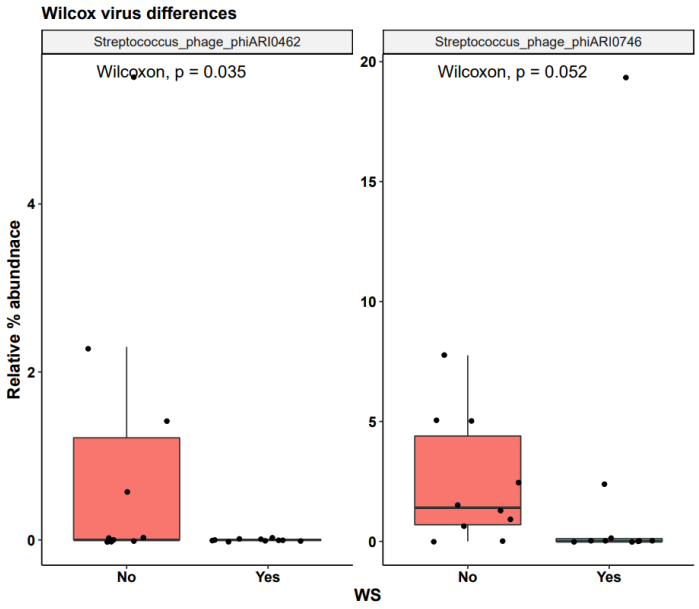


Table 1: Subject Demographics of all 92 samples.

White Spot	Age	Gender		Race			Ethnicity (Hispanic/Latino)	
		Female	Male	African American	Caucasian	Asian	Yes	No
Yes	14.17± 1.55	23	23	6	36	4	9	37
No	14.28± 1.83	23	23	5	38	3	12	34
p-value	0.908	1.00		0.866			0.619	

Table 2: Secondary Outcome measures of all 92 samples.

		White Spot	No White Spot	p-value
Fluoridated Toothpaste	Yes	46	45	1.00
	No	0	1	
Number of Snacks	<3	32	38	0.222
	≥3	14	8	
DMFT Score (pts)		1.04 ± 1.63	0.59 ± 1.02	0.214
DMFT Total Possible Score (pts)		26.91 ± 1.75	26.83 ± 2.00	0.732
Saliva pH		6.85 ± 0.37	6.93 ± 0.40	0.392
Saliva Buffer Capacity (pts)		**8.70 ± 1.62**	**9.54 ± 1.90**	**0.00715**†
Salivary Flow (mL)		7.48 ± 3.24	7.91 ± 3.37	0.736
BOP Score (pts)		**29.9 ± 28.8**	**11.5 ± 12.3**	**0.0000608**‡
BOP Total Possible Score (pts)		162 ± 10.2	160 ± 12.2	0.966
Plaque Score (pts)		**33.7 ± 13.9**	**26.9 ± 16.0**	**0.0202***
Plaque Total Possible Score (pts)		53.9 ± 3.51	53.5 ± 4.08	0.966
Time in Braces (months)		**21.7 ± 9.99**	**18.1 ± 9.17**	**0.0431***

*$p < 0.05$; †$p < 0.01$; ‡$p < 0.001$

Table 3: PCA and PERMANOVA of clinical factors compared to OTU.

	p-value	Adjusted p-value (FDR)
Presence of White Spot	**0.001**	**0.009**
Saliva pH	**0.001**	**0.009**
Race	**0.006**	**0.036**
Saliva Buffer Capacity	**0.011**	**0.0495**
BOP	**0.014**	**0.0504**
Age	0.040	0.12
Time in Braces	0.085	0.205
Plaque Score	0.091	0.205
Salivary Flow	0.109	0.218
Gender	0.242	0.436
Ethnicity	0.315	0.515
Number of Snacks	0.399	0.599
Fluoridated Toothpaste	0.590	0.738
DMFT	0.615	0.738

Table 4: OTUs significantly different between no white spot and white spot groups as determined by 16S rRNA gene sequencing.

		pvalue	padj	
2	OTU289.Fusobacterium	3.49E-27	6.91E-25	
3	OTU157.Prevotella	5.29E-07	5.24E-05	
4	OTU94.Selenomonas	8.53E-07	5.63E-05	
5	OTU130.Streptococcus	4.81E-06	0.00019	
6	OTU232.Dialister	4.60E-06	0.00019	
7	OTU20.Eikenella	5.84E-06	0.000193	
8	OTU149.Leptotrichia	2.89E-05	0.000817	
9	OTU55.Prevotella	6.55E-05	0.00162	
10	OTU243.Prevotella	0.000103	0.002264	
11	OTU304.Bifidobacterium	0.000179	0.003545	
12	OTU131.Actinomyces	0.000237	0.004261	
13	OTU334.Lachnoanaerobaculum	0.000305	0.00504	
14	OTU78.Actinomyces	0.000634	0.009649	padj < 0.01
15	OTU83.Lachnoanaerobaculum	0.000827	0.010916	
16	OTU222.Schwartzia	0.000825	0.010916	
17	OTU216.Actinomyces	0.000994	0.012304	
18	OTU9.Rothia	0.001886	0.020847	
19	OTU329.Prevotella	0.001895	0.020847	
20	OTU1.Corynebacterium	0.002069	0.021502	
21	OTU212.Prevotella	0.002172	0.021502	
22	OTU191.Veillonella	0.004187	0.039482	
23	OTU23.Streptococcus	0.004729	0.04138	
24	OTU174.Prevotella	0.004807	0.04138	
25	OTU117.Actinomyces	0.005041	0.041589	
26	OTU297.Selenomonas	0.005606	0.044398	
27	OTU230.Streptococcus	0.006334	0.048237	padj < 0.05
28	OTU102.Campylobacter	0.007879	0.057777	
29	OTU116.Megasphaera	0.009139	0.064627	
30	OTU165.Actinomyces	0.010141	0.069241	
31	OTU22.Lautropia	0.011358	0.072545	
32	OTU474.Atopobium	0.011143	0.072545	
33	OTU186.Oribacterium	0.016124	0.099767	
34	OTU38.Actinomyces	0.0173	0.099986	
35	OTU12.Rothia	0.017566	0.099986	
36	OTU211.Streptococcus	0.017674	0.099986	
37	OTU5.Neisseria	0.020723	0.110896	
38	OTU228.Capnocytophaga	0.02054	0.110896	
39	OTU234.Fusobacterium	0.022048	0.114879	
40	OTU26.Actinomyces	0.023139	0.117475	
41	OTU219.	0.023949	0.118546	

Table 5: Demographics of sample subset for mWGS sequencing.

White Spot		No	Yes	p-value
Age		14.4 ± 1.71	14.5 ± 1.18	0.88
Gender	Female	4.00	7.00	
	Male	6.00	3.00	
Tooth	UL2	7.00	5.00	
	UR2	3.00	5.00	
Number of Snacks	<3	8.00	6.00	
	≥3	2.00	4.00	

DMFT		0.6 ± 1.34	1.4 ± 2.12	0.327
DMFT Total Possible		27.6 ± 1.26	27.2 ± 1.69	0.556
Saliva pH		6.96 ± 0.45	6.90 ± 0.29	0.726
Saliva Buffer Capactiy (pts)		9.4 ± 2.12	8.6 ± 1.35	0.327
Salivary Flow (mL)		8.6 ± 4.09	7.3 ± 2.71	0.413
BOP#		**11.8 ± 14.17**	**41.7 ± 46.0**	**0.0651**
BOP Total Possible		163.2 ± 10.12	163.2 ± 10.12	1.00
Plaque #		29.0 ± 19.67	38.0 ± 15.43	0.27
Plaque Total Possible		54.4 ± 3.37	54.4 ± 3.37	1.00
Race	African American	1.00	2.00	
	Caucasian	9.00	8.00	
Ethnicity (Hispanic/Latino)	Yes	1.00	3.00	
	No	9.00	7.00	
Time in braces (months)		24.81 ± 14.0	21.42 ± 8.43	0.52

Table 6: *Strepotococcus* species OTU abundance of mWGS subsample between no WS and WS groups.

White Spot	No	Yes	p-value
OTU2 *S. mitis*	6.69 ± 3.78	2.88 ± 4.53	0.796
OTU130 *S. mutans*	**0.03 ± 0.09**	**2.88 ± 4.53**	**0.008**
OTU29 *S. gordonii*	0.76 ± 0.74	0.72 ± 1.36	0.334
OTU23 *S. sanguinis*	**0.75 ± 1.53**	**0.28 ± 0.8**	**0.039**
OTU225 *S. parasanguinis*	**0.4 ± 1.27**	**0.64 ± 0.95**	**0.012**
OTU230 *S. anginosus*	**0.05 ± 0.11**	**0.33 ± 0.48**	**0.043**
OTU211 *S. thermophilus*	0.01 ± 0.02	0.72 ± 1.56	0.284
OTU137 *S. cristatus*	0.01 ± 0.03	0.12 ± 0.37	1.000
OTU453 *S. anginosus*	0.00 ± 0.00	0.41 ± 1.22	0.168
OTU188 *S. anginosus*	0.01 ± 0.03	0.003 ± 0.01	1.000

Table 7: DESeq p-values for metabolic pathways for difference between no white spot and white spot groups.

	p	p.adjust(unlist(p), method = "fdr")
PWY.5100..pyruvate.fermentation.to.acetate.and.lactate.II	4.330035e-05	0.02100067
PWY.3781..aerobic.respiration.I..cytochrome.c.	1.504687e-03	0.09975319
PWY.7279..aerobic.respiration.II..cytochrome.c...yeast.	1.504687e-03	0.09975319
PWY.6692..Fe.II..oxidation	1.504687e-03	0.09975319
PWY.5083..NAD.NADH.phosphorylation.and.dephospho...	2.089242e-03	0.09975319
PWY.7431..aromatic.biogenic.amine.degradation..bacte...	2.089242e-03	0.09975319
PWY.5188..tetrapyrrole.biosynthesis.I..from.glutamate.	2.879473e-03	0.09975319
PWY.5121..superpathway.of.geranylgeranyl.diphosphat...	2.879473e-03	0.09975319
FOLSYN.PWY..superpathway.of.tetrahydrofolate.biosynt...	2.879473e-03	0.09975319
PWY.7392..taxadiene.biosynthesis..engineered.	2.879473e-03	0.09975319
POLYISOPRENSYN.PWY..polyisoprenoid.biosynthesis..E....	2.879473e-03	0.09975319
PWY.6612..superpathway.of.tetrahydrofolate.biosynthesis	2.879473e-03	0.09975319

	p	p.adjust(unlist(p), method = "fdr")
PWY0.1241..ADP.L.glycero..beta..D.manno.heptose.biosynthesis	2.879473e-03	0.09975319
GLYCOL.GLYOXDEG.PWY..superpathway.of.glycol.metabolism.and.degradation	2.879473e-03	0.09975319
PWY.6859..all.trans.farnesol.biosynthesis	3.886207e-03	0.11087119
PWY.6471..peptidoglycan.biosynthesis.IV..Enterococcus.faecium.	3.886207e-03	0.11087119
PWY0.1261..anhydromuropeptides.recycling	3.886207e-03	0.11087119
PENTOSE.P.PWY..pentose.phosphate.pathway	5.196042e-03	0.11850379
PWY.5994..palmitate.biosynthesis.I..animals.and.fungi.	5.196042e-03	0.11850379
PWY.6992..1.5.anhydrofructose.degradation	5.196042e-03	0.11850379
PWY.6823..molybdenum.cofactor.biosynthesis	5.196042e-03	0.11850379
METHGLYUT.PWY..superpathway.of.methylglyoxal.degradation	6.841456e-03	0.11850379
RHAMCAT.PWY..L.rhamnose.degradation.I	6.841456e-03	0.11850379
PWY.5896..superpathway.of.menaquinol.10.biosynthesis	6.841456e-03	0.11850379
PWY.5845..superpathway.of.menaquinol.9.biosynthesis	6.841456e-03	0.11850379
PWY.5862..superpathway.of.demethylmenaquinol.9.biosynthesis	6.841456e-03	0.11850379
UBISYN.PWY..superpathway.of.ubiquinol.8.biosynthesis..prokaryotic.	6.841456e-03	0.11850379
PWY.5508..adenosylcobalamin.biosynthesis.from.cobyrinate.a.c.diamide.II	6.841456e-03	0.11850379
P381.PWY..adenosylcobalamin.biosynthesis.II..late.cobalt.incorporation.	7.161610e-03	0.11977175
PWY.2941..L.lysine.biosynthesis.II	8.930698e-03	0.12739378
BIOTIN.BIOSYNTHESIS.PWY..biotin.biosynthesis.I	8.930698e-03	0.12739378
PWY.6519..8.amino.7.oxononanoate.biosynthesis.I	8.930698e-03	0.12739378
PWY.5384..sucrose.degradation.IV..sucrose.phosphorylase.	8.930698e-03	0.12739378
PWY.7007..methyl.ketone.biosynthesis	8.930698e-03	0.12739378
PWY0.1586..peptidoglycan.maturation..meso.diaminopimelate.containing.	1.149624e-02	0.15069401
PWY.5103..L.isoleucine.biosynthesis.III	1.149624e-02	0.15069401

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