The Effect of Alcohol and Tobacco Consumption on the Microbial Composition of the Oral Microbiome

By,
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B.S., University of Miami, 2019

Thesis

Submitted in partial fulfillment of the requirements for the degree of Master of Science in the Graduate Program of Biotechnology at Brown University

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Table of Contents

Table of Figures

Table of Table

Abstract

Abstract of The Effect of Alcohol and Tobacco Consumption on the Microbial Composition of the Oral Microbiome, by Gyles Ward, ScM, Brown University, August, 2021.

The oral cavity is home to the second largest microbiome in the human body. Its size is partly due to the large surface area, and multi-compartmentalization of the oral cavity. Oral microorganisms are often the intermediaries between illness and health. Fluctuations in the number of microbial species can trigger immune response locally and distally through microbial translocation. For example, *Neisseria* and *P. gingivalis* are pathogens of interest, linked to several oral cancers and Alzheimer's disease respectively.

Alcohol and tobacco consumption has proven to be a driving force towards this problem. Prior research has shown that chronic alcohol use has deleterious effects on the species richness and evenness of an oral community. Despite the growing popularity of this field, there is conflicting data on exactly how alcohol consumption and tobacco use shapes, or rather reshapes, the oral microbiome. This study aims to characterize the oral microbiome of young, orally healthy individuals who differ on drinking status (light/ heavy) and smoking status (smoker/non-smoker). In particular, relative abundances of *Neisseria* and *P. gingivalis* within those groups will be compared.

This study included 24 participants (12 light drinkers, 12 heavy drinkers; 6 smokers, 18 non-smokers) with a median age of 22 from a National Institute on Alcohol Abuse and Alcoholism (NIAAA)-funded alcohol administration study, conducted at Brown University. DNA was extracted form saliva samples and sequenced using 16S rRNA sequencing.

14 Phyla, 151 genera and 109 species were identified. Light and heavy drinkers did not differ significantly in alpha diversity (Shannon evenness index) but did on beta diversity (Bray-Curtis Dissimilarity). Smoking groups did not differ in alpha or beta diversity. *Neisseria* trended higher in heavy drinkers and non-smokers. *P. gingivalis* was not detected in any of the samples. In light of this, the three most abundant species in both drinking groups (*Prevotella melaninogencia, Haemophilus parainfluenzae and Rothia mucilaginosa)* were statistically tested. Only *Haemophilus parainfluenzae* was statistically more abundant in non-smokers compared to non-smokers. Overall, oral microbiomes for healthy young adults were successfully characterized, even though differences on the basis of drinking and smoking were generally insignificant.

Background

What is the microbiome?

The code to human life can be found within a single cell organism. Birth, growth, survival and death are stories written by our ancestral author, the microbe [1]. Microbes are microscopic unicellular organisms found in nature. They are subdivided into six major categories: Archaea, Algae, Bacteria, Fungi and Viruses [2]. These six microbes power the fundamental processes of life and are integral to growth and evolution. Most exist in their single cell form but some form colonies. In fact, most microbes must colonize to survive. Through the years, the biochemical makeup of microbes has changed drastically. As the environment changes, so do they. Reproductive mutation and population divergence has birthed new species [1, 3-4]. Now there are approximately one trillion different species of microbes on earth [5]. These species cluster together to form integrated communities that occupy every part of the environment. While some of these communities succumb to adverse environments, others grew stronger, co-evolving with the environment. Most co-evolved with humans, developing a mutual relationship.

Now, bacteria, fungi and viruses are sewn into human physiology, mediating homeostasis of biochemical processes. In fact, 20% of our blood is made up of microbial products [6-7]. All humans, animals and plants contain unique and robust communities of non-pathogenic microorganisms called a microbiome. Microbiomes are not only host specific but they're also habitat-specific. They exist in all parts of our body: the gastrointestinal tract, the oral cavity, the lungs, the genitalia and much more. Interestingly, the total number of microbial cells within our microbiome outweigh the number of human cells by a single order to magnitude [1,

8]. This collection of species originates at birth and diversifies as we get older. It bends and shifts per our adaptive and innate immune systems [9].

Only about one third of the genes found in the total microbiome in every human is conserved. The rest is varied depending on several different factors [10]. The most significant difference is seen between healthy and disease state people. Multiple studies have shown that cancers, gastrointestinal diseases, and neurological disorders reshape the structure of our microbiomes [11]. Gender, race, age and even socioeconomic status have also been identified as markers for certain microorganisms [12-14].

Nutrition also plays a tremendous role in determining the condition of our microbiomes. What we eat and how much we eat can often be the thin line between well-being and illness. Difference in the microbiome can even be explained by where we eat and how we eat it. For instance, Western diets that are rich in meats and fats may alter the number of species present in the gut microbiome. Also, infants who were breastfed vs formula-fed may have slight or major discrepancies in microbial diversity [15-16].

The microbiome field has generated significant interest in the biomedical community. Scientists have discovered links between microbiome integrity and fundamental physiological processes. For example, energy production and homeostasis, metabolism and nutrient reabsorption, gastrointestinal epithelial health, and immunological activity and immune system response [17]. Some microorganisms within our bodies have even evolved unique functions that we can rely on, saving humans the evolutionary stress [18]. Scientists have also discovered links to various diseases. The relative abundance of certain bacterial species can predispose us to disease. Therefore, now more than ever, it's paramount to know exactly what's in our microbiome, how it differs from person to person, and how it interacts with diseases [12, 17].

A monumental first step to this goal was the Human Microbiome Project. Similar to the Human Genome Project, this research aimed to identify microbial taxa shared between human hosts. The study used a meticulous set of inclusion and exclusion criteria to compose a taxonomic profile for a normal or healthy state microbiome. Today, this taxonomic profile acts as a reference genome for advanced metagenomics analysis and the development of diagnostic biomarkers for different diseases [18-19]. Many studies have used data from the Human Microbiome Project and others akin to delineate connections between the gastrointestinal microbiome, which is the largest microbiome in the human body, and various diseases. More recently, the oral microbiome has become the most studied human microflora and hot spot for biomedical research.

The Oral Microbiome

The oral cavity is home to the second largest microbiome in the human body. Its size is partly due to the large surface area, and multi-compartmentalization of the oral cavity. Microbes can occupy space on our teeth, tongues, cheeks, gingival sulcus, tonsils and hard and soft palates. Each of these habitats have their own unique landscape of microbiota. The composition and architecture of these communities are largely dependent on nutrient availability, toxic metabolites, pH level, shear forces, and the health state of the host [20].

Many studies have delved deeply into the types of bacterial taxa that inhabit each of these compartments and the mouth in general. A major study was the Human Oral Microbiome Project, which much like the Human Microbiome Project provided genomic reference for oral microflora research [21-22]. The oral microbiome is considered more heterogeneous than its larger counterpart, the gut microbiome. Currently, 1000 bacterial taxa have been identified at the

species level, stemming from the phyla, Actinobacteria, Bacteroidetes, Chlamydiae, Chloroflexi, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes, Synergistetes, Tenericutes. Yet still, 53% of the bacterial species present in the oral cavities have not been properly denominated and 35% of them haven't even been cultivated [22-23]. Additionally, the oral cavity acts as a transient home for allochthonous species, which enter the mouth through eating, drinking and inhaling. Altogether, these species make up a micro-ecosystem of over 20 billion microorganisms [24].

Technology has developed alongside the oral cavity. Conventional laboratory cultivation techniques such as pyrosequencing, polymerase chain reaction, anaerobic cultures and proteomics are responsible for progress in this field. Culture-independent sequencing methods like 16S rRNA have become the gold standard for microbiome metagenomics. Using the 16S ribosomal RNA gene, which is a gene sequence exclusive to prokaryotes, allows us to identify bacteria at the species level in an efficient, cost-effective and more high-throughput manner [20, 25-26]. Figure 1 is a schematic of the process. The V3 and V4 regions of the ribosomal RNA are sequenced. The V4 region is a variable region used to distinguish between bacterial species. This method allows us to recognize both culturable and non-culturable microbes. Some studies are becoming more focused and using custom DNA microarrays to directly target microbes or microbial communities.

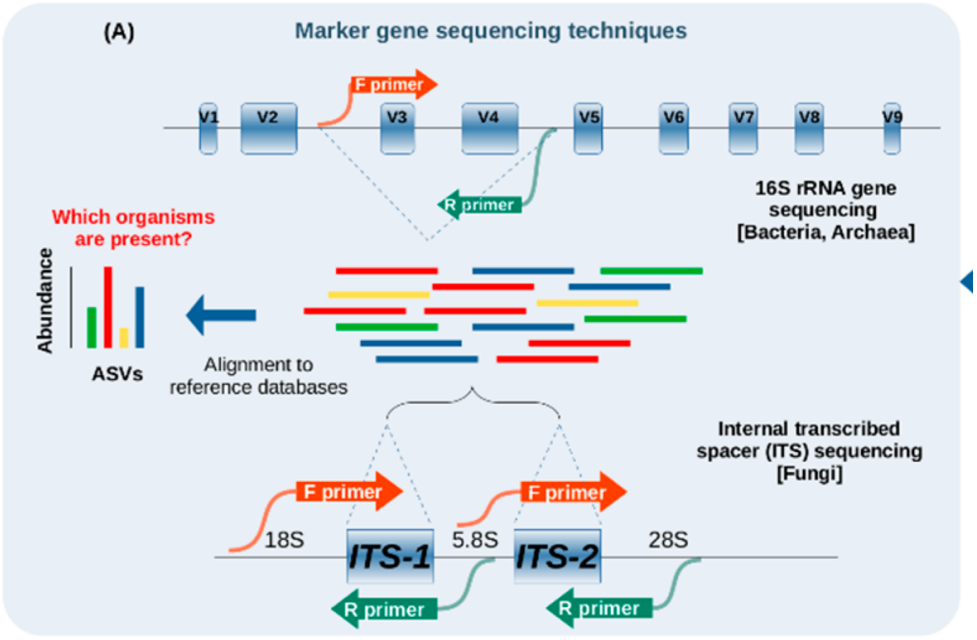

Figure 1. Taken from Willis and Gabaldón (2020). A schematic of 16S rRNA sequencing for bacteria, archaea, and fungi. This is a widely used sequencing technique that uses the V4 variable region of 16S ribosomal RNA to distinguish species.

The onset of these advanced technologies has propelled oral microbiome research to the population level [28]. Species richness and relative abundance can be analyzed across a population, emphasizing differences in gender, race, nutrition, oral health, age and other clinical characteristics. Species richness and relative abundance can be also linked to diseases such as diabetes, obesity, cancer, and nutritional deficiencies [16, 29].

Oral Microbiome and Disease

Oral microorganisms are often the intermediaries between illness and health. Periodontitis, dental carries, and oral cancers have been directly correlated to interactions between host and oral microbiota [30]. Under normal conditions, oral microorganisms live in a

dynamic equilibrium with the host immune system. External influences alter the integrity and the composition of the micro-ecosystem, which leads to pathogenic infection. For example, taking antibiotics can eliminate commensal bacteria with immunological properties and increase the risk of pathogenic bacterial colonization. Secondly, changes in diet can cause nutritional and metabolic stress to the structure of the micro-environment. This alteration in structure and bacterial composition is summed up in the term "dysbiosis" [31-32].

Oral dysbiosis triggers a host immune response, which attacks the different habitats within the oral cavity. A major consequence is the expansion of the gingival sulcus due to the deterioration of the connective tissue structures between the tooth and the gingiva. This creates an oxidant-rich space for aerobic bacteria to colonize, called the periodontal pocket. Eventually the host will develop gingivitis and later, periodontitis [32]. Also, pathogenic bacteria in the periodontal pocket and in the oral cavity in general may also produce acidic metabolites that can destroy tooth enamel and lead to dental caries, and tooth loss [33].

Moreover, the periodontal space becomes inflamed due to florid immune system responses. This inflammation degenerates the epithelial tissues and releases bacteria and inflammatory mediators into systemic circulation. Oral hygiene practices ameliorate this process in affected individuals. Many studies have proven that tooth brushing increases the risk of bacteremia in children who have either suffered from tooth loss, tooth decay, or been treated for tooth decay. This may not be a concern for otherwise healthy individuals, but immunocompromised individuals may not be able to defend their body against infection. Some of these bacteria travel down to the lungs and result in bacterial pneumonia [32]. The etiology of heart disease is also closely linked to bacteremia. One study in 2004 showed that patients with periodontitis has a 400% greater risk of stroke than patients with dental carries [34]. Another

study found a connection between atherosclerosis in the carotid arteries and periodontal pathogens. Atherosclerosis is the gradual occlusion of a blood vessel due to inflammation [35]. Researchers have even uncovered relationships between the oral microbiome and the gut microbiome. Pathogenic bacteria from the mouth can be swallowed into the gut and incite dysbiosis. Consequently, gut bacteria can escape through the epithelial lining of the small intestine into peripheral circulation. Patients with this problem have been cited to suffer from diabetes, obesity and fatty liver disease [14, 36].

Oral dysbiosis may even be one etiology of Alzheimer's disease. Alzheimer's disease is an irreversible, neurodegenerative disorder that leads to dementia. Dementia is fundamentally the loss of cognitive abilities such as reasoning, memory, and thinking [37]. Lately, periodontal pathogens, birthed by oral dysbiosis have been linked with Alzheimer's disease. Leira et al. completed a meta-analysis of data on the subject and found a strong association between severe periodontitis and Alzheimer's (Odds Ratio: 2.98; Confidence Interval: 1.58-5.62) [37]. Similarly, Chen et al. found that patients who had periodontitis for more than 10 years had a 1.707 greater chance of developing the disease [21].

The key player in this relationship between Alzheimer's and Periodontitis may be *Porphyromonas gingivalis* (P. gingivalis). P. gingivalis is a gram-negative, anaerobic bacteria found to be a major driver towards chronic periodontitis. The bacterium can work directly or indirectly through altering inflammatory systems that break down periodontal tissue [37-39]. A study using transgenic mice induced periodontitis through P. gingivalis and saw that cognitive functions were severely impaired relative to mice in the control group. They also saw an increase in amyloid-beta (AB) proteins which are hallmarks for the Alzheimer's. These AB proteins are cytotoxic to neurons in the brain [39-40]. Another study done by Dominy et al. found P.

gingivalis ribosomal RNA in the cerebral cortex of Alzheimer's patients. They also found P. gingivalis DNA in the cerebral spinal fluid and saliva of clinical Alzheimer's patients, which might indicate pathogenic translocation [41].

One theory to explain this data is the dislocation of oral bacteria to the gut microbiome mentioned earlier. Patients who suffer from periodontitis swallow P. gingivalis pathogens every day, relocating it to the gut. P. gingivalis can survive in acidic conditions, which makes it an ideal resident of the stomach. The pathogen can escape into circulation due to gut dysbiosis and travel to the brain, where it can cross the blood brain barrier. Once in the brain, the pathogen might secrete virulence factors called gingipains. Gingipains are proteases that are essential to nutrition, colonization and host tissue deterioration. Once released, they may evoke an inflammatory response. AB proteins might be a part of this immune response. These proteins may localize to the site of infection because of their antimicrobial properties [36, 39, 41]. However, much more research needs to be done to corroborate this theory and previous findings. Interestingly, time has also been dedicated to exploring the effects of chronic alcohol consumption and smoking on the abundance of P. gingivalis and other bacteria and its related effects.

The schematic below provides a snapshot of diseases linked oral microbiome composition. Many bacterial species have been identified as intermediaries between health state and disease state. Interestingly, P. gingivalis and Neisseria have been linked to more than one disease and in some cases they are linked to the same disease (rheumatoid arthritis).

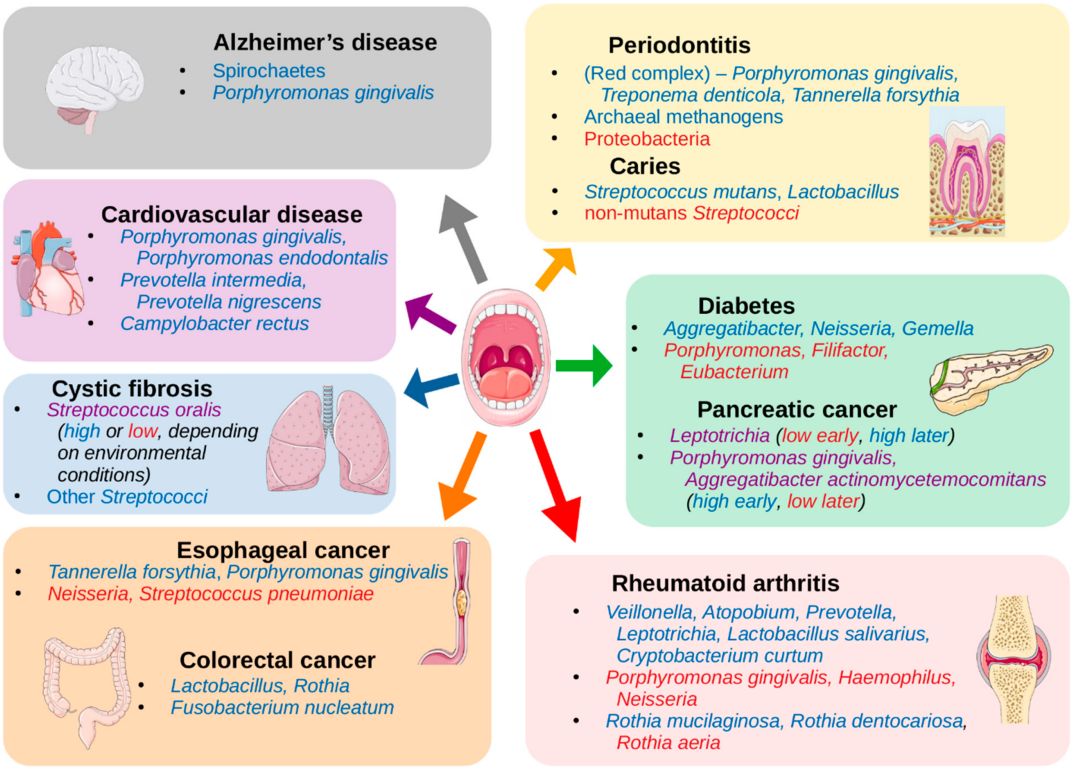

Figure 2. Taken from Willis and Gabaldón (2020). A schematic of systemic disease linked to the changes in the oral microbiome. The bacterial species listed under each disease have been found in in either high (blue) or low (red) abundances in patients suffering from those diseases.

Oral Microbiome and Alcohol Consumption

Alcohol consumption, or rather the presence of ethanol has proven a driving force towards oral dysbiosis. Acute or chronic alcohol consumption can even have a deleterious effect on the relative abundance of species and the species richness within an oral microbiome. Many studies that dive into this phenomenon come up with conflicting data. Some studies find significant differences in the abundance of oral taxa between drinkers and non-drinkers while others only see a slight difference [13,42].

A typical case is with *P. gingivalis.* Some studies show an increase in the relative abundance of *P. gingivalis* in the saliva of alcohol dependent participants. In a study of 49 alcohol dependent and 49 non-alcohol dependent individuals, subgingival biofilm samples were collected and analyzed with the checkerboard technique. Alcohol dependent individuals had an overall higher mean count of *P. gingivalis* compared to the other group [43]. See Appendix A.

On the contrary, in a different study, a list of pathogens and cytokines were probed for in four experimental groups, Alcohol dependent with periodontitis (ADP), Non-alcohol dependent with periodontitis (NAP), alcohol dependent without periodontitis (ADNP), and non-alcohol dependent without periodontitis (NANP). Subgingival samples were collected, and there was no significant difference in abundance of P. gingivalis between groups [44].

A study performed by Zhou et al observed an inverse relationship between alcohol drinking and P. gingivalis presence. Patients suffering from moderate and severe acute alcoholic hepatitis with long time drinking histories were examined. Two *P. gingivalis* strains were detected for using antibody tests. Interestingly, in both moderate and serve groups, participants with the least amount of drinking years in their drinking history saw the highest levels of both strains. So, there is a lot of uncertainty surrounding this subject, which prevents experts in the field from delineating a cause and effect relationship with absolute confidence [45].

Moreover, cancers of the mouth, head, neck and digestive track are also connected to alcohol consumption and in particular, alcohol dependence. These diseases and their etiology may be mediated by commensal bacterial species in the oral cavity [46]. An observational study run by Fan, et al. analyzed the oral microbiome of participants selected from various cancer studies. Oral washes were selected and participants were characterized as light, moderate or heavy drinkers. Distinctions were also made based on whether they drank beer, liquor or wine.

The authors found that the relative abundance of *Lactobacillales*, and order of bacteria decreased with high alcohol consumption. *Lactobacillales* promotes oral health and its absence is noted by an increase in pH levels. Elevated pH levels were speculated to increase levels of *Neisseria* [47]. *Neisseria* are a genus of bacteria with 11 known species that colonize humans. They are highly transformable bacteria that produce high levels of acetaldehyde, a probable human carcinogen. This suggests that ethanol consumption could promote oral carcinogenesis [46, 48].

In another study with orally healthy participants, alcohol consumption also led to increased acetaldehyde production. Participants ranging from age 20 to 60 years old, were required to rinse for 30 seconds with an alcoholic beverage. Participant rinsed with several different types of alcoholic beverages (beer, wine, cider, liquor, spirits). Saliva samples were taken 30 seconds, 2 minutes, 5 minutes and 10 minutes after the initial rinse. Acetaldehyde production was monitored using gas chromatography or enzymatic analysis. Researchers observed acetaldehyde levels above even what previous studies deemed carcinogenic [49]. Comparable observations were made in a study with orally unhealthy participants. Acetaldehyde volume was evaluated in 132 men and women. Of those participants, 68 were light/non-drinkers, 58 were moderate drinkers and 6 were heavy drinkers. The heavy drinking group had the highest volume of salivary acetaldehyde followed by moderate, then light/non-drinkers [50].

Dissimilarly, in an experiment by Yokoyama et al. the oral microbial compositions of healthy men were tested for acetaldehyde production ability in the presence of ethanol. The men were split into two groups, high and low acetaldehyde production ability. Bacterial communities were divided into two populations, type 1 and type 2. Type 2 had a dominance of Neisseria bacteria, which was found to produce high levels of acetaldehyde in vitro in the presence of

ethanol in previous research [48, 51]. In this study, acetaldehyde production was inversely correlated to the relative abundance of Neisseria.

In another study, Moritani et al examined microflora abundance in normal conditions. They took 166 orally healthy individuals, determined the abundance of microflora components using 16sRNA pyrosequencing, and evaluated acetaldehyde production using gas chromatography. Results dictated that Neisseria produced copious levels of acetaldehyde in healthy oral cavities [52]. Thus, these findings contradicted Yokoyama et al.'s claim that Neisseria abundance is inversely correlated to acetaldehyde production.

Oral Microbiome and Tobacco Consumption

Tobacco consumption may also play a significant role in oral microbiome health. Although it's not as widely studied as alcohol, some literature suggests that it decreases diversity and can lead to general bad oral health and in extreme cases, oral cavity squamous cell cancer [53-54].

In studies focusing on tobacco consumption alone, oral microbiome composition differed not only by smoking status but also by method of tobacco consumption. For example, in a Middle Eastern study where 330 smokers and nonsmokers were examined, while cigarette and dokha smokers had significantly different oral microbiome structures relative to nonsmokers, shisha smokers did not. Interestingly, there were no significant differences in oral composition between smoking groups with respect to the type of tobacco product used. Furthermore, oral diversity seemed to be comparable between all smokers and non-smokers [55].

Similar results were seen in a study analyzing the effects of both smoking and drinking on bacteria richness, evenness, and diversity. Twenty-two subjects were divided into a control

group (no tobacco consumption and no or little alcohol consumption), heavy tobacco and alcohol consumers, and only tobacco consumption. After collecting saliva samples through swabs, there was no reportable difference between species diversity and evenness between any of the groups. Nonetheless, the group that only consumed tobacco had less species richness than the control group. In particular, Neisseria was found at lower levels in the groups that only consumed tobacco and heavy consumers of both tobacco and alcohol, which might suggest that when paired with tobacco consumption, alcohol has, in some cases, the opposite effect on Neisseria abundance [56]. That being said, this study featured relatively small groups, which is a major limitation to consider. Below is a figure taken from the study. It shows Neisseria at statistically lower abundances in both the smoking group and the smoking and drinking groups.

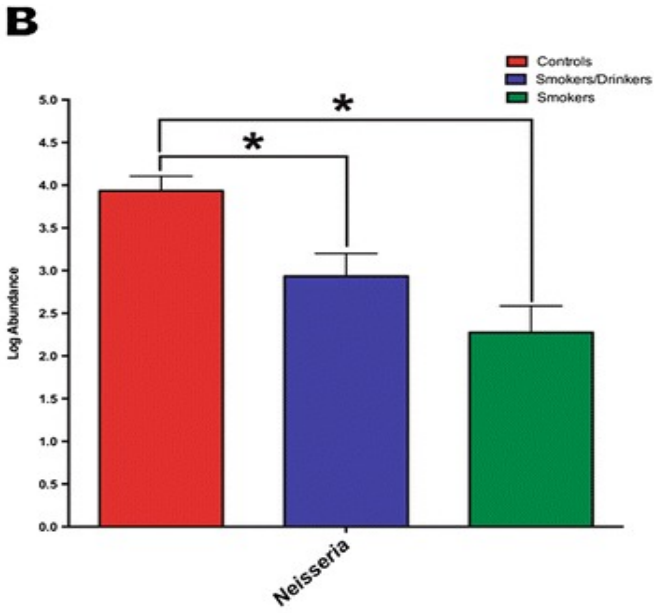

Figure 3. Taken from Thomas et al (2014). The Log abundances of Neisseria across control and experimental groups (smokers/drinkers, smokers). Neisseria is statistically lower in smokers/drinkers and smokers.

Perhaps smoking and drinking have a compound effect on oral microflora composition altogether. In another study, 35 males were split into three groups, healthy social drinkers, alcohol-dependent non-smokers and alcohol-dependent smokers. Alcohol dehydrogenase (ADH) activity was determined using the photometric method. ADH is the enzyme that Neisseria uses to convert ethanol into acetaldehyde. Alcohol dependent groups (both smoking and non-smoking) showed a lower level of ADH activity relative to the control group [57].

Aims and Hypotheses

Aims

Conflicts between the aforementioned studies have provided the framework for this research. This thesis aims to advance progress in this field forward by examining the role of alcohol and tobacco use in oral microflora composition. This study specifically aims to:

1. Determine the difference in oral species diversity between healthy light and heavy drinkers.
2. Evaluate the implications of light and heavy drinking on microbiota composition with respect to bacterial species, *Neisseria* and *P. gingivalis*.
3. Determine the effect of smoking on microbiota composition with respect to bacterial species, *Neisseria* and *P. gingivalis.*

Hypotheses

1. Species richness and evenness will differ significantly between heavy and light alcohol drinkers.
2. Species richness and evenness will differ significantly between smokers and non-smokers
3. Relative abundance of *Neisseria* and *p. gingivalis* will differ significantly between heavy and light drinkers
4. Relative abundance of *Neisseria* and *P. gingivalis* will differ significantly between smokers and non-smokers.

Materials and Methodology

Study overview

This study included 24 participants from a National Institute on Alcohol Abuse and Alcoholism (NIAAA) funded alcohol administration study, operated in the School of Public Health at Brown University. Participants were accepted into the study based on an extensive list of inclusion/exclusion criteria followed in a series of screening interviews. During screening, participants were separated into two drinking status groups, heavy drinker and light drinker, smoking status was recorded and salivary samples were collected and stored for analysis. Final sample characteristics are shown in Table 1. All participant information was classified and each of them were given personal identification numbers, by which they were referred to throughout the duration of the study.

Drinking and Smoking Status Determination

All participants provided informed consent before beginning study procedures. Individuals who were actively seeking or receiving treatment for drug and alcohol use or experiencing withdrawal symptoms after withholding from alcohol use for 48 hours were excluded from the study.

Drinking groups were determined by recent drinking behavior. Heavy drinkers were denoted as individuals who have had at least five heavy drinking episodes in four consecutive weeks in the 90 days leading up to their screening interview. A heavy drinking episode for women would be 4 drinks or more in one day and 5 drinks or more for men. Alternatively, heavy drinkers must have an average weekly drinking pattern of more than 7 drinks for women and more than 14 drinks for men. Light drinkers were denoted as individuals who drink on at least a

monthly basis and have had no more than 3 heavy drinking episodes in the 90 days prior to their screening interview. They must also have no history of heavy drinking in their lifetime that lasted more than 6 months. Participants were assigned a smoking status based on whether they had smoked a cigarette within the 30 days prior to their screening session.

An initial pre-screening interview was conducted over the telephone where potential participants were either accepted or denied based on the inclusion/exclusion criteria. Accepted participants then had an in-person screening interview where eligibility was confirmed. During the in-person screening, urine samples were tested for drug metabolites. Women were also required to take a pregnancy test. Blood alcohol level was also recorded and expected to be 0.00 g% (participants were required to withhold alcohol use for 48 hours prior to screening) Additionally, participants were given a medical history assessment where they reported any medication use. Basic vital functions were assessed. Salivary samples were collected and stored as described below.

Inclusion/Exclusion Criteria:

All study participants must:

1) Be 21-55 years
2) Be Fluent in English
3) Have reported a BAC ≥ 0.07 g/dL at least once in the last year.
4) Have reported monthly alcohol consumption (or more) in the past 6 months.
5) Report no use of amphetamines, methamphetamines, cocaine, opioids, or benzodiazepines in the last 30 days.
6) Begin every visit with a .00 g% breath alcohol concentration.

All study participants must not:

1) Be receiving treatment for alcohol or drug use, save smoking cessation.
2) Have any chronic diseases that require daily use of medication.
3) Have used antibiotics or probiotics in the last month
4) Have a GI tract disorder that requisites treatment or discomforts the participant.
5) Use non-steroidal anti-inflammatory drugs (NSAIDs) daily. All Participants should refrain from any NSAIDs 48 hours before each visit.
6) Produce a positive urine toxicology for amphetamines, methamphetamines, cocaine, opioids, or benzodiazepines. Participants will still be included in the study with a positive cannabis test.
7) Have a history of alcohol withdrawal symptoms after short-term abstinence.
8) Produce a score ≥ 10 on the Clinical Institute Withdrawal Assessment for Alcohol Revised.
9) Have a history of seizures.
10) Suffer from a major psychiatric disorder determined by,
    a. Depressive symptoms/ a score ≥ 20 of the Center of Epidemiological Studies Depression Scale – Revised.
    b. Possible bipolar disorder/ positive screening on the Mood Disorder Questionnaire
    c. Possible psychotic disorder/ positive screening on the psychosis module of the Structured clinical interview for DSM-IV-TR axis I disorders, research version (SCID-I-RV).

11) Have a history of fainting, infection, or severe psychological discomfort when blood is drawn.
12) An inability to abstain from tobacco consumption for less than 8 hours.
13) An inability to abstain from cannabis consumption for less than 48 hours.
14) Be pregnant, nursing, planning a pregnancy or refuse to use reliable birth control for the study's duration if sexually active with a male partner (females only).

Salivary Sample Collection

Salivary samples were collected from eligible participant's after obtaining informed consent. Drinking and eating was prohibited for at least 30 minutes prior to saliva sample collection. Samples were collected using an Omnigene Oral saliva kit. Participants were instructed to swish a mouthful of water in their mouth and spit before filing up (to the black line) a collection tube with saliva. The saliva had to be bubble-free. After that, a research assistant wearing gloves took the collection tube and sealed it. The funnel lid fitted to the collection tube contained space stabilizing solution that was mixed with the saliva after closure. Next, the funnel lid was replaced by a plastic cap. The tube was gently inverted for a proper incorporation of stabilizing solution and to ensure no saliva was leaking out. Lastly. a cryogenic label with the identification number of the participant was pasted on the side of the tube and the sample was stored at -80 degrees Celsius until analysis.

DNA Extraction

Before DNA extraction, the saliva samples were removed from -80 Degree Celsius storage, thawed in a cooler and spun down for one minute on a vortex shaker. DNA extraction was done using the ZymoBIOMICS DNA Miniprep Kit per the protocol detailed in the instruction manual.

The samples were transferred from saliva tubes to the ZR BashingBead Lysis Tubes, combined with 750 microliters of ZymoBIOMICS Lysis Solution and closed securely. The tubes were then centrifuged at 10,000xg for 1 minute. After centrifugation, the tubes were taken out and 400 microliters of the supernatant from each sample was removed and placed in Zymo-Spin III-F Filters fitted with collection tubes. The supernatant was then centrifuged again at 8,000xg for one minute. After that, the filters were discarded and 1,200 microliters of ZymoBIOMICS DNA Binding Buffer was added to each of the collection tubes and mixed well.

When mixing was done, 800 microliters of the mixtures in the collection tubes were transferred to Zymo-Spin IICR Columns fitted with new collection tubes and centrifuged once again at 10,000xg for 1 minute. Flow through from the collection tubes were thrown away and another 800 microliters of the mixture were transferred from the collection tubes to the columns and centrifuged again at 10,000xg for 1 minute.

Next, 400 microliters of ZymoBIOMICS DNA Wash Buffer 1were added to the column fitted with a new collection tube and centrifuged at 10,000xg for 1 minute. The flow through was discarded then 700 microliters of ZymoBIOMICS DNA Wash Buffer 2 was added to the column with collection tubes and centrifuged again at 10,000xg for 1 minute. The flow through was discarded again. After that, 200 more microliters of ZymoBIOMICS DNA Wash Buffer 2 were added to the column with collection tubes and centrifuged at 10,000xg for 1 minute. Then, the

columns were transferred to clean 1.5 ml microcentrifuge tubes, combined with 100 microliters of ZymoBIOMICS DNase/RNase Free Water, incubated for 1 minute, and centrifuged at 10,000xg for 1 minute to elute the DNA.

Meanwhile, the Zymo-Spin III-HRC Filters were fitted with new collection tubes. 600 microliters of ZymoBIOMICS HRC Prep Solution was added to each filter and centrifuged at 8,000xg for 3 minutes. Lastly, the eluted DNA from the microcentrifuge tubes was added to the prepared filters and fitted with new 1.5 ml microcentrifuge tubes and centrifuged at 16,000xg for 3 minutes.

16s rRNA Amplicon Generation

After DNA isolation, samples were sequenced using 16s ribosomal RNA sequencing. All microbes have a conserved 16S ribosomal RNA gene with a hypervariable region known as V4. This region was amplified using 806R and 515F barcoded primers taken from the Earth Microbiome Project and Phusion High-Fidelity polymerase [58]. Amplicons were generated using a thermos cycler. Initial denaturation of DNA happened at 98 degrees Celsius for 30 seconds. Complete denaturation happened after 34 cycles at 98 degrees Celsius for 10 seconds. Annealing happened at 57 degrees Celsius for 30 seconds and extension happened at 72 degrees Celsius for 30 seconds. Final incubation was at 72 degrees Celsius for 5 minutes.

Polymerase Chain Reaction

Sequencing was performed by the Rhode Island Genomics and Sequencing Center at the University of Rhode Island (Kingston, RI, USA). Amplicons were stored and shipped to the sequencing location. Amplicons were pair-end (2x250 bp) sequenced using a 500-cycle kit with

standard protocols on the Illumina MiSeq platform. The average read depth was 5,238 ± 1,744 reads per sample.

Sequence Filtering and Statistical Analysis

Forward and reverse sequences were filtered, trimmed, de-noised and merged using the R (version 3.5.0) DADA2 (version 1.14.1) package [59-61]. Taxonomic assignments were also made with the DADA2 function *assignTaxonomy* using the RDP Classifier algorithm with training set 18 [61-62]. Alpha (Shannon Evenness Index) and beta diversity (Bray-Curtis Dissimilarity) were calculated using the Phyloseq package [63-64]. Statistical analysis and graph construction were done in GraphPad Prism (version 9.1.2 (225)). For all alpha diversity measures and relative abundance calculations, an unpaired Welch's t-test was used with a significance level of $p=0.05$. For the Bray-Curtis dissimilarity index, a non-parametric permutational analysis of variance (PERMANOVA) test was conducted to determine the spatial variance and the effect of drinking status on dissimilarity [65]. The significance level was $p=0.05$.

Results

Participant Characteristics

Table 1: Participant Characteristics

Characteristics	All Participants (N=24)	Light Drinkers (n=12)	Heavy Drinkers (n=12)	Smokers (n=6)	Non-smokers (n=18)
Median Age	22	21.5	25.5	23	22
Min Age	21	21	21	21	21
Max age	33	29	33	27	29
Male (%)	50	50	50	67	44
Female (%)	50	50	50	33	56

Participants were separated by drinking and smoking status. Those whose samples produced low quality or low sequence reads or were not assigned to a drinking group at screening were removed, resulting in a final sample size of 24. Light drinkers and heavy drinkers were represented equally (12 participants per group). Additionally, sex was balanced overall and across drinking groups (6 participants of each sex within each drinking group). Moreover, participants were healthy young individuals. Individuals up to 55 years old were eligible to participate in the study. The observed age range was 21 to 33 years. The median age was 22 for all participants and differed slightly across assigned drinking and smoking groups. Most participants were 25 years old or younger which leaves older groups underrepresented. Smokers were also underrepresented with only 6 participants reporting use of tobacco. Sex is balanced within the non-smoker group but not the smoker group.

Overall Taxonomy

Sequences were assigned taxonomies using the RDP classifier algorithm with training set 18, first at the phylum level, then at the genus level and finally at the species level. Figures 4 and 5 show the relative abundance of each phylum and genus respectively as a function of drinking status. Percentage values for each phylum are presented in Appendix B. In terms of phylum (figure 4), both groups appear to have a relatively similar collection of microorganisms (species richness). Proteobacteria, bacteroidetes, firmicutes, fusobacteria, and actinobacteria dominate while synergistetes, verrucomicrobia, tenericutes, and plantae are nearly undetectable. However, there are marginal differences between the two groups upon closer inspection. Heavy drinkers have a slightly higher abundance of Bacteroidetes and fusobacteria while light drinkers have higher abundance in proteobacteria (25.62%), firmicutes (29.09%), and actinobacteria (7.02%). As mentioned earlier, these phyla are among those most prevalent in the oral microbiome [22-23]. In general, many of the phyla present in light drinkers may exist in higher abundances in heavy drinkers.

Figure 5 reveals a similar pattern. Overall, both groups represent many of the same genera but the abundances of those genera differ. The dominating genera are Prevotella, *Haemophilus, Neisseria*, *Streptococcus*, *Veillonella*, and *Fusobacterium*. Light drinkers rank higher in *Prevotella* (24.09%), *Haemophilus* (10.74%)*, Veillonella* (7.85), *and Fusobacterium* (4.84), while heavy drinkers have more *Streptococcus* (11.22%) and *Neisseria* (8.70%). Interestingly, the two groups are most different across less abundant genera. Rothia and Porphrymonas are more abundant in light drinkers (3.16% and 1.99%), yet heavy drinkers are more abundant in *Megasphaera* and *Leptotrichia* (1.06% and 3.25%). Furthermore, heavy

drinkers have relatively strong presences by Duncaniella, Paramuribaculum, and much more (represented by the other category) that are either negligible or non-existent in light drinkers. Overall, heavy drinkers show lower abundance of some of the more dominant genera but seem to have higher abundances across most of the less prevalent genera. Full table of genera abundances can be found in Appendix C.

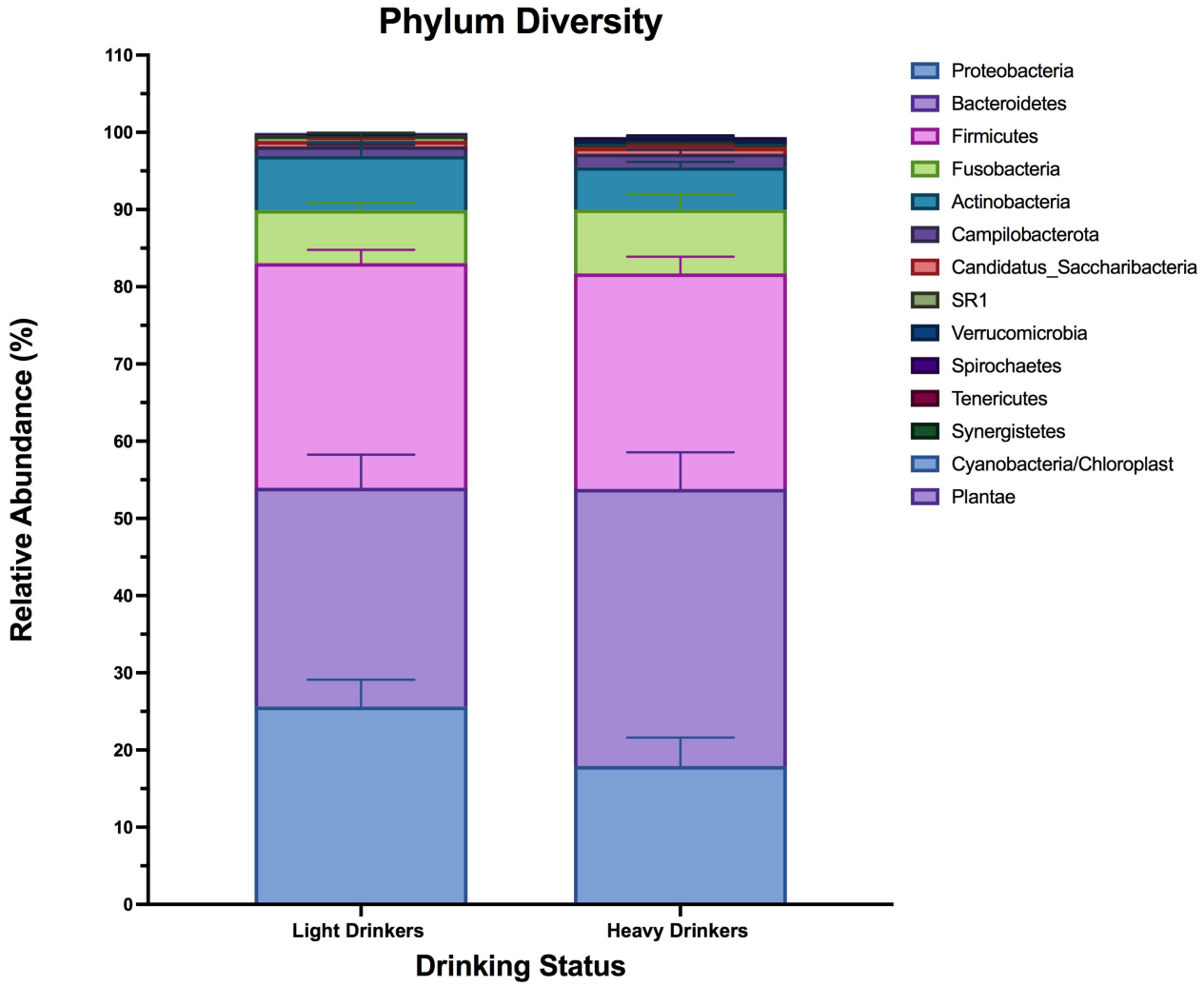

Figure 4. 16s rRNA sequencing identified 14 phyla across 24 saliva samples. The relative abundances of each phyla within each drinking group were calculated and represented in this stacked bar graph. Error bars represent SEM (n= 12 per drinking group).

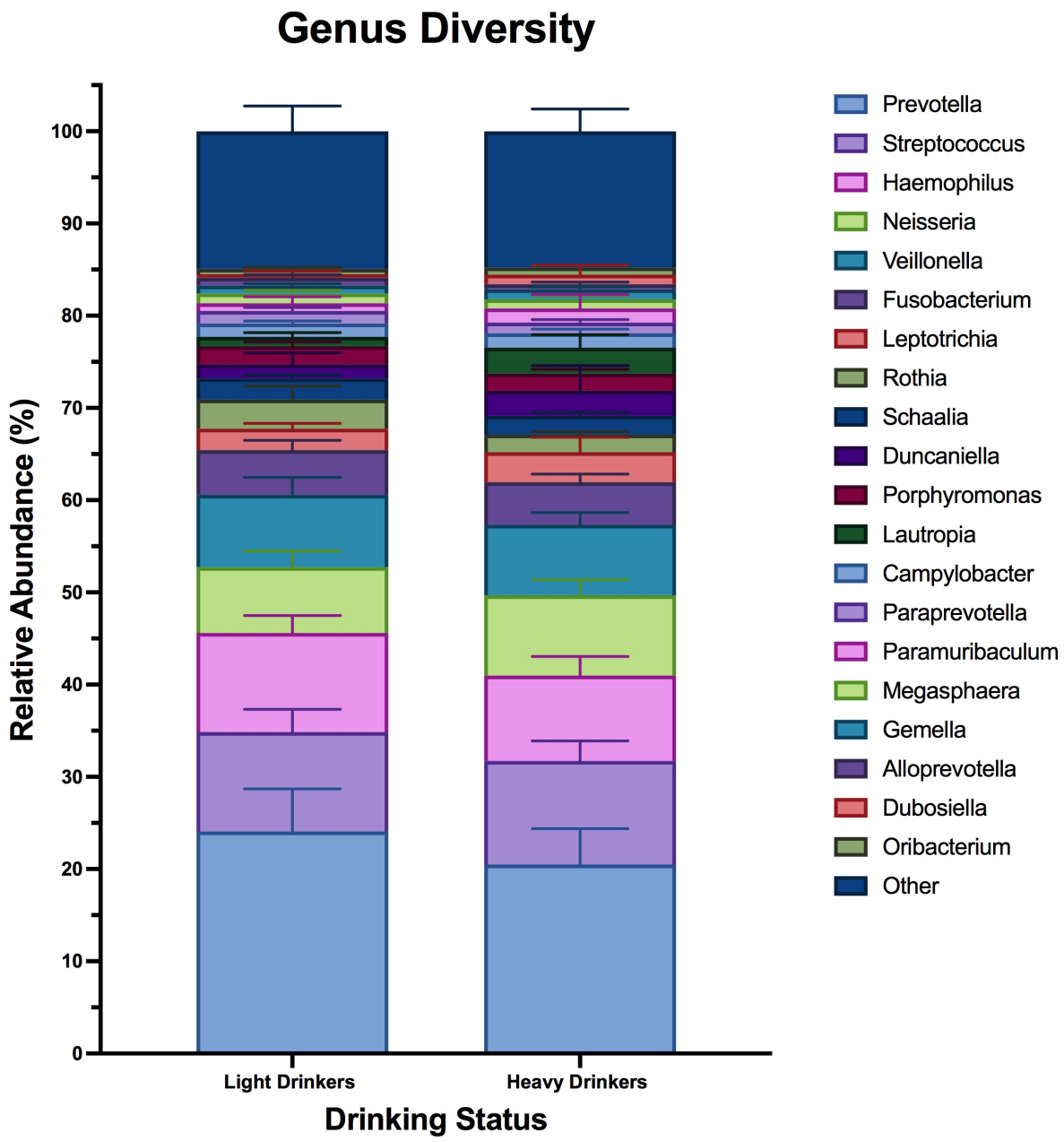

Figure 5. 16S rRNA sequencing identified 126 genera across 24 saliva samples. The relative abundances of the 20 most abundant genera within each drinking group were calculated and represented in this stacked bar graph. The error bars represent SEM (n= 12 per drinking group).

The species diversity graph gives a snapshot of the most prevalent species across samples. Again, the species richness of both drinking groups seem to be the same across the top 20 species. Where they differ is species evenness, particularly in the "other" species category. The most prevalent genus in both drinking groups was *Prevotella,* so as expected, many species from Prevotella belong to the top 20 most prevalent species. On the contrary, although *Neisseria* was highly abundant in both drinking drinks, none of its species occupy the top 20. The most dominant species are *Prevotella melaninogencia, Haemophilus parainfluenzae and Rothia mucilaginosa.* Of those species, *Prevotella melaninogencia* and *Haemophilus parainfluenzae* are more abundant in light drinkers (13.25% and 10.24%) whereas *Rothia mucilaginosa* is slightly more abundant in heavy drinkers (2.61%). A full table of abundances can be found in Appendix D. Interestingly, *P. gingivalis* was not assigned to any sequences. This could be due to several factors as discussed below.

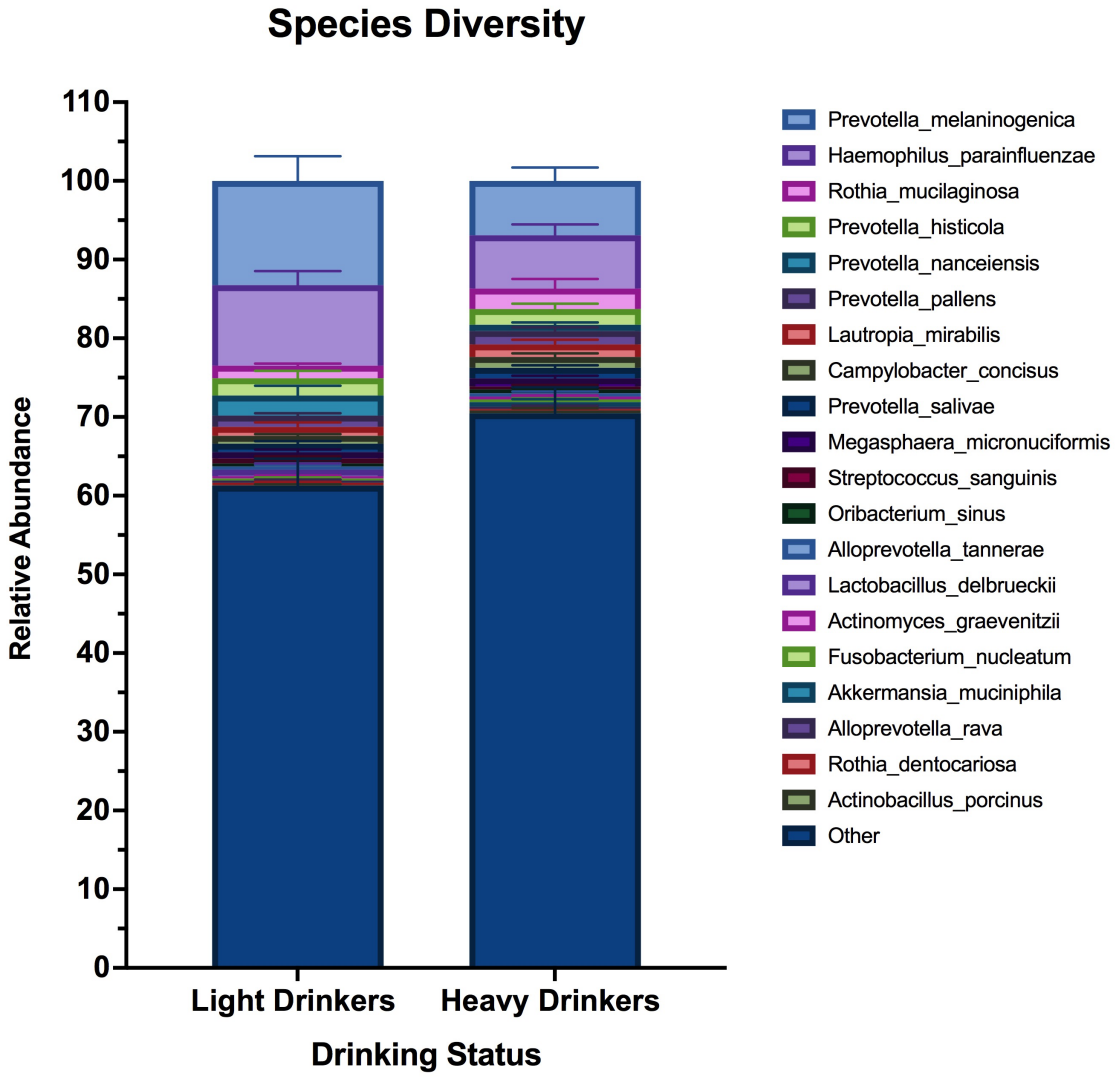

Figure 6. 16S rRNA sequencing identified 109 species across 24 saliva samples. The relative abundances of the 20 most abundant species within each drinking group were calculated and represented in this stacked bar graph. The error bars represent SEM (n= 12 per drinking group).

Alpha Diversity

Alpha diversity is a measure of both species richness and evenness within each sample. In other words, it quantifies the number of different species present in each saliva sample and their abundance relative to the total number of species. The specific alpha diversity metric used in this study is the Shannon Diversity Index, which assigns a diversity value to each saliva sample. The average diversity index was determined for each drinking group and smoking group and revealed in the box plots below (Figure 6 and 7). An unpaired Welch's t-test was performed to determine the difference between the two averages.

The box plots are relatively short, meaning the wasn't much variation in alpha diversity between samples within each drinking group. However, heavy drinkers did have slightly more variation, made apparent by the longer box shape and longer whiskers. Interestingly, heavy drinkers have a longer minimum line, which suggests that most of the variation happened around the lower quartile. Both groups have shorter maximum lines and median lines positioned closer to the top of the box, which suggests the distribution on data is negatively skewed. Visually, heavy drinkers seem to have a more diverse community of bacteria; however, there wasn't a statistically significant difference in alpha diversity between the drinking groups.

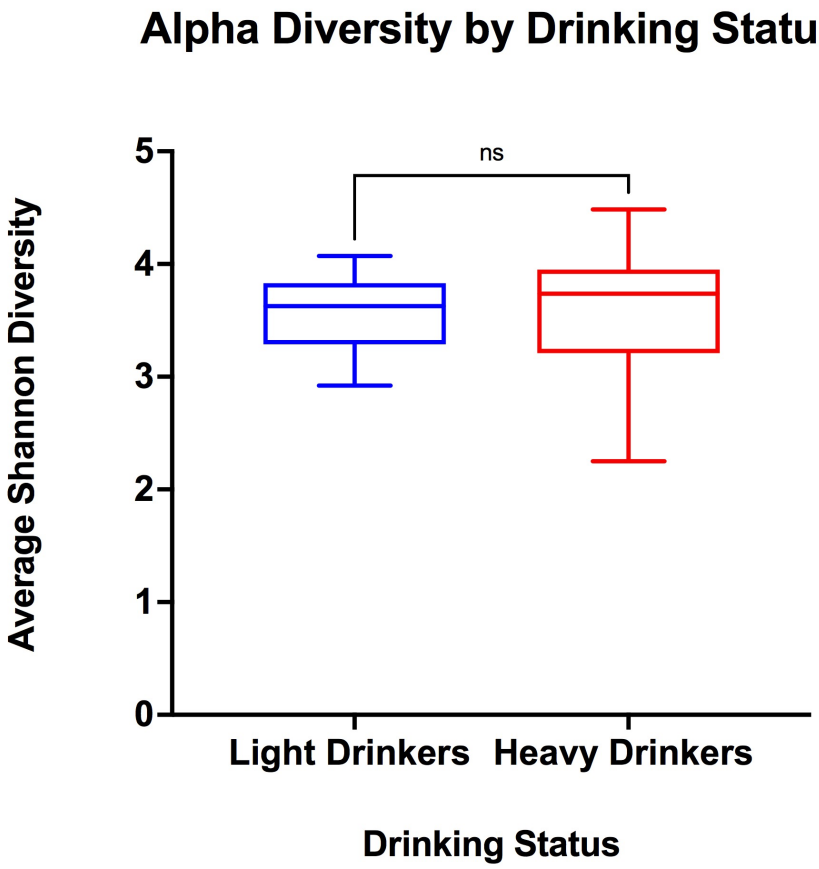

Figure 7. A box plot of average Shannon diversity by drinking status. A Welch's t test shows there is no significant difference between light drinkers and heavy drinkers ($t(17.29) = 0.02644$, $p = 0.9792$).

The same pattern is seen between smoking groups. For both groups, the boxes and whiskers are short, which suggests little overall variation. However, the smoking group has more comparative variation. The median lines for both groups are closer to the top of the box, which again suggests a negatively skewed distribution. Similar to the heavy drinker group, most of the variation for both smokers and non-smokers happens at the lower quartile. Most samples with higher diversity indexes were closely related. Once again, the smokers had more diversity visually, but statistically there was no significant difference between the two groups. Sex was not

examined as a variable of alpha diversity within drinking and smoking groups because sex was balanced within drinking groups and the smoker group had such a small sample size.

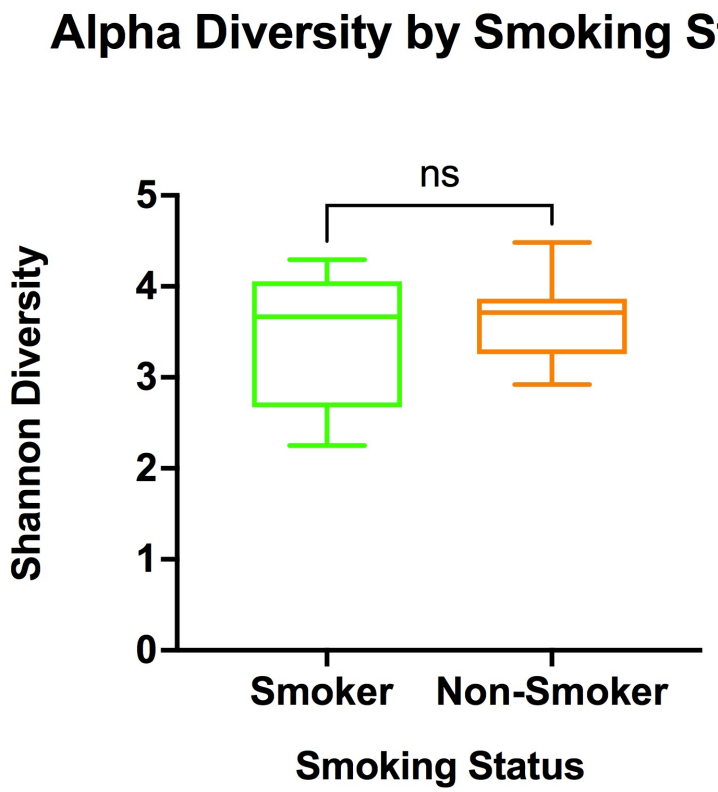

Figure 8. A box plot of average Shannon diversity by drinking status. A Welch's t test shows there is no significant difference between smoking groups ($t(5.869) = 0.6741$, $p=0.5872$).

Beta Diversity

The Bray-Curtis Dissimilarity Index [63] was used to determine the dissimilarity between each saliva sample (Figure 9). There is considerable overlap between the two drinking groups, which is expected in human samples. Most samples within each drinking group are clustered together yet some samples that fall far outside the ellipses. Visually, there aren't any clear clusters that would denote a conclusive difference between the two drinking groups. However, a PERMANOVA test did determine a statistically significant distance ($f(23)=$, $p = 0.05$) between centroids related to drinking group status. This suggests that although the alpha diversity was not statistically different by drinking group, there are profound differences in the species richness and evenness of both groups. In other words, groups are sufficiently dissimilar. Sex was not examined as a variable of beta diversity because it was balanced within drinking groups.

The same metric was used to measure dissimilarity between smoking groups. Unlike drinking groups, smoking groups did seem to cluster but there wasn't a statistical difference between centroids. Perhaps the size of the smoking group was too small to result in any statistically significant results.

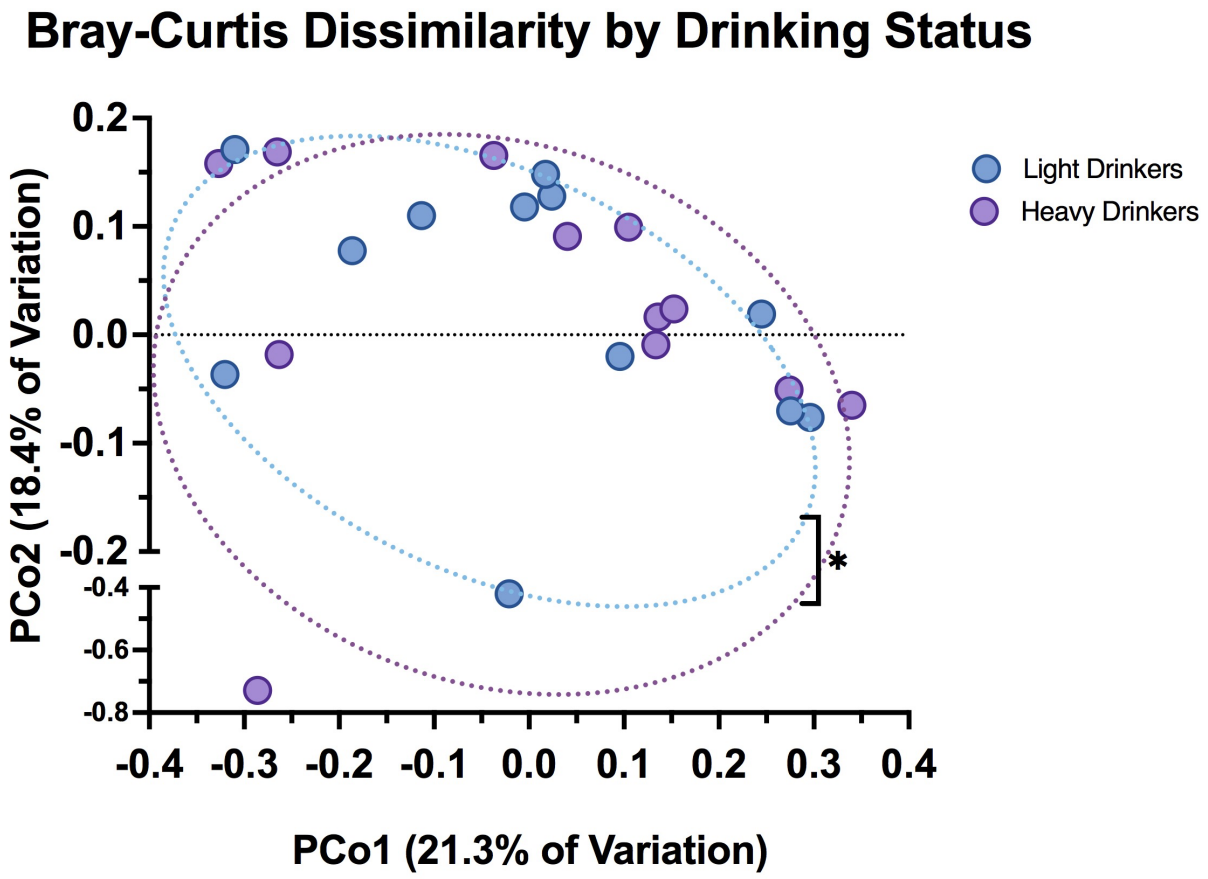

Figure 9. A principle coordinate analysis plot with Bray-Curtis dissimilarity distances between light and heavy drinkers. Each point represents a single saliva sample (n=24) Ellipses represent the 95% confidence interval spread from centroids of each drinking group cluster. PERMANOVA tests determined the distance between the centroids to be statistically significant ($f(23)=1.7097$ $p= 0.037$). Graph produced by Jenna Wurster.

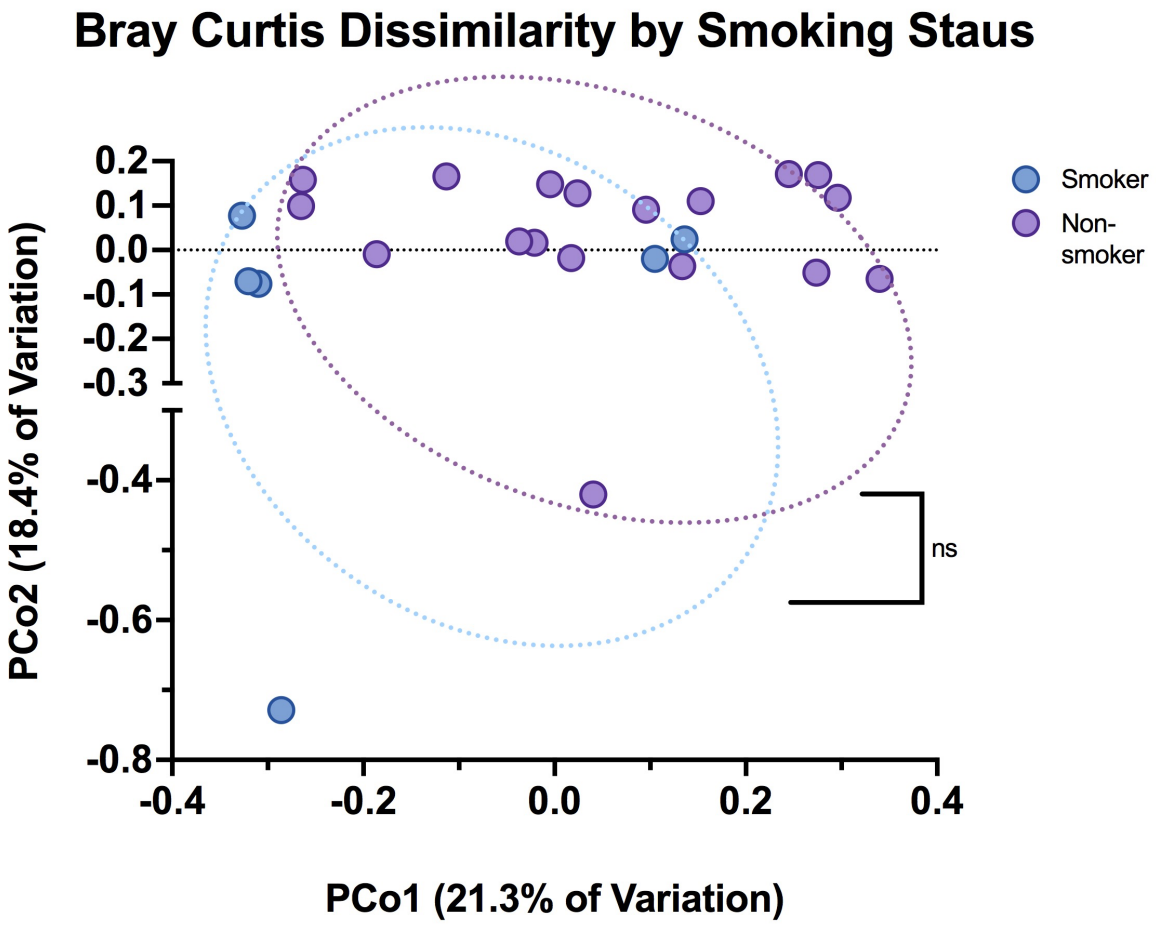

Figure 10. A principle coordinate analysis plot with Bray-Curtis dissimilarity distances between smokers and non-smokers. Each point represents a single saliva sample (n=24) Ellipses represent the 95% confidence interval spread from centroids of each drinking group cluster. PERMANOVA tests determined the distance between the centroids to not be statistically significant ($f(23)=1.2777$, $p = 0.197$).

Analyzing Drinking and Smoking Effect on *Neisseria*

Neisseria is a genus closely associated with oral cancers. Many studies have found higher levels of *Neisseria* in alcohol users that suffer from oral cancers. The average relative abundances on Neisseria was calculated for each drinking group and smoking group and presented in the bar graphs below (Figure 11 and 12). An unpaired Welch's t test was conducted to determine the significant difference between the averages. Overall, *Neisseria* abundance was relatively low within all samples. There was no significant difference between drinking groups but the genus did trend higher in heavy drinkers. There seemed to be large variation within both drinking groups. Similarly, there wasn't a significant difference in *Neisseria* abundance between smokers and non-smokers but non-smokers tended to have higher abundances. Again, there was a lot of variation within smoking groups.

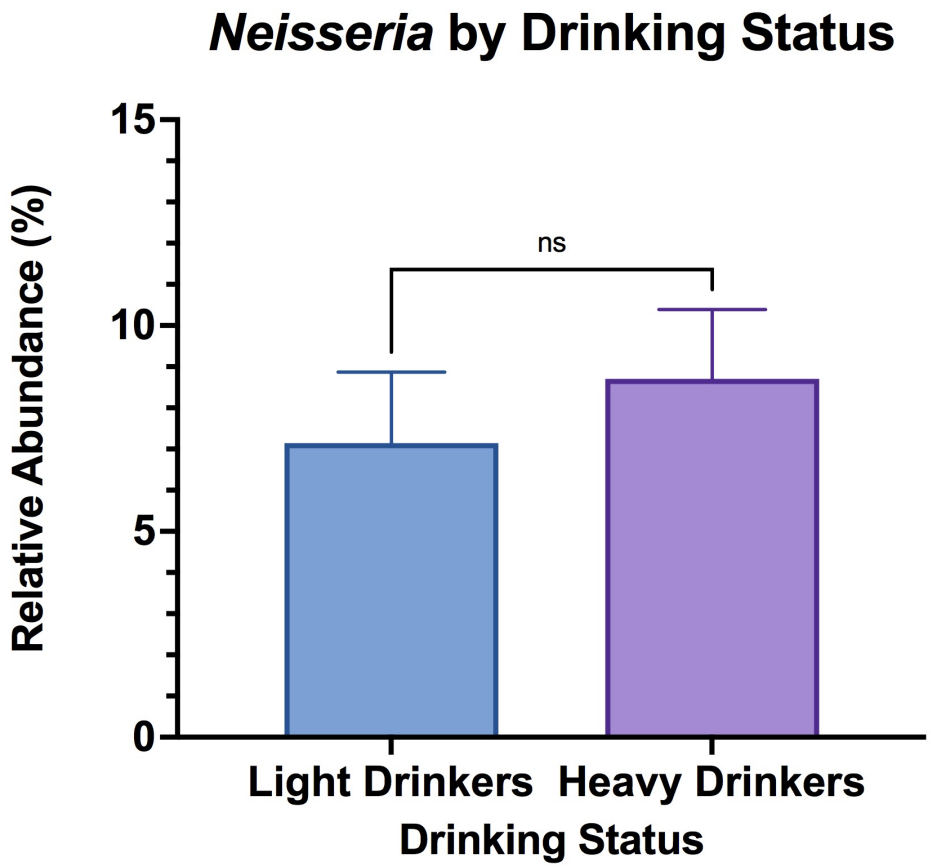

Figure 11. A bar graph of average relative abundance of Neisseria by drinking status. Bars represent SEM (n=12 per drinking group). A Welch's t test determined no significant difference ($t(21.98) = 0.6474$, $p = 0.5241$,) between light drinkers and heavy drinkers.

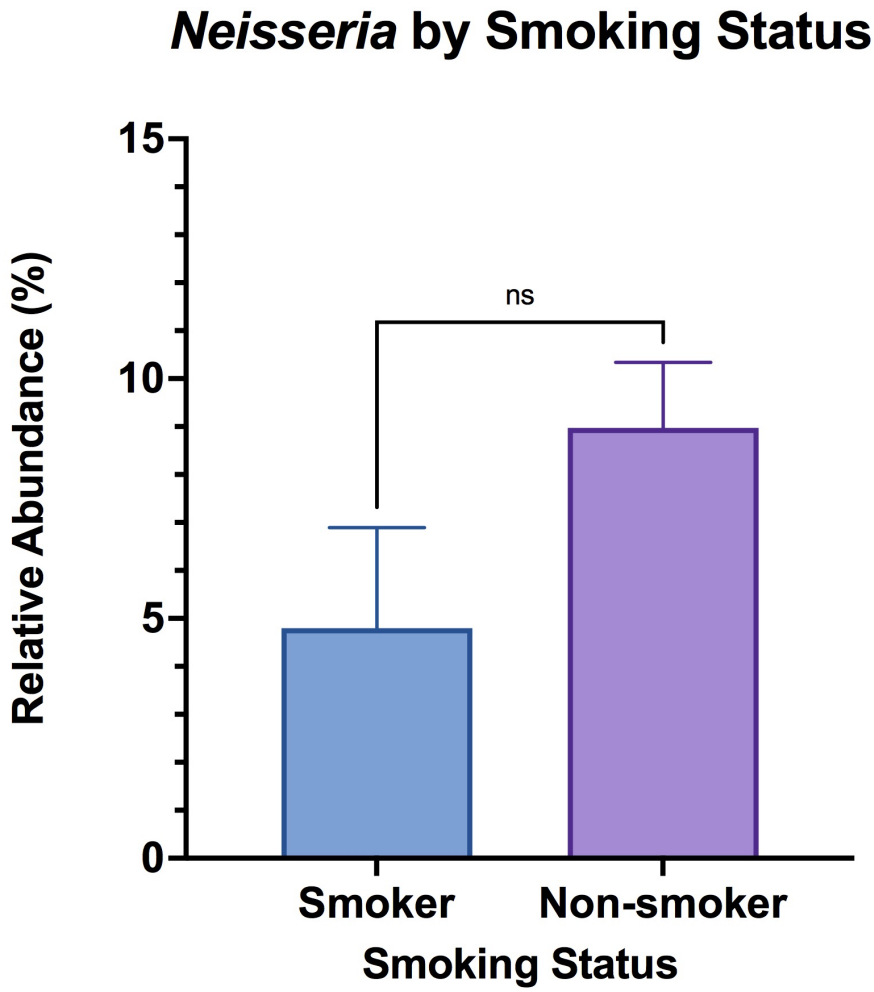

Figure 12. A bar graph of average relative abundance of Neisseria by smoking status. Bars represent SEM (n=6 for smoker, n= 18 for non-smoker). A Welch's t test shows no significant difference between smokers and non-smokers ($t(9.631) = 1.664$, $p=0.1283$).

Analyzing Drinking and Smoking Effect on *Porphyromonas*

Porphyromonas is a gram-negative obligate anaerobic genus found in both light and heavy drinkers. Some reports link it to oral cancers (Chattopadhyay et al, 2019). *Porphyromonas* was chosen as a specific genus of interest because it is the genus to *P. gingivalis* and is linked to oral health and some of the same disease states as *Neisseria*. The average relative abundance of the genus was calculated and visualize on a bar graph. There was no significant relationship between relative abundance and drinking status nor between relative abundance and smoking status. Despite that, light drinkers and non-smokers tended to have higher abundances of the genus.

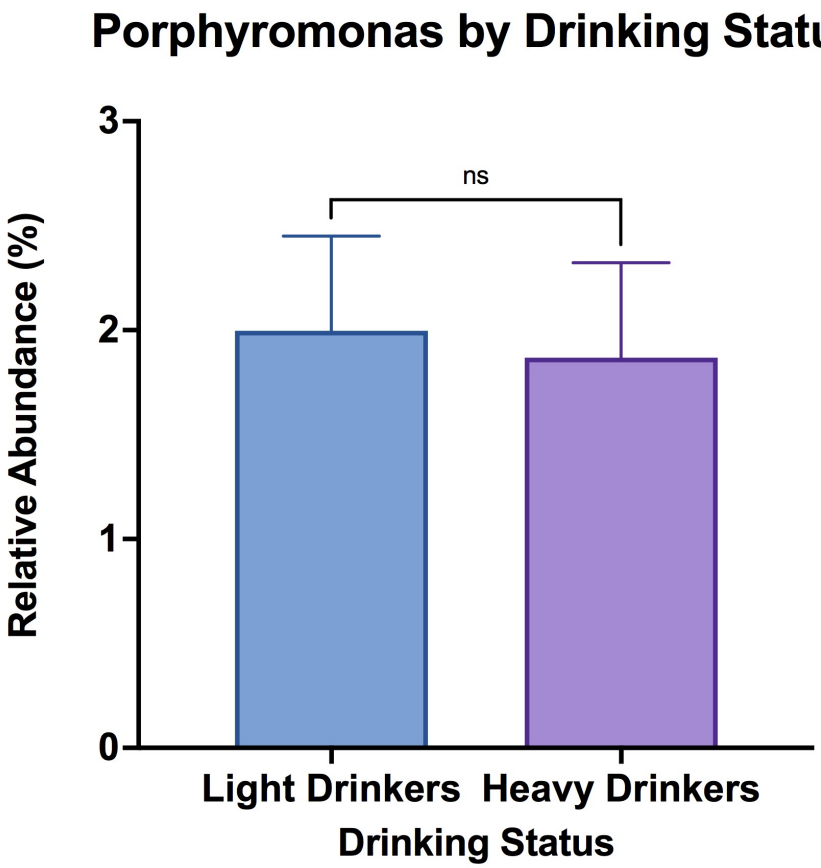

Figure 13. A bar graph of average relative abundance of Porphyromonas by drinking status. Bars represent SEM (n=12 per drinking group). A Welch's t test shows no significant difference ($t(22) = 0.1993$, $p = 0.8438$) between light drinkers and heavy drinkers.

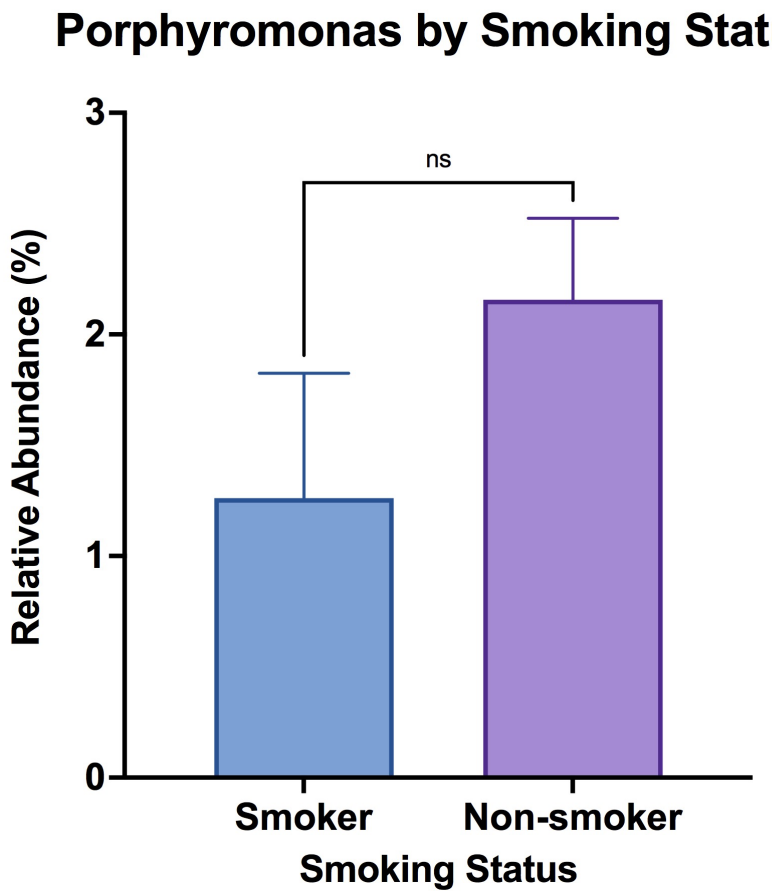

Figure 14. A bar graph of average relative abundance of *Porphyromonas* by smoking status. Bars represent SEM (n=6 for smoker, n= 18 for non-smoker). A Welch's t test shows no significant difference between smokers and non-smokers (t(9.644) = 1.330, p = 0.2140).

Determining Lactobacillales Abundance in Light Drinkers

Lactobacillales is an order found in both light and heavy drinkers. A suggested reason for Neisseria abundance increasing in heavy alcohol consumers relies on Lactobacillales lactic acid production. As alcohol consumption increases, abundance of the order decreases which means less lactic acid is being produced in oral cavities. This increases the pH of the oral cavity which in turn may increase Neisseria. Lactobacillales was significantly higher in light drinkers which would suggest light drinkers have a healthier oral microbiome. Furthermore, that heavy drinkers had less Lactobacillales might account for qualitatively, but not statistically, higher Neisseria in heavy drinkers.

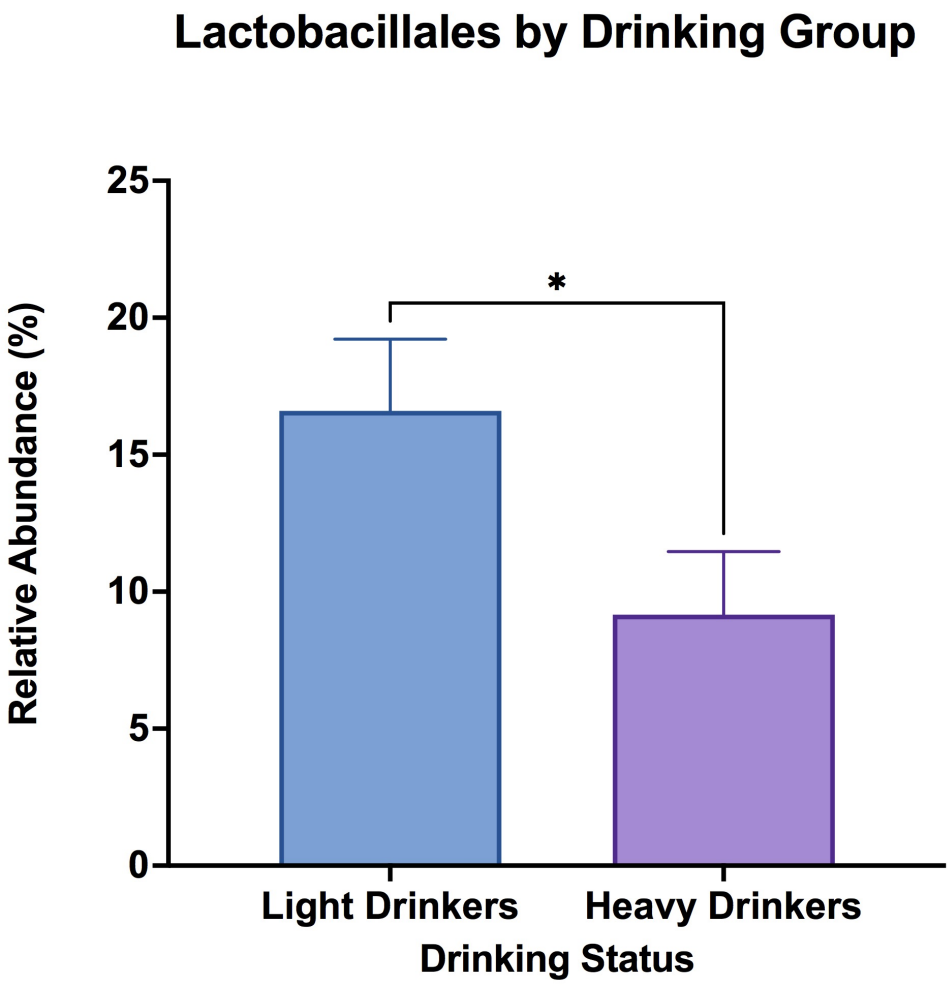

Figure 15. A bar graph of average relative abundance of Lactobacillales by drinking status. Bars represent SEM (n=12 per drinking group). A Welch's t test shows a significant difference ($t(21.64) = 2.136$, $p = 0.0443$,) between light drinkers and heavy drinkers.

Analyzing Drinking and Smoking Effect on *Prevotella melaninogencia, Haemophilus parainfluenzae* and *Rothia mucilaginosa*

The top three most abundant species in both drinking groups were *Prevotella melaninogencia*, *Haemophilus parainfluenzae* and *Rothia mucilaginosa.* Further analysis was done to determine whether there was a significant difference in abundances of all three species between drinking groups and smoking groups. There were no significant differences between drinking groups for all three species. Smoking groups differed on *Haemophilus parainfluenzae* but not on *Prevotella melaninogencia* and *Rothia mucilaginosa.*

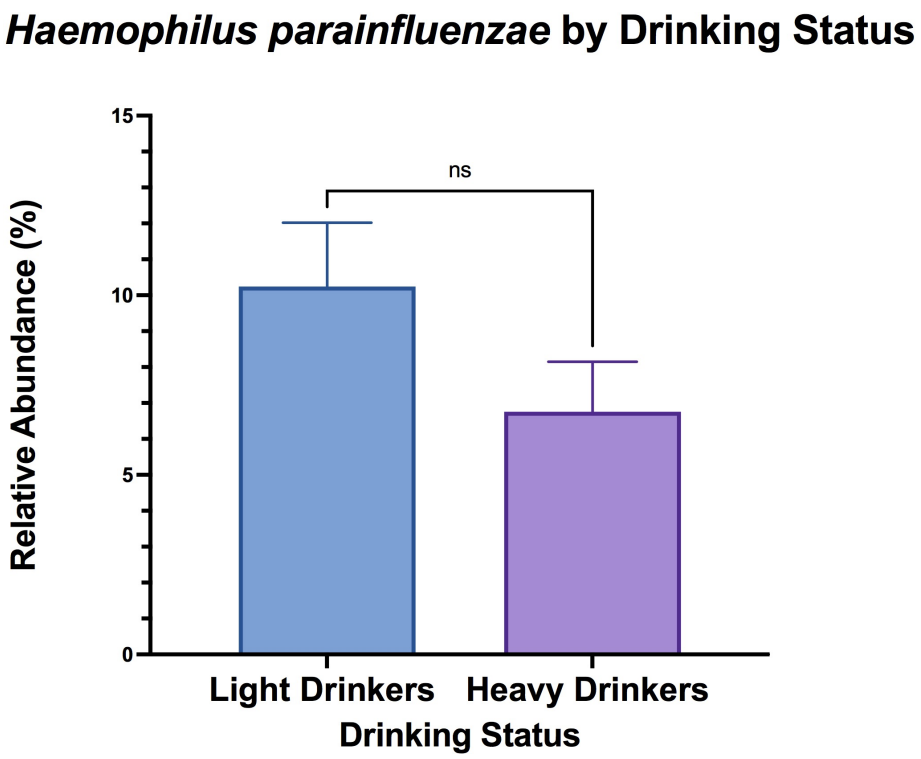

Figure 16. A bar graph of relative abundance of *Haemophilus parainfluenzae* by drinking status. The error bars represent SEM (n=12 per drinking group). A Welch's t test found no significant difference between light and heavy drinkers (t(20.80) = 1.542, p= 0.1380).

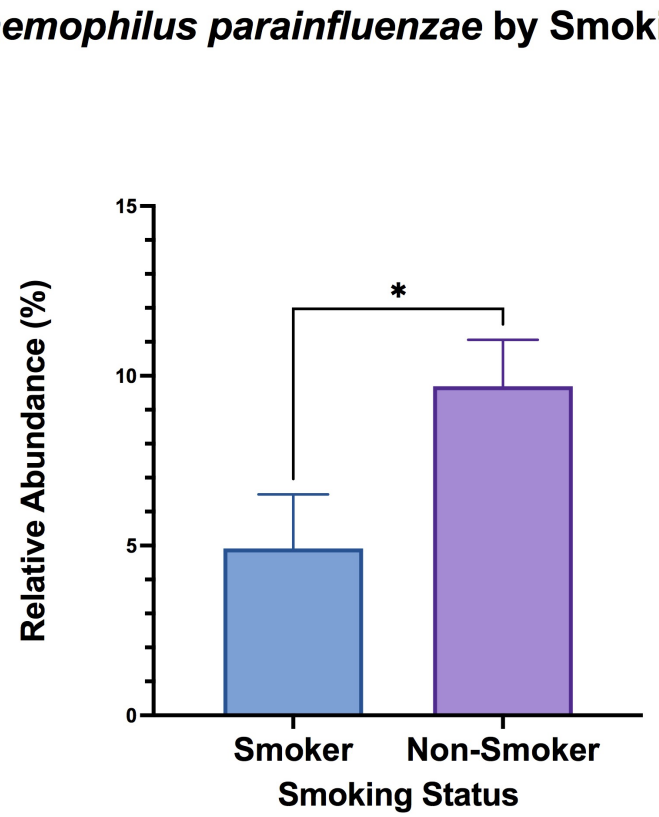

Figure 17. A bar graph of average relative abundance of *Haemophilus parainfluenzae* by smoking status. Bars represent SEM (n=6 for smoker, n= 18 for non-smoker). A Welch's t test shows a significant difference (t(12.99) = 2.281, p= 0.0400) between smokers and non-smokers.

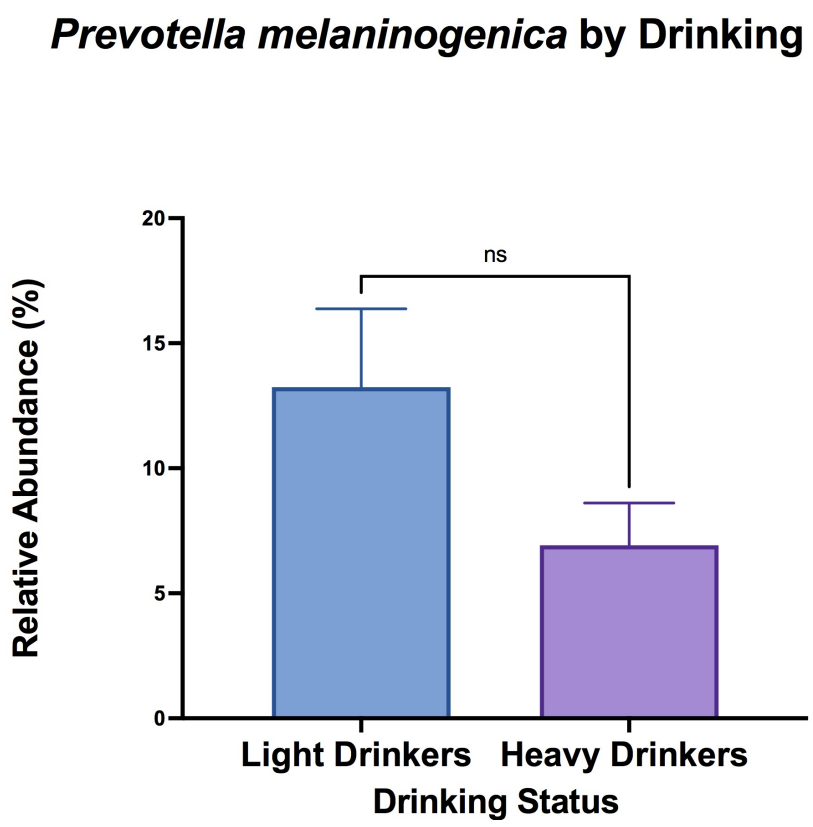

Figure 18. A bar graph of relative abundance of *Prevotella melaninogenca* by drinking status. The error bars represent SEM (n=12 per drinking group). A Welch's t test found no significant difference between light and heavy drinkers (t(16.94) = 1.777, p= 0.0935).

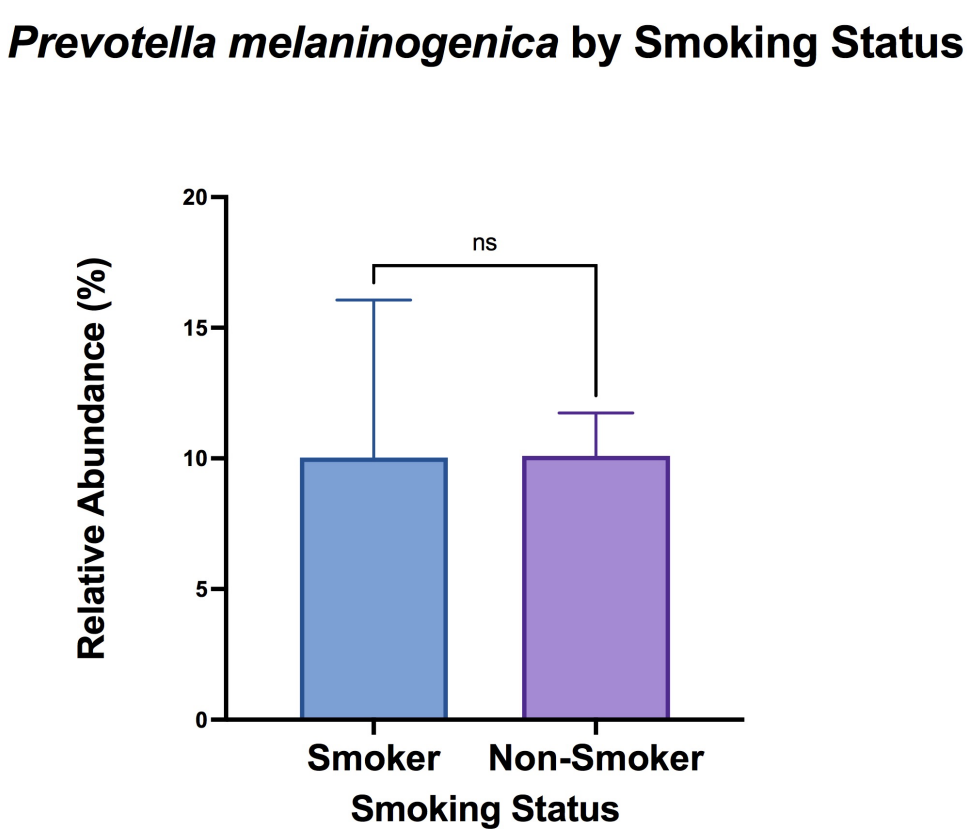

Figure 19. A bar graph of average relative abundance of *Prevotella melaninogenica* by smoking status. Bars represent SEM (n=6 for smoker, n= 18 for non-smoker). A Welch's t test shows no significant difference between smokers and non-smokers (t(5.755) = 0.01140, p = 0.9913).

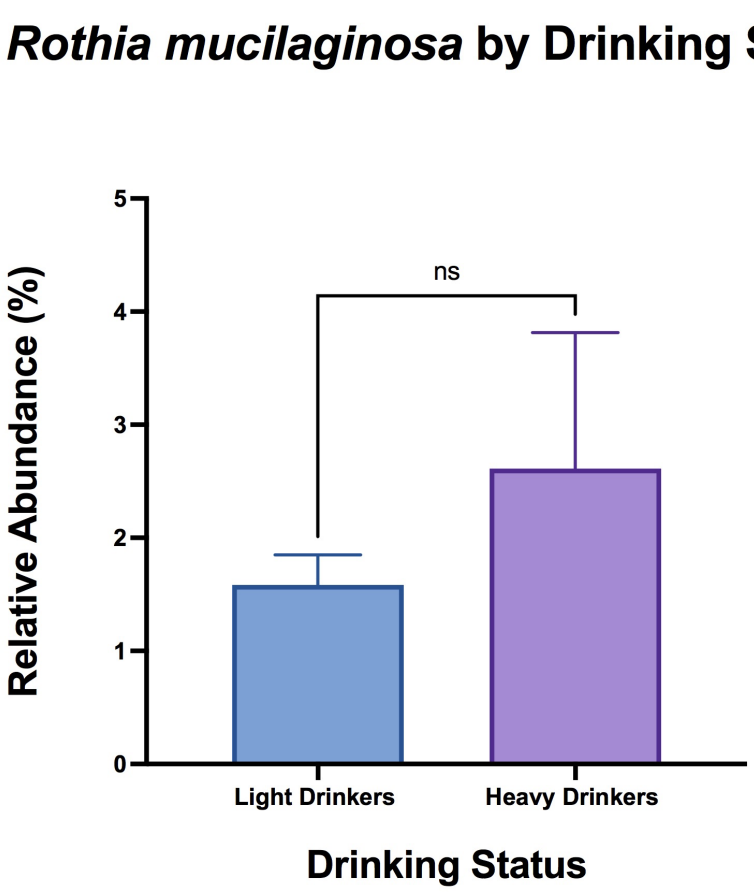

Figure 20. A bar graph of relative abundance of *Rothia mucilaginosa* by drinking status. The error bars represent SEM (n=12 per drinking group). A Welch's t test found no significant difference between light and heavy drinkers (t(12.07) = 0.8351, p=0.4199).

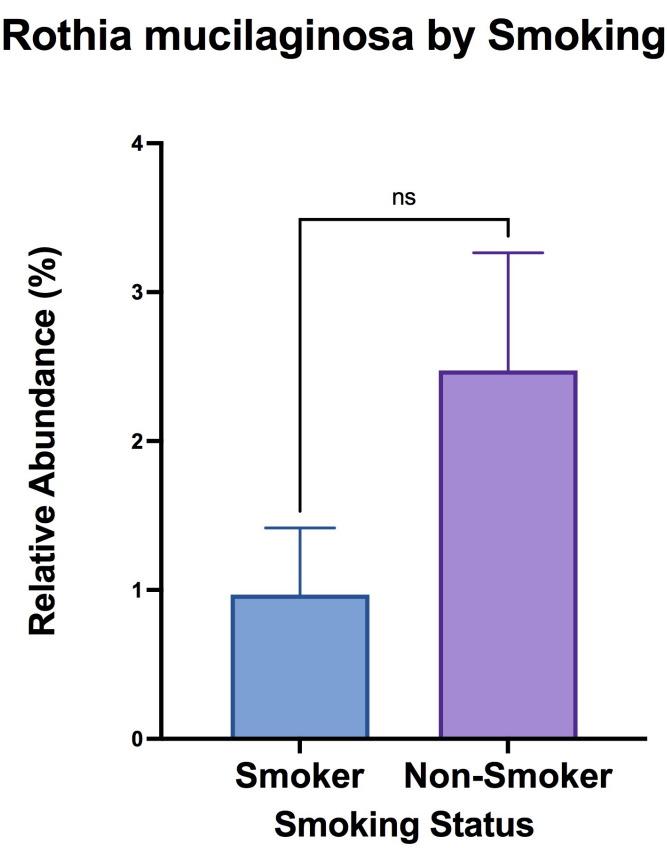

Figure 21. A bar graph of average relative abundance of *Rothia mucilaginosa* by smoking status. Bars represent SEM (n=6 for smoker, n= 18 for non-smoker). A Welch's t test shows no significant difference between smokers and non-smokers (t(21.97)=1.660, p=0.1110).

Discussion

Conflicting reports make it challenging to deduce whether diversity in the oral microbiome is a good thing- and if it is, then how much diversity is too much diversity? The goal of this study was to characterize the oral microbiome of light and heavy drinkers, noting key differences in the abundance of certain microbes that might lead to dysbiosis and ultimately disease states. Overall, samples from light and heavy drinkers were dominated by Proteobacteria, Bacteroidetes, Firmicutes, Fusobacteria, and Actinobacteria. On average, those phyla made up approximately 97% of light drinker oral microbiomes and 95% of that of heavy drinkers. Although the abundance of each of those phyla differed somewhat between drinking groups, the difference was only marginal. One might say at the phylum level, light drinkers and heavy drinkers are statistically indistinguishable. The fact that they both comprise what are deemed normal and healthy bacteria in the appropriate amounts could suggest that alcohol consumption as it relates to this group of participants is not enough to disorder the oral microbiome.

Light and heavy drinking groups were similar on the genus level as well. Both groups were dominated by *Prevotella*, *Haemophilus, Neisseria*, *Streptococcus*, *Veillonella*, and *Fusobacterium.* Similar to the distribution of phyla, among the most abundant genera, both drinking groups are virtually indistinguishable. What's interesting is that the groups differ more by less abundant genera. Differences in *Porphrymonas* (Figure 13) and *Neisseria* (Figure 11) were not statistically significant ($p=0.8438$ and $p=0.5241$, respectively), Qualitatively, however, Neisseria was more abundant in heavy drinkers. Both genera are gram negative, anaerobic bacteria linked to oral cancers [66-67]. High levels of *Neisseria* are linked to increased acetaldehyde production, which results in heightened oral cancer risk [46, 49, 68]. Fan, et al. suggested that that alcohol consumption mediates a negative correlation between Lactobacillales

and Neisseria. Lactobacillales is an order that holds species that produce lactic acid, which is a phenomenon believed to mediate, in part, the pH levels of the oral cavity. Heavy drinking is associated with reduced levels of Lactobacillales in the microbial environment. Less Lactobacillales leads to less lactic acid production which causes an increase in pH levels. *Neisseria* prefers high pH environments so it is believed to thrive in heavy drinkers [47]. In this study, Lactobacillales was significantly lower in heavy drinkers ($p = 0.0443$; Figure 15). This not only supports Fan et al.'s findings but it also raises questions about how alcohol consumption may lower Lactobacillales abundance.

Neisseria within light drinkers was considerably varied. There were major outliers with high abundances that may have increased the average abundance and negated a significant difference between the two drinking groups. Moreover, the participants in this study were generally and orally healthy. Most studies that linked *Neisseria* to cancer risk included patients that suffered from oral cancers or were at risk of developing oral cancers. Thirdly, studies that looked at *Neisseria* abundance had higher age ranges and heavy drinking groups consisted of older folks with more drinking years. The participants in this study were young. The median age is 22 for all participants and 25.5 for heavy drinkers specifically. Most young adults only have a few years of drinking experience. Perhaps alcohol induced Neisseria abundance is a long-term effect of drinking. Given these inconsistencies, one might even suggest that normal microbiomes have higher levels of Lactobacillales and therefore lower levels of Neisseria. Over time, as alcohol consumption increases and becomes more acute, the trend reverses and Neisseria becomes more abundant, producing more metabolites that are harmful to the human body.

Neither *Neisseria* nor *Porphyromonas* was significantly affected by smoking status though non-smokers tended to have a higher abundance of both (Figures 11 and 13). Previous

studies reported Neisseria to be statistically lower in smokers than in smoker/drinker and control groups (Figure 3). Thus, these findings resemble previous research [55] albeit without statistical significance. *Neisseria* and *Porphyromonas* also had a lot of variation within smoking groups. There were outliers within each group that could have increased the overall average abundance on each genus within smoking groups and reduced statistical significance. Even if there were a statistically significant effect of smoking on both genera, it would be difficult to predict any solid associations in the current study due to the small sample size of smokers (n =6). If smoking and drinking do have a collaborative impact on *Neisseria* and *Porphyromonas*, further studies must be done with a larger smoking group to reveal more trustworthy patterns and test statistical interactions.

On the species level (figure 6), drinking groups had comparable species richness. Every species in the top 20 most abundance species was present in at least one sample from each drinking group. Nevertheless, there were clear differences in species evenness. Similar to the trend in genera, light drinkers had higher abundances of the most prevalent species but heavy drinkers seemed to be considerably more representative of less common species included in the "other" category. The top three most abundant species in both drinking groups were *Prevotella melaninogenica (P. melaninogenica), Haemophilus parainfluenzae (H. parainluenzae)* and *Rothia mucilaginosa (R. mucilaginosa)*. *P. melaninogenica* is a pathogenic anaerobic bacterium closely associated with respiratory infections when in high abundance in the respiratory microbiome [69-70]. Its function in the oral microbiome is contested to say the least. It has been linked to healthy oral cavities when in high abundances [71]. In that regard, it makes sense that it is present in high abundances in both drinking groups because both groups contain orally healthy individuals. There was no significant difference between its relative abundance in light and

heavy drinkers or smokers and non-smokers (Figures 17 and 18) but light drinkers tended to have a higher abundance. Perhaps in a study with a larger sample size, *P. melaninogenica* abundance might be indicative of light drinkers having a healthier oral microbiome. Interestingly, *P. melaninogenica* has also been linked to oral lichen planus, a T-cell mediated autoimmune disease that causes chronic inflammation of the mucous membranes in the mouth [66]. Whether this is a commensal or harmful bacteria is still unclear.

There was also no significant difference in the relative abundance of *H. parainfluenzae* between drinking groups but light drinkers tended to have higher abundance of it (Figure 16). On the contrary, a significant association was found between the species and smoking status (Figure 17). Non-smoking groups had much higher abundances of the bacterium. *H. parainfluenzae* is an opportunistically pathogenic bacterium that was found in high abundances in HIV patients [72-73]. There is clearly some hidden relationship between smoking and this species. Perhaps non-smokers have a more welcoming environment to *H. parainfluenzae.* Again, a more robust sample size is needed to codify these results. *R. muciliaginosa* is a gram-positive opportunistic pathogen that typically causes infection in immunocompromised hosts and has been linked to prosthetic infections [73-74]. In the oral cavity, this bacterium can also reinforce immune system response. Uranga et al. suggests that *R. muciliaginosa* releases enterobactin, which in turn reduces the proliferation of carcinogenic bacterial strains [75]. So, in some regards this bacterium may be useful to heavy drinkers who have higher levels of Neisseria, a carcinogen producing genus. There was no significant association with it and drinking status or smoking status (Figure 20 and Figure 21). However, heavy drinkers and non-smokers tended to have higher abundances. As previously stated, heavy drinkers and non-smokers also tended to have higher abundances of Neisseria. One could suggest that *R. mucilaginosa* and *Neisseria* abundance are related in that

when *Neisseria* abundance increases, *R. mucilaginosa* multiplies and combats *Neisseria's* proliferation. This is just a theory, however. More research should be done one to determine the relationship between the two microorganisms.

A species that was noticeably absent from both drinking groups was *P. gingivalis (*other species belonging to the Porphyromonas genus were found). This might be because it wasn't properly captured during the saliva collection stage. P. gingivalis mainly inhabits the subgingival sulcus and requires rigorous methods of removal [38]. This study relied on oral swishing and perhaps this method wasn't enough to loosen the bacterium and promote translocation into the collection tube. Future studies might employ a swabbing or scrapping method.

Alpha and beta diversity metrics also resulted in peculiar findings. The Shannon evenness index was used to calculate alpha diversity. This index considers both the number of species present in each sample as well as the abundance of those species. There was no significant difference in alpha diversity based drinking status or smoking status (Figures 7 and 8). However, samples from heavy drinkers and non-smokers trended towards greater diversity. Samples from heavy drinkers also had wider variation. The highest (H= 4.48) and lowest (H= 2.25) diversity measures came from the heavy drinking group. This finding might suggest that heavy drinking doesn't have the same effect on all drinkers. Perhaps this effect relies on other factors such as overall oral health. Previous studies focused mainly on specific species and this study would suggest that drinking has no effect on overall alpha diversity but that's hard to say definitively with such a small sample size of drinkers. This study also focused on healthy non-dependent drinkers. Much like Neisseria abundance, perhaps alcohol's overall effect on alpha diversity is long-term and best observed longitudinally. In terms of tobacco consumption, smokers tended to have a wider range of H values than non-smokers. One might suggest that heavy drinking and

smoking as it relates to the participants in this study trended toward more diversity but a larger sample size of drinkers that also smoke is needed to make significant predictions.

Interestingly, beta diversity differed significantly between the two drinking groups was statistically significant (Figure 9). The Bray-Curtis dissimilarity metric was used to determine the dissimilarity between the two drinking groups. This method takes two samples and identifies the least abundant species present in both samples. Then it sums up the total abundance of those least abundant species and divides by the total abundance of all species present in both samples. This results in an index that can plotted on a principle coordinate axis. Although there was overlap of drinking groups, a PERMANOVA test did determine that there was a significant difference between the two experimental groups by drinking status. This is interesting given that there was no significant difference in alpha diversity. Perhaps within this study, level of alcohol consumption does affect diversity on the community level but that effect is harder to see on the individual level. In other words, drinking status does in fact affect oral microbiota diversity but it would take a large population to visualize that difference. Smoking groups were not statistically dissimilar. That being said, smokers did tend to cluster together clearly but for one data point that fell outside the ellipsoid. This could suggest a trend towards dissimilarity. Perhaps it was the small size of the smoking group that hindered a significant finding. On the other hand, many studies have reported that oral diversity is independent of smoking status. Therefore, this study might provide further evidence of tobacco consumption being ineffective on over diversity but effective on particular species (smokers tended to have lower abundances of all species examined in this study, Haemophilus parainfluenzae was statistically lower in non-smokers).

Conclusion

In closing, due to the lack of statistical significance in alpha diversity between drinking groups we must accept the null hypothesis for hypothesis number one in terms of alpha diversity but not beta diversity. For now, we can say that while light drinkers tended to have higher abundances of dominant taxa, heavy drinkers overall trended towards more diversity. For hypothesis number two we must accept the null hypothesis, although we can say that smokers trended toward more alpha diversity. As for hypothesis number three, the relative abundance of Neisseria did not differ significantly between drinking groups and smoking groups. Furthermore, P. gingivalis was not assigned to any of the sequences found in both light and heavy drinker samples so we must accept the null hypothesis for hypotheses three and four. Notwithstanding, several other species of interests were identified and explored. Despite failure to reject most of the null hypotheses, this study did succeed in providing a framework for the what the oral microbiome composition of light and heavy drinkers looks like in young smoking and non-smoking adults. The main limitation of this study was its small sample size (N=24). In that regard, this study should act as a preliminary investigation into the effect of drinking status on oral microbiota.

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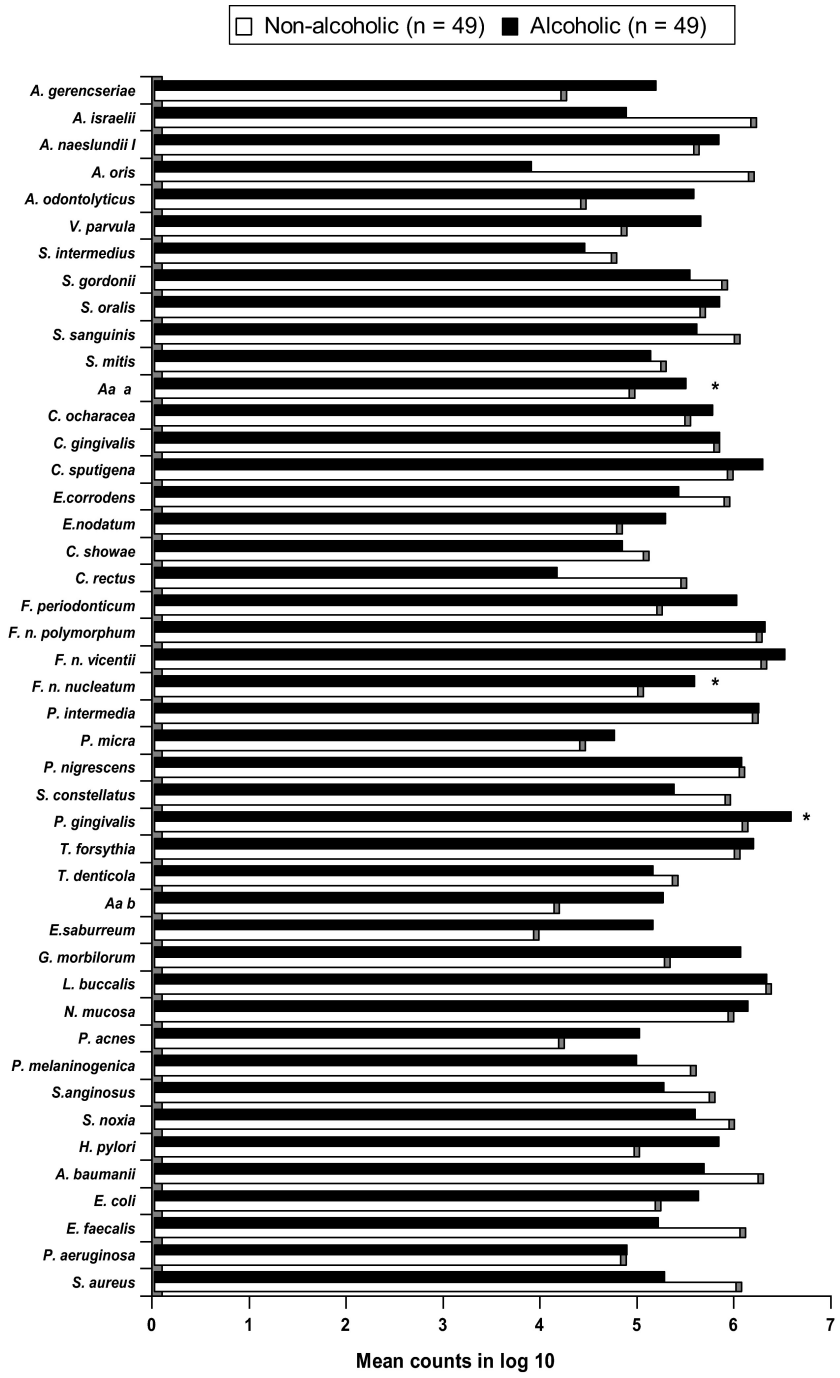
Appendix A



Appendix A. Taken from Amaral et al (2011). A stacked bar graph of bacterial log-transformed bacterial mean counts in alcoholics and non-alcoholics. P. gingivalis is statistically higher in alcoholics.

Appendix B

Phylum	Mean Relative Abundance in Light Drinkers (%)	Mean Relative Abundance in Heavy Drinker (%)
Proteobacteria	25.62	17.93
Bacteroidetes	28.34	35.88
Firmicutes	29.09	27.93
Fusobacteria	6.88	8.26
Actinobacteria	7.02	5.50
Campilobacterota	1.24	1.74
Candidatus_Saccharibacteria	0.67	0.77
SR1	0.74	0.39
Verrucomicrobia	0.02	0.56
Spirochaetes	0.19	0.38
Tenericutes	0.02	0.03
Synergistetes	0.03	0.02
Cyanobacteria/Chloroplast	0.05	0.02
Plantae	0.01	0.00

Appendix B. A table of the 14 phyla identified within each sample. The mean relative abundance of each phyla found within light and heavy drinkers is presented in percentages.

Appendix C

Genus	Mean Relative Abundance in Light Drinkers (%)	Mean Relative Abundance in Heavy Drinker (%)
Prevotella	24.09	20.49
Streptococcus	10.77	11.22
Haemophilus	10.74	9.27
Neisseria	7.14	8.70
Veillonella	7.85	7.64
Fusobacterium	4.84	4.62
Leptotrichia	2.32	3.25
Rothia	3.16	1.91
Schaalia	2.29	2.04
Duncaniella	1.51	2.72
Porphyromonas	1.99	1.87
Lautropia	1.01	2.79
Campylobacter	1.43	1.55
Paraprevotella	1.35	1.17
Paramuribaculum	0.84	1.54
Megasphaera	1.06	0.98
Gemella	0.85	1.09
Alloprevotella	0.86	0.57

Dubosiella	0.38	1.03
Oribacterium	0.58	0.79
Other	14.92	14.76

Appendix C. A table of the top 20 most abundant genera identified within each sample. The mean relative abundance of each genus found within light and heavy drinkers is presented in percentages. The other category represents all other genera found in relatively low abundances.

Appendix D

Species	Mean Relative Abundance in Light Drinkers (%)	Mean Relative Abundance in Heavy Drinker (%)
Prevotella melaninogenica	13.25	6.92
Haemophilus parainfluenzae	10.24	6.76
Rothia mucilaginosa	1.58	2.61
Prevotella histicola	2.19	1.99
Prevotella nanceiensis	2.56	0.78
Prevotella pallens	1.44	1.71
Lautropia mirabilis	1.13	1.59
Campylobacter concisus	0.99	1.39
Prevotella salivae	1.07	1.31
Megasphaera micronuciformis	0.81	1.05
Streptococcus sanguinis	0.63	0.36
Oribacterium sinus	0.39	0.49
Alloprevotella tannerae	0.39	0.42
Lactobacillus delbrueckii	0.81	0.00
Actinomyces graevenitzii	0.31	0.37
Fusobacterium nucleatum	0.27	0.34
Akkermansia muciniphila	0.00	0.58
Alloprevotella rava	0.26	0.29

Rothia dentocariosa	0.29	0.17
Actinobacillus porcinus	0.09	0.36
Other	61.27	70.48

Appendix D. A table of the top 20 most abundant species identified within each sample. The mean relative abundance of each species found within light and heavy drinkers is presented in percentages. The other category represents all other species found in relatively low abundances.