ABSTRACT

Title of Dissertation: CHARACTERIZING BACTERIAL COMMUNITIES ACROSS DIVERSE TOBACCO PRODUCTS AND DYSBIOSIS IN THE ORAL MICROBIOME RESULTING FROM TOBACCO USE

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To evaluate the adverse health effects associated with tobacco smoking, the majority of studies have largely focused on the impact of chemical constituents in tobacco products and less on the impact of microbial contaminants within these products. Similarly, the United States (U.S.) Food and Drug Administration's (FDA) Family Smoking Prevention and Tobacco Control Act (FSPTCA) requires tobacco manufacturers and importers to test and report on chemical constituents included on their list of harmful and potentially harmful constituents (HPHCs) in tobacco products and tobacco smoke; however, this HPHC list excludes microorganisms. Nevertheless, over the past decade, an increasing number of studies have explored the bacterial microbiome of tobacco products (e.g., cigarettes, little cigars, cigarillos, hookah and smokeless tobacco) and mainstream smoke. These studies have yielded robust data on bacterial diversity

and bacterial community composition within individual tobacco product types. However, to date, there are no comparative characterizations of the bacterial microbiome across diverse tobacco products. In particular, there have been no studies that have characterized the metabolically-active (live) bacterial communities across these products; bacterial communities that might be transferred to the user's oral cavity and cause subsequent adverse health effects. Furthermore, from an oral health perspective, while smoking/using tobacco products causes significant oral dysbiosis (bacterial community disturbances), there is a lack of data on the potential transfer of bacteria from tobacco products to user's oral cavities or transient temporal changes in the oral microbiome that might result from smoking these tobacco products. Moreover, there are limited data comparing oral microbiome differences between cigarette smokers and smokeless tobacco users.

To address these knowledge gaps, my dissertation research involved a comparative analysis of the bacterial microbiome of commercially-available tobacco products and an evaluation of the impacts of tobacco use on the oral microbiome of users. My specific aims were: 1) To evaluate the compositional differences in the bacterial microbiome between conventional tobacco products; 2) To evaluate transient changes in the oral microbiome of cigarette smokers after a single use of a little cigar; and 3) To conduct a comparative characterization of the oral microbiome between cigarette users, smokeless tobacco users, and non-users over time.

Overall, I found that each type of conventional tobacco product harbored a significantly different bacterial community, with mentholation and/or flavoring being a significant driver of bacterial community changes. However, across all products the majority of the metabolically-active bacterial community was identified as *Pseudomonas*, along with members of the phylum Firmicutes, as well as a few pathogenic species previously associated with animal/human

respiratory diseases. In analyzing the oral microbiome of cigarette smokers, I found that a single use of a little cigar product did not cause transient changes in the oral microbiome. In analyzing longer temporal effects of smoking on the oral microbiome, I found that, even though total bacterial diversity and composition did not change over time, multiple bacterial taxa were significantly different, with regard to their relative abundance, after four months. In addition, I found that dysbiosis of the oral microbiome was dependent on the type of tobacco product used (cigarettes or smokeless tobacco), and that tobacco smokers/users' oral microbiomes harbored a more diverse set of bacterial species when compared to that of non-users.

In summary, this work provides a comparative analysis of the total and metabolically-active bacterial microbiome of tobacco products, as well as rich findings regarding the relationship between tobacco use and oral microbiome dysbiosis. These data address major research priorities of the FDA relating to furthering our understanding of the adverse health risks associated with tobacco smoking. Specifically, my data will add to the current body of knowledge regarding the potential role of tobacco bacterial communities in the development of smoking-related diseases. My data also can be leveraged by tobacco regulatory bodies to make future evidence-based policy changes that help reduce risks associated with microorganisms in tobacco products and protect public health.

CHARACTERIZING BACTERIAL COMMUNITIES ACROSS DIVERSE TOBACCO PRODUCTS AND DYSBIOSIS IN THE ORAL MICROBIOME RESULTING FROM TOBACCO USE

by

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Dedication

To the apple of my eye, Subhang.

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Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

Tobacco smoking and secondhand smoke lead to almost half a million deaths per year in the United States (U.S.) [1,2]. Accounting for almost 90% of lung cancer cases, smoking is also responsible for causing chronic obstructive pulmonary disease, strokes, and heart attacks [3]. The majority of the studies that have investigated adverse health impacts associated with tobacco use have focused on the impact of harmful chemical compounds on the respiratory system [4,5]. During the past decade, multiple studies have also characterized the microbial community of commercially available tobacco products such as traditional cigarettes, little cigars, cigarillos, smokeless tobacco and hookah [6–9,9–12]. However, even though characterizing the microbial community of traditional tobacco products is on the rise, limited studies have carried out a comprehensive comparative analysis of the bacterial microbiome across differing tobacco products, which could include potential human pathogens. Therefore, comprehensively evaluating the microbial communities of diverse commercial tobacco products is an important emerging research area.

Similarly, there are significant knowledge gaps regarding the viability of tobacco bacterial communities and the potential for these bacteria to be transmitted to tobacco users. While one early culture-based study recovered viable bacteria from smoked tobacco filters [13], few have detected bacterial endotoxins or peptidoglycan markers in tobacco smoke [14,15]. Recently, however, our group demonstrated for the first time that viable bacterial species originating from cigarette tobacco, including *Bacillus, Paenibacillus* and *Terribacillus* can be aerosolized in mainstream cigarette smoke [16], and potentially be transmitted to cigarette users. Nevertheless, with the consensus among microbiologists being that only 2% of bacterial species

in the environment are culturable [17], knowledge gaps in characterizing the viable or metabolically-active bacterial microbiome in commercial tobacco products remain.

From a public health perspective, the transmission of tobacco bacterial communities, as well as harmful chemical constituents, from tobacco products to users could result in significant changes in the tobacco user's oral bacterial community (or oral microbiome) [18,19]. Use of tobacco has been associated with dysbiosis (deviation from symbiosis or community disturbance) in oral bacterial community composition, which is also dependent upon multiple intrinsic (e.g., physiology and genetics) [20,21] and extrinsic (e.g., product type or length of smoking) [22,23] factors of the user and the product. Given that oral microbiome dysbiosis is linked to a multitude of diseases (e.g., gingivitis, periodontitis, cardiovascular diseases, and diabetes) [24,25] it is important to further our understanding of the dysbiosis in oral bacterial communities of tobacco users, as well as the temporal stability of these oral microbiome changes.

To address the above-mentioned knowledge gaps, the overall goal of my dissertation research was to comprehensively characterize and compare the bacterial microbiomes of different types of tobacco products and evaluate the influence of smoking and smokeless tobacco use on the oral microbiome of smokers. My specific aims were as follows:

1. Evaluate the compositional differences in the bacterial microbiomes between conventional tobacco products (Chapter 3).
2. Compare the transient changes in the oral microbiome of cigarette smokers after a single use of a little cigar product (Chapter 4).
3. Conduct a comparative characterization of the oral microbiome between cigarette users, smokeless tobacco users, and non-users over time (Chapter 5).

Each of the above-mentioned specific aims is addressed in three manuscripts that are presented in Chapters 3-5 in this dissertation. To provide additional context for these manuscripts, Chapter 2 includes background on the importance of studying the bacterial communities present in tobacco products, as well as changes in their composition with the addition of chemical additives such as menthol and/or flavors. Chapter 2 also includes an overview of changes in the temporal stability of the oral microbiome associated with smoking different types of tobacco products; linkages between oral dysbiosis and adverse health outcomes; and the effects of smoking tobacco on the oral microbiome. Finally, this chapter also discusses the relationships between demographics (e.g., racial diversity, age, gender) and the oral microbiome.

Chapter 3 then comprises a manuscript, titled "*Conventional tobacco products harbor unique but heterogenous microbiome*". In this chapter, a detailed characterization of bacterial community composition (diversity and abundance) across diverse commercial and research tobacco products is presented, along with the effects of mentholation and/or flavoring of tobacco products on tobacco bacterial communities. Additionally, the metabolically-active (live) fraction of total tobacco bacterial communities is characterized, leveraging the coupled use of a DNA labeling technique with 16S rRNA gene sequencing. This chapter has been submitted to a peer-reviewed journal for publication.

Chapter 4 follows with a second manuscript entitled "*Single use of a little cigar does not result in transient changes to the oral microbiome*". This chapter explores the transient changes occurring within the oral microbiome after a one-time exposure to a tobacco product. Because of the prevalent use of flavored tobacco products in the U.S., we compare oral bacterial community

changes resulting from the use of flavored little cigars versus their non-flavored counterparts. Furthermore, we compare variability in the transient stability of the oral microbiome within and between the study participants.

Chapter 5 follows with a third manuscript titled "*Dysbiosis of the oral microbiome varies between cigarette smokers, smokeless tobacco users, and non-users*". This chapter presents a comparison of oral bacterial communities across three groups (cigarette smokers, smokeless tobacco users, and non-users), as well as an evaluation of the temporal stability of the oral bacterial communities across these three groups. Finally, Chapter 6 presents conclusions, future research directions, and the public health significance of my work.

In closing, it is important to note that the majority of my dissertation was formulated, designed, and written during the ongoing COVID-19 pandemic (2020-2022). Given the multitude of intrinsic and extrinsic factors that play a role in exacerbating COVID-19 symptoms, the effect of tobacco smoking on the risk of COVID infection was also researched by many. Even though a few studies provide evidence of smokers being protected from developing COVID-19 symptoms, the majority of studies to date demonstrate a positive association between smoking tobacco, and the risk of contracting COVID-19, the severity of COVID-19, and even death. To comprehensively evaluate and summarize this body of literature, I engaged in a side project and conducted a mini-review, entitled "*The predisposition of smokers to COVID-19 infection: A mini-review of global perspectives.*" This mini-review is included in Appendix A and has been submitted to a peer-reviewed journal.

Chapter 2: Background

2.1 Tobacco products

A variety of tobacco products are used globally, ranging from combustible to non-combustible and heat-not-burn electronic nicotine delivery systems (ENDS) products. The most common combustible tobacco product used in the United States (U.S.) is cigarettes [26] which consist of finely cut tobacco filler that is cured and rolled into paper. Pipe tobacco is generally loose-leaf tobacco that is burned in a traditional smoking pipe. Little cigars look like cigarettes and contain pipe tobacco wrapped with tobacco-infused paper. Tobacco used in cigars is aged for a year before fermentation, giving a characteristic flavor and smell that is different from cigarettes [27]. Unlike cigarettes and little cigars, cigars do not include a filter in their construction and can deliver ten times more nicotine, two times the tar, and five times the carbon monoxide of a filtered cigarette [28]. Hookah tobacco (also known as shisha or waterpipe) is usually smoked through an apparatus where the tobacco smoke travels via water before being inhaled by the user.

Non-combustible products include smokeless tobacco (chew, snuff, nicotine gels, and dissolvable tobacco). Although a few forms of non-combustible tobacco can be inhaled, most are placed under the tongue, or in the lower labial groove [29]. While nicotine gels are absorbed through the skin on the hand, dissolvable tobacco is usually sold as lozenges, sticks, strips, or orbs. Lastly, ENDS products include vape pens, hookah pens, electronic cigarettes (e-cigarettes), e-cigars, e-pipes, and vaporizers. These products usually have a heating coil that primarily heats electronic liquids (e-liquids) or vape juice.

2.1.1 Federal regulations on the manufacturing and sale of tobacco in the U.S.

Tobacco smoking remains the single most preventable cause of death in the U.S, with close to half a million Americans dying annually from tobacco-related diseases, and over 35,000 non-smokers dying annually from secondary tobacco exposure [30]. The year 2020 saw the largest single rise (by 800 million units) in sales of the number of cigarettes within the past two decades in the U.S. [31]. With the primary aim of regulating tobacco manufacturing, distribution, and sales, the U.S. Food and Drug Administration (FDA) signed the Family Smoking Prevention and Tobacco Control Act (FSPTCA) in 2009. Under this Act, tobacco manufacturers and importers are required to test and report on the FDA-established list of 93 harmful and potentially harmful constituents (HPHCs) in tobacco products. The 2009 FSPTCA also banned the use of *all other* flavorings in commercial cigarettes (including bidis and kreteks), leaving mentholated cigarettes to be investigated in greater detail [32]. With the 2009 ban on the marketing and sale of flavored cigarettes, consumption of flavored non-cigarette products has been on the rise. In 2016, the FDA extended its regulatory authority to cover all smokeless tobacco products including dissolvables, nicotine gels, and hookah tobacco [33]. While specific warning labels were mandated for certain smokeless tobacco products by the FDA in 2019 [34], in 2021 FDA announced plans to propose bans on all characterizing flavors (including menthol) in all tobacco products [28]. Last year (2021), FDA blocked the sale of 55,000 flavors (including mint) of e-cigarettes, excluding menthol varieties [35].

2.1.2 Chemical constituents of tobacco products

In traditional tobacco products such as cigarettes, there are over 4,000 chemical constituents irrespective of their method of production, the most prevalent being nicotine,

acetaldehyde, benzene, acrolein, 1,3-butadiene, and tobacco-specific nitrosamines (TSNAs) [36]. Other tobacco products also contain thousands of chemical contaminants. For example, when comparing between products, one session of water pipe (hookah) or bidi smoking delivers higher concentrations of nicotine, tar, and carbon monoxide compared to one session of smoking conventional cigarettes sold in the U.S.[37,38]. Moreover, ratios of TSNAs to nicotine content in commercial little cigars were found to be higher than that of commercial cigarettes [39]. Generally, in comparison to conventional cigarettes, e-liquids contain relatively fewer chemicals such as a stabilizing agent (propylene glycol/glycerol), nicotine solution, and additives/flavors (e.g., menthol, strawberry, chocolate, caramel, and many more). There are also a few nicotine-free e-liquids available on the market.

Apart from nicotine, other major components of tobacco products are the additives, which can account for up to 10% of the product's weight [40]. Approximately 600 different types of additives have been documented, and these primarily mask the harshness of smoke and affect the color, odor, and flavor of tobacco [40]. The major additives are sugars, humectants, ammonia compounds, preservatives, and flavorings [41]. The most popular flavor that has long been used by the tobacco industry to attract users is menthol [42–44]. Menthol addition in tobacco products started back in the 1920s, promising a smoother inhalation, reduced airway irritation, and suppressed cough, making it easier overall for the smoker to breathe [45,46]. Today, 90% of all tobacco products contain varying amounts of menthol [47] and menthol cigarettes account for 36% of the cigarette market [48]. While menthol decreases nicotine metabolism and lowers nicotine metabolic clearance [49], it does not alter total nicotine consumption [50]. The 2011 FDA's Tobacco Products Scientific Advisory Committee (TPSAC) report on menthol cigarettes concluded that smoking

mentholated cigarettes is associated with increased initiation, progression of smoking, increased dependence, and a decreased likelihood of smoking cessation[1].

Use of mentholated and flavored tobacco products is popular in the U.S. For example, while a recent survey documented that mint and fruit-flavored e-cigarettes were the most popular e-cigarettes [51], menthol-flavored smokeless tobacco products comprised more than half (54.5%) of all smokeless tobacco sales revenue in the year 2020 [52]. Although cigarette use has been declining in the U.S. by 26% since 2009, 91% of this decline was in the non-menthol market [53]. With increasing sales of menthol products, there are an estimated 18.6 million menthol cigarette smokers [54]. But the use of menthol cigarettes is not uniform. Following decades of selective marketing by tobacco companies [53,55], adolescents, youth, and 85% of African American smokers use mentholated cigarettes and are characterized by a significantly lower likeliness to quit [56]. To summarize the damage caused by menthol cigarettes in the U.S. population, a simulation model showed that menthol cigarettes accounted for an extra 10.1 million smokers during the years 1980-2018 [57].

2.1.3 Microbiological contaminants in tobacco products

Beyond chemical constituents, over the past 50 years, researchers have begun to characterize the microbial communities present in commercially-available tobacco products (cigarettes, little cigars, cigarillos, water pipe/hookah, and smokeless tobacco) [58]. While early studies relied on culture-dependent techniques [15,59–62], more recent studies have relied on 16S rRNA gene-based microarray and next-generation sequencing approaches to characterize over 89 unique bacterial genera and 19 fungal genera in the microbiome of commercial tobacco products [8–11,63–65], and custom-made research cigarettes [66].

For example, a number of studies identified *Bacillus, Pseudomonas,* and *Staphylococcus* in commercial cigarettes, little cigars, and cigarillos, including some potentially pathogenic strains capable of causing respiratory infections among smokers [7–9,9,65]. The recent identification of thermophiles, such as *Anoxybacillus*, *Schlegella*, and *Silanimonas,* in commercial cigarettes has raised additional concerns because these bacteria can withstand higher temperatures such as those generated within a cigarette during the combustion process [65]. Along with cigarettes, previous studies that have characterized the bacterial microbiome of little cigars and cigarillos have documented the presence of potentially pathogenic *Pseudomonas pseudoalcaligenes* and *Staphylococcus sciuri* [9,63] across the majority of tested products. Although very few studies have evaluated the bacterial microbiome of hookah tobacco, several pathogenic bacteria including *Halomonas*, *Staphylococcus*, and *Pseudomonas* have been reported [12]. Additionally, a multitude of bacterial genera such as *Tetragenococcus, Bacillus, Staphylococcus, and Pseudomonas* have been identified in both domestic (U.S.) and international smokeless tobacco products [10,11,64].

In contrast to the multitude of bacterial microbiome characterizations of commercial tobacco products, only a few studies have researched the fungal microbiome of these products. The most common fungal genera identified in cigarettes have been *Mucor, Humicola, Aspergillus, Penicillium*, and *Fusarium*[58]. Specifically, domestic U.S. snuff has been found to harbor *Humicola lanuginosa*, *Thielavia albomyces*, *M. pulchella* var. *sulfurea*, and *Talaromyces thermophilus,* while smokeless tobacco products from Pakistan have been shown to contain *Aspergillus, Penicillium, Mucor, Sepedonium, and Trichophyton* [67].

2.1.4 Effects of chemical additives (menthol or flavors) in tobacco products on the tobacco bacterial microbiome

Studies from our group have shown that mentholated cigarettes harbor a lower diversity of bacteria when compared to their non-mentholated counterparts; however, the mentholated products contain bacterial taxa which can survive harsh conditions [8,65]. To our knowledge, these are the only two studies that have compared mentholated with non-mentholated varieties of commercially available cigarettes. However, an earlier study from our group compared mentholated little cigars and demonstrated statistically significantly lower bacterial diversity (similar to mentholated cigarettes) in these products when compared to non-mentholated varieties [9]. In contrast, flavoring in little cigars has been shown to increase bacterial diversity when compared to non-flavored counterparts [9,63]. With limited data on compositional differences in bacterial microbiome between mentholated/flavored tobacco products and non-flavored products, more in-depth studies are needed in this area with regard to non-cigarette products such as little cigars, cigarillos, and smokeless tobacco.

2.2 Oral microbiome

The oral cavity is the first to encounter mainstream smoke/aerosols from tobacco products and hence, has the greatest potential to be affected by them. The total human microbiome can be divided into the *core* microbiome (which remains relatively constant in composition for all individuals and the *variable* microbiome (which is dependent on an individual's behavior and lifestyle and, therefore, changes in composition) [68,69]. While the bacteria found in the oral cavity are uncommon in other habitats of the human body, an average human mouth harbors over 700 species [70–73]. Predominant species (88%) found in the oral cavity

belong to *Streptococcus, Veillonella, Rothia, Prevotella, Neisseria, Fusobacterium, Leptotrichia,* and *Porphyromonas* [71,74,75]. Dysbiosis of the oral microbiome has been associated with the initiation of multiple adverse health issues including periodontal, respiratory, cardiovascular, and systemic cancers [25,76,77].

2.2.1 Heterogeneity of the oral microbiome across locations inside the oral cavity

Various oral receptors and surface adhesins in the oral cavity present a multitude of surface types (soft like the tongue and cheeks or hard like the teeth and palates) on which bacteria can adhere. Previous studies of the oral microbiome have collected and tested samples from various surfaces in the oral cavity (dental surfaces, inner cheeks, palates, the tongue, and saliva) [18,78–81]. Comparisons across oral sampling sites [71,82–84] and collection methods [85] during these studies have demonstrated significant differences in the diversity and relative abundance of operational taxonomic units (OTUs) (basic units to classify groups of closely related bacteria) within the oral cavity. For example, dental samples have been characterized by the highest diversity in bacteria while inner cheeks have had the lowest [81]. However, because of the variable sampling sites used across multiple oral microbiome studies, comparisons across studies can be difficult. A recent comparative study on e-cigarette users showed higher alpha bacterial diversity in the saliva samples from users compared to non-users, while a significant shift in bacterial community composition was observed only in buccal swab samples when comparing between users and non-users [79]. Saliva has a low nutrient content and high flow rate (0.3 ml/min) [86], hence some controversies exist regarding its true indigenous bacterial community which may be thought of as mixed populations shed from other oral surfaces such as the tongue or the buccal mucosa. With the average saliva volume in the mouth being only 0.74mL [87], a constant bacterial

exchange likely needs to occur to maintain a stable salivary microbiome. With limited data on mechanisms of stability in the salivary microbiome, there remains much to be explored regarding bacterial community dysbiosis between different oral sites among tobacco users and non-users.

2.2.2 Intrinsic factors affecting the oral microbiome

While the above-mentioned studies have identified differences in bacterial diversity and composition across different oral sites, there remain other intrinsic factors that play a role in defining one's microbiome. After birth, bacteria colonize multiple niches within the oral cavity and form a stable and diverse microbiome. Ten out of 15 body sites tested in the Human Microbiome Project (HMP) showed significant associations between host genetic variation and microbiome composition [88], and researchers agree that both environmental and host genetic factors influence the microbial composition in niches that come in contact with the environment (skin, lungs, mouth, and gut) [89–93].

With previous studies demonstrated associations between racial diversity and susceptibility to diseases, research has been carried out to compare oral microbiome diversity among populations from various geographic locations [20,94–96]. While previous studies have focused on studying the oral microbiome of populations across continents [20,94,95,97], there remains controversy regarding the role of race and ethnic origin as a significant determinant of oral bacterial community composition. One previous study reported that even though a high bacterial diversity was shown within and between individuals from 12 locations worldwide, the geographic location had little to no effect on oral bacterial community compositions [95]. In contrast, the salivary microbiome from five different populations (Germans, Alaskans, Africans, African Americans, and Caucasians) has been shown to be significantly different in terms of oral

bacterial community profiles [20,94,98]. However, each of the above-mentioned studies included a small sample size (<200), which may have impacted the findings. Subsequently, another study included over 1,000 participants in a comparison of oral microbiomes between African Americans and European-Americans [96]. With higher species richness among the African Americans, 32 bacterial taxa were significantly differentially abundant among the two above-mentioned groups. Even though there have been a few studies focusing on the role of race as a driver of oral bacterial community composition, there are limited data specifically including smokers. When a group of African American smokers was compared to Caucasian smokers, no significant differences were found in bacterial community profiles [19], potentially indicating that smoking tobacco products supersedes the influence of factors like race on the oral microbiome.

Furthermore, gender and age-specific differences in the oral microbiome have also been documented [99–101]. For example, alpha diversity in the salivary microbiome was shown to be higher in boys than in girls [102]. Specifically, *Lactobacillus lactis* and *Alloprevotella* spp. were abundant in caries-active girls, while *Neisseria flavescens, Rothia aeria,* and *Haemophilus pittmaniae* were found to be significantly higher among boys [100–102]. Bacterial community composition was also shown to be dependent on the body weight of an individual child [100]. Comparing between ages, adult oral microbiomes have been associated with an increase in the relative abundance of periodontal pathogens [103–106]; specifically, the "red complex" (a bacterial aggregate responsible for severe clinical manifestations of periodontal diseases) pathogen *Treponema* was predominant in adults' oral microbiomes when compared to that of children [101,106].

2.2.3 Effects of smoking tobacco on the oral microbiome

While intrinsic factors such as race and genetics affect the oral microbiome, extrinsic factors like one's lifestyle, behavior, tobacco, and alcohol consumption also affect the oral microbiome. Previous studies have characterized the oral microbiome of smokers/tobacco users to be substantially different from that of non-smokers [18,19,23,79,107–109]. While early in-vitro studies used culture-based techniques to show growth inhibition of the common oral bacteria *Neisseria* in the presence of cigarette smoke [13], more recent studies using molecular techniques have also shown decreased *Neisseria* and *Porphyromonas* in oral wash samples from smokers when compared to non-smokers [18,110]. Dysbiosis of the oral microbiome has been linked to periodontal, respiratory, and multiple types of cancers [111–113]. Smoking can initiate several possible changes in the host, such as immunosuppression, oxygen deprivation, and biofilm formation which can lead to the loss of beneficial bacteria, and perpetuation of pathogenic bacteria colonization in the oral cavity [107,114–116].

Tobacco smoking has been attributed to severe health effects ranging from lung cancer and chronic bronchitis to emphysema. Smokeless tobacco chewing has been linked to oral cancers [117]. Since bidis and kreteks are used less in the U.S., existing research has mainly been generated by international groups. Research from India indicates that smoking bidis places users at a greater risk for nicotine addiction [118–120], which can ultimately lead to greater bidi usage and associated adverse health risks. For instance, the increased use of bidis is associated with oral, lung, stomach, and esophageal cancers [118,121–123], as well as a threefold increased risk of coronary heart disease and a fourfold increased risk of chronic bronchitis. Studies from Indonesia demonstrate that smoking tobacco products (like kreteks) is associated with an increased risk of abnormal lung function and acute lung injury[124,125].

2.2.4 Compositional changes in the oral microbiome associated with tobacco smoking

Comparing cigarette smokers and non-users, changes in the relative abundance of more than 172 bacterial OTUs have been recorded with a decrease in species such as *Neisseria, Porphyromonas,* and *Gemella* on mucosal surfaces among smokers, and an increase in *Streptococcus*, *Veillonella*, and *Eubacterium* among smokers [19]. A higher relative abundance of anaerobes and a lower relative abundance of aerobes have been associated with plaque samples from cigarette smokers when compared to non-smokers [18,19]. A recent study comparing the oral microbiome among adult smokeless tobacco users showed a significantly lower relative abundance of *Lactobacillus* and *Haemophilus*, and a higher abundance of *Fusobacteria, Porphyromonas, Desulfobulbus, Enterococcus*, and *Parvimonas* among users [108].

With 20.4 million users of ENDS [126–128], recent studies have also focused on ENDS' effect on the oral and respiratory systems [23,129–131]. Higher alpha diversity was recorded for bacterial communities in saliva from e-cigarette smokers' oral cavities when compared to that from non-users [79]. In the same study, a significantly higher relative abundance of *Veillonella*, *Haemophilus,* and *Staphylococcus* was also found among ENDS users when compared to non-users. While significantly higher alpha diversity and shifts in beta diversity in the salivary microbiome of e-cigarette users (compared to controls) were seen [79], another study found no significant differences in beta diversity among e-cigarette users vs. controls [131]. Comparing cigarette and e-cigarette users, e-cigarette users were shown to harbor a unique periodontal microbiome enriched with *Fusobacterium* and *Bacteroidales* (G-2) [132].

2.2.5 Temporal changes in the oral microbiome associated with tobacco use

The disturbances affecting the stability of any ecosystem can be short-term pulses or long-term presses [133]. Apart from the intrinsic factors of the human body (e.g., age, race, and health condition), extrinsic factors also affect the relative abundance of bacterial species present in the oral microbiome (e.g., the number of products smoked/used per day, years of smoking). Since these factors can significantly change over both the short and long term, it becomes pertinent to study the stability/changes in the oral microbiome with time. In general, the HMP demonstrated temporal stability of the heterogenous oral microbiome [134]. Additionally, the salivary microbiome was shown to be consistent for up to a year [135–137] and even remained stable with the use of antibiotics [81]. A shorter span of four months has also not been associated with significant changes in the oral microbiome [138]. In contrast, the composition of the salivary microbiome has been shown to change rapidly over time (within 1 min) in a single individual when compared to that of the tongue [139]. Looking specifically into bacterial genera, although the levels of predominant genera like *Streptococcus, Neisseria*, and *Veillonella* were stable over seven days for non-smokers, lower stability was observed for smokers during the same period including the colonization of pathogens associated with periodontitis within 24 hours of biofilm development [107]. With limited data on the stability of the microbiome in different oral sites, additional studies are needed to focus on oral microbiome changes after one-time and/or long-term smoking exposure.

2.2.6 Effects of smoking mentholated/ flavored tobacco products on the oral microbiome

As mentioned above, while tobacco use significantly alters the user's oral microbiome, the type of tobacco product (e.g., cigarette, little cigar, smokeless tobacco or hookah), and

chemical additives present in the tobacco product also play a role in altering bacterial community diversity and composition in the oral cavity and have an impact on ultimate health outcomes. A study published earlier last year (2021) looked at the harm from menthol cigarettes in the U.S. and estimated a loss of 3 million life-years with 378,000 premature deaths during the period 1980 - 2018 [57]. With studies demonstrating the increasing use of menthol cigarettes among minors and the associated lower quit rates, higher nicotine dependence scores, increased craving/ withdrawal symptoms, increased odds of stroke, and increased propensity to initiate smoking [140,141], FDA is working towards banning menthol as a characterizing flavor in cigarettes within this year (2022) [142]. Hypothetically banning menthol in cigarettes suggested that 11-46% of U.S. smokers would consider switching to another tobacco product [143]. Moreover, the 2009 Tobacco Control Act did not include a statutory ban on the use of menthol and/or flavors in other tobacco products such as little cigars and hookah [142].

From an oral health perspective, few studies have tested the effects of mentholated tobacco products on the oral bacterial microbiome. Culture-based assays testing menthol and other flavored e-liquids showed greater detrimental effects on common oral commensals when compared to non-flavored counterparts [144]. While the role of flavors in e-liquids, hookah, and little cigars has not been studied in-depth, a few studies have shown that flavored e-liquids are potent stimulators of a variety of mammalian tissues and cell lines [145–147]. In a recent study on e-liquids, flavoring was associated with higher antimicrobial and antioxidant activity, which was enhanced with the addition of nicotine [148]. While systemic intake of nicotine was not affected, smoking mentholated cigarettes significantly impaired nicotine metabolism [50], increased serum nicotine and cotinine levels, and increased exhaled carbon monoxide levels (when compared to non-mentholated cigarettes) [149]. An extensive review from FDA TPSAC on the effects of

menthol addition in cigarettes concluded that mentholation does not inherently affect the toxicity of cigarette smoke or increase the risk to humans in comparison to non-menthol cigarette smokers [1], but data on the effects of smoking mentholated/ flavored tobacco products on smokers' oral microbiomes is limited.

2.2.7 Effects of smoking different tobacco products on the oral microbiome

Considering the multiple tobacco product types available in the market, few studies have focused on a comparative characterization of the oral microbiome between users of different tobacco product types. A multivariate analysis from one study showed significant differences in oral bacterial community composition between e-cigarette users, smokers, and non-users, with higher virulence signatures and pathogen colonization among tobacco users [23], and a significantly higher relative abundance of *Porphyromonas* and *Veillonella* among vapers [150]. Another recent study compared subgingival plaques from cigarette smokers, shisha users, and non-smokers, showing specific pathogens, *Streptococcus sanguinis* and *Tanerella forsythia,* to be at a higher relative abundance among shisha users, while *Streptococcus mutans* and *Veillonella dispar* were at a higher relative abundance among cigarette users [22]. Another pilot study demonstrated that, while the relative abundance of multiple bacterial genera was significantly altered with cigarette smoking, only that of *Actinobacillus, Porphyromonas, Lautropoa*, and *Bifidobacterium* were altered significantly with dokha use and no changes were seen with shisha users [151]. While these are the only studies comparing the oral microbiome between users of different tobacco products, to our knowledge, there are no side-by-side comparisons of the bacterial microbiomes of multiple oral cavity sites and between cigarette and smokeless tobacco product users.

2.3 Oral health and tobacco-related disparities

Irrespective of intrinsic (e.g., physiology and genetics) [20,21] and extrinsic (e.g., product type or length of smoking) factors, using tobacco products perturbs the oral health of users and these impacts are not equal across differing groups. While biological and behavioral differences impact oral health [152–154], disparities in oral health stem from socioeconomic disparities, influenced by social and cultural beliefs. For example, oral diseases like caries disproportionally affect ethnic minorities, rural populations, and women [155–159], and the prevalence of periodontitis and untreated caries is the highest among non-Hispanic Blacks, followed by Mexican Americans [160]. A recent review suggests that the disproportionate development of some periodontal diseases among men compared to women is due to hormonal differences, poorer oral hygiene, and greater tobacco use [161].

Tobacco-related disparities include a higher prevalence of product initiation with lower rates of cessation, disproportionate access to treatments, and an increased burden of tobacco-related diseases among underrepresented groups. For example, tobacco use plays a major role in fatalities due to heart disease, cancer, and stroke among African Americans [162]. Data from the Centers for Disease Control and Prevention (CDC) demonstrate that lifestyle behaviors like smoking are also associated with economic status, education, access to health insurance, and marginalized groups [163]. Biological differences also have been shown to impact the inhalation of nicotine and its metabolism in the body. For example, nicotine metabolism is higher among whites when compared to African Americans and Asians [164,165], potentially putting African Americans at a substantially higher risk of nicotine-related lung cancers for similar levels of tobacco consumption [165]. Lower socioeconomic status (SES) groups (including African

Americans) have been shown to smoke at three times the rate of higher SES groups [166], with men smoking at a higher rate than females [167].

The following three chapters present manuscripts reporting novel data that address multiple knowledge gaps that have been identified above.

Chapter 3: Conventional tobacco products harbor unique but heterogenous microbiomes

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3.1 Abstract

While an increasing number of studies have evaluated tobacco microbiomes, comparative microbiome analyses across diverse tobacco products are non-existent. Moreover, to our knowledge, no previous studies have characterized the metabolically-active (live) fraction of tobacco bacterial communities and compared them across products. To address these knowledge gaps, we compared bacterial communities across four commercial products (cigarettes, little cigars, cigarillos and hookah) and one research cigarette product. After total DNA extraction (n=414) from all samples, the V3V4 region of the 16S rRNA gene was sequenced on the Illumina HiSeq platform. To identify metabolically-active bacterial communities within these products, we applied a coupled 5-bromo-2'-deoxyuridine labeling and sequencing approach to a subset of samples (n=56). Each tobacco product was characterized by its signature microbiome, along with a shared microbiome across all tobacco products consisting of *Pseudomonas aeruginosa, P. putida, P. alcaligenes, Bacillus subtilis*, and *Klebsiella pneumoniae*. Comparing across products, a statistically significantly higher ($p < 0.05$) relative abundance of *Klebsiella* and *Acinetobacter* was observed in commercial cigarettes, while a higher relative abundance of *Pseudomonas* and *Pantoea* was observed in research cigarettes. *Methylorubrum* and *Paenibacillus* were higher in hookah, and *Brevibacillus, Lactobacillus, Bacillus, Lysinibacillus*, and *Staphylococcus* were higher in little cigars and cigarillos. Across all products, the majority of the metabolically-active bacterial communities belonged to the genus *Pseudomonas,* followed by several genera within the Firmicutes phylum (*Bacillus, Terribacillus*, and *Oceanobacillus*). Identification of some metabolically-active pathogens such as *Bacillus cereus* and *Haemophilus parainfluenzae* in commercial products is of concern because of the potential for these microorganisms to be transferred to users' respiratory tracts via mainstream smoke. Future work

is warranted to evaluate the potential impact of these tobacco bacterial communities on users' oral and lung microbiomes, which play such an important role on the spectrum from health to disease.

3.2 Introduction

Since the implementation of the 2009 Family Smoking Prevention and Tobacco Control Act, tobacco manufacturers in the United States (U.S.) have been required to test and disclose the ingredients in tobacco products under section 904(a)(3) of the Food, Drug, and Cosmetic Act (FD&C Act) [168]. To facilitate this process, the Food and Drug Administration (FDA) published a list of 93 harmful and potentially harmful constituents (HPHCs) in tobacco products and selected 20 of these HPHCs to be tested, with the findings reported back to FDA [36]. While these 20 HPHCs include a comprehensive list of chemicals that are potentially present in commercial tobacco products, microbiological constituents that could be harmful to tobacco users' health are not addressed.

Nevertheless, over the past 50 years, multiple research groups have detected and characterized a plethora of microorganisms (bacteria and fungi) in commercial tobacco products using both culture-dependent and -independent techniques [7,8,10–12,15,59,61,169]. For example using culture-based techniques, Eaton et al. (1995) cultured *Mycobacterium* spp. from cigarettes, and Kurup et al. (1983) isolated *Aspergillus* spp. from cigarette tobacco [59,61]. Subsequently, using culture-independent approaches, multiple bacterial genera (e.g., *Bacillus, Pseudomonas aeruginosa, Klebsiella*, and *Methylobacterium*) were identified in cigarettes [169], while *Halomonas, Staphylococcus*, and *Pseudomonas* were detected in hookah tobacco [12], and *Bacillus, Staphylococcus,* and *Lactobacillus* were identified in smokeless tobacco products [10,11,170]. More recently, our group and others have extensively characterized tobacco microbiomes using next-generation sequencing approaches, demonstrating differences in bacterial diversity and abundance across brands, lots, and additives (menthol/flavors) of traditional cigarettes, little cigars, cigarillos, hookah, and smokeless tobacco [7–9,11,12,63,65] (Malayil et al., 2022 under review).

The high-throughput sequencing approaches used in these studies have enabled us to characterize total bacterial diversity (including low abundant members) in tobacco products; however, DNA sequencing approaches cannot differentiate between live and dead bacteria within these products. To overcome this challenge, DNA labeling methods have been coupled with sequencing approaches to tease out the metabolically-active or live fraction of microbial communities present in complex environmental samples [171–176]. Specifically, the DNA labeling dye 5-bromo-2'-deoxyuridine (BrdU), a synthetic thymidine analog capable of incorporating into replicating DNA or dividing cells, has enabled researchers to identify live, replicating bacterial community members within complex communities. Previous studies have coupled BrdU labeling with next-generation sequencing, quantitative PCR, or immunocytochemistry assays to identify metabolically-active bacterial communities in soil and water, as well as to evaluate the response of live fungal species to plant litter substrates [174,177–186]. However, to our knowledge, there are no published studies that have applied coupled BrdU labeling and 16S rRNA gene sequencing approaches to evaluate the metabolically-active fraction of tobacco bacterial communities. Moreover, there are no data comparing overall tobacco microbiomes across diverse products such as cigarettes, little cigars and hookah.

To address these knowledge gaps, we performed a detailed comparative analysis of the bacterial microbiomes present across different types of tobacco products. Additionally, we utilized a coupled BrdU labeling and 16S rRNA gene sequencing approach to identify the metabolically-active fraction of the total heterogeneous bacterial communities present across these products.

3.3 Methods

3.3.1 Selection of tobacco products

Commercially-available cigarettes, little cigars, cigarillos, and hookah, as well as custom made research cigarettes were included (**Table 1**). The commercially-available tobacco products were purchased from stores in the College Park, MD area, as well as online. Tobacco products were selected based on their market availability, accessibility, and popularity among U.S. tobacco users. Each type of tobacco product included brands with or without additives (menthol or flavors). Commercially-available cigarettes included Marlboro menthol (MMB), Marlboro red (MRB), Newport non-menthol (NNM), Newport menthol (NMB2) and Newport menthol gold (NMG); and the research cigarettes were custom-made SPECTRUM cigarettes (obtained from the National Institute of Drug Abuse, MD (NIDA)). The little cigars included Swisher Sweets Cherry (SSC), Cheyenne menthol (CMB), and Cheyenne Full Flavor (CFF); and the cigarillo product was Swisher Sweets Original cigarillos (SSO). Finally, the hookah products included Al Fakher mint (AFMF), Al Fakher watermelon (AFWF), Al Fakher Two Apple (AFTA), Fumari Ambrosia (FAMB), Fumari Mint Chocolate Chill (FMCC), and Fumari White Gummy Bear (FWGB).

3.3.2 Extraction of total DNA and sequencing

Total DNA was extracted from 414 samples (90 cigarettes, 108 hookahs, 108 little cigars, 36 cigarillos, and 72 research cigarettes), which included six replicates of each type of product, using a previously published protocol [7,187,188]. Briefly, after dissecting the tobacco product aseptically, 0.2 g of tobacco was measured and placed into Lysing Matrix B tubes, and 1 mL of

ice-cold 1X Phosphate buffer solution (PBS; Gibco-Life Technologies, NY) was added. Samples were then incubated twice at 37°C and 55°C with enzyme cocktails (Cocktail A: lysozyme from chicken egg white (Sigma-Aldrich, St. Louis, MO), lysostaphin from *Staphylococcus staphylolyticus* (Sigma-Aldrich, St. Louis, MO), and mutanolysin from *Streptomyces globisporus* ATCC 21553 (Sigma-Aldrich, St. Louis, MO); and Cocktail B: Proteinase K (Invitrogen-Life Technologies, Grand Island, NY, USA) and 10% (w/v) sodium dodecyl sulfate (SDS) (BioRad, Hercules, CA)), before mechanical lysis of cells using the MP Biomedical Fastprep 24 (Santa Ana, CA). Extracted DNA was purified using the Qiagen DSP DNA Mini Kit according to the manufacturer's protocol, and then PCR-amplified targeting the V3V4 hypervariable region of the 16S rRNA gene using the universal primers 319F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT). Amplicons were visualized on a 1% agarose gel and then sequenced on an Illumina HiSeq2500 (Illumina, San Diego, CA) using a previously published protocol [187,188].

3.3.3 BrdU treatment and immunocapture of BrdU

For a subset of tobacco samples (n=56), each tobacco product was aseptically dissected, and 0.2 g of each product was weighed out into Lysing Matrix B tubes (MP Biomedicals, OH). The samples were then incubated at room temperature in the dark for 48 h with 26 µL of 7.69 mM BrdU for incorporation of BrdU into replicating DNA. Concurrently, control samples without the addition of BrdU (no treatment (NOTRT)) were incubated similarly. After incubation, total DNA was extracted and purified following the above-mentioned procedures. After DNA extraction, immunocapture and isolation of BrdU-labeled DNA were performed according to a previously published protocol [174,189] and sequenced as mentioned above.

3.3.4 Sequencing reads analysis

16S rRNA reads were screened for low quality and short length and assembled using PANDAseq, demultiplexed, and chimera trimmed using UCHIME. Quality reads were then incorporated into QIIME v1.9 and clustered de-novo using VSEARCH. Taxonomies for BrdU and NO TRT samples were assigned using the Greengenes database, using a 0.97 confidence threshold. The resulting operational taxonomic unit (OTU) table, reference sequences, and phylogenetic tree files were imported into R Statistical computing software (v. 0.99.473) using the *Phyloseq* R package (v. 1.38.0) for downstream analysis. Taxonomic assignments and relative abundance of bacterial taxa (for each sample) used for comparative analyses were determined with the Kracken 2 classification tool [190] and Bracken abundance estimator [191] from quality trimmed QIIME pair-end reads. Downstream statistical analysis and visualization of data were performed in RStudio, Microbiome Analyst [192,193], Power BI (v 3.0.3.0), and Cytoscape (v 3.7.2). Core and shared microbiomes were analyzed using network analyses of the relative abundance of bacterial species within products (visualized at > 5% relative abundance) and brands (visualized >1% relative abundance). A chord plot was used to visualize bacterial species between commercial and research cigarettes above 5% relative abundance, and a Sankey plot was used to visualize bacterial taxa between BrdU treated and non-treated samples above 10% relative abundance.

3.3.5 Statistical analysis

After quality trimming and removing samples that had a Goods coverage value of ≤ 0.95, 365 samples (out of 414) were included in downstream analyses. Alpha diversity was computed using the *Vegan* R package and the Dunn's test was performed as a *post-hoc* test for the Kruskal-Wallis test to evaluate differences between products: *p*-values were adjusted using the Holm method, and $p < 0.05$ was considered statistically significant for all statistical tests performed in this study. Beta diversity analysis was performed using non-metric multidimensional scaling (NMDS) on the Bray-Curtis distance matrix (using *Vegan* R package) and statistical significance was computed using permutational multivariate analysis of variance (ADONIS) with 999 permutations after Wisconsin double standardization. A pairwise ADONIS test (on 999 permutations) was performed to compute adjusted *p*-values between pairs of tobacco products. Bacterial abundance profiles were compared with Linear Discriminant Analysis (LDA) Effective Size analysis (LEfSe) using Kruskal-Wallis sum-rank test [194] to detect differentially abundant taxa and their effect size. The FDR-adjusted *p*-value was set to < 0.05 and the LDA was set to >2.0.

3.3.6 Data availability

Data concerning the samples included in this study are deposited under the following NCBI BioProject accession numbers: PRJNA635703, PRJNA473598, PRJNA601146, and PRJNA641233.

3.4 Results

3.4.1 Bacterial microbiome across products

Comparing alpha diversity (measured with the Shannon diversity index) across products, hookah had the lowest diversity, while cigarillos had the highest diversity. Statistically significant differences ($p < 0.05$) in bacterial genera diversity were found between all pairs of compared products (**Figure 1a**). Our beta diversity comparison showed that 19% (ADONIS $R^2 = 0.19$; $p < 0.001$) of the variation in bacterial community composition was explained by product type (**Figure 1b**). The greatest variation in bacterial community composition structure was found among the little cigar samples, while the least was among the commercial cigarettes. A pairwise ADONIS test of significance also demonstrated significant variability in bacterial community composition between all pairs of tobacco products (**Table S1**). While the least effect size was between little cigars and cigarillos (ADONIS $R^2 = 0.02$), the greatest effect size was found between cigarettes and research cigarettes (ADONIS $R^2 = 0.24$).

Since both alpha and beta diversity metrics are unable to differentiate between the specific bacterial taxa that may be significantly different across tobacco products, the average relative abundance of bacterial genera identified across products was compared (**Figure 1c)**. While the average relative abundance of *Acinetobacter* and *Klebsiella* was higher (FDR adjusted $p < 0.05$) in commercial cigarettes, relative abundance of *Brucella, Enterobacter, Micrococcus, Pantoea* and *Pseudomonas* was higher in research cigarettes (FDR adjusted $p < 0.05$). Little cigars had a significantly higher relative abundance of *Moorella, Lactobacillus, Clostridium* and *Brevibacillus*, and cigarillos had a higher relative abundance of *Staphylococcus, Lysinibacillus, Corynebacterium, Blautia, Bacillus* and *Aerococcus* **(Figure 1c)**.

At the species level, both commercial cigarettes and research cigarettes had a higher relative abundance of *Pseudomonas putida, Pseudomonas oryzae, Pseudomonas stutzeri*, *Pantoea agglomerans, Klebsiella pneumoniae, Enterobacter hormaechei,* and *Sphingomonas* sp. LK11 compared to that in the little cigar, cigarillo, and hookah products (**Figure 2**). *Bacillus subtilis, Brevibacillus formosus*, *Clostridium botulinum*, *Moorella thermoacetica*, and *Lysinibacillus sphaericus* had a lower relative abundance in cigarettes (commercial and research) compared to hookah, cigarillos, and little cigars. *Lactobacillus fermentum, Lactobacillus salivarius*, *Staphylococcus saprophyticus*, *B. formosus*, *C. botulinum*, *M. thermoacetica*, and *Aerococcus urinaeequi* were at a higher relative abundance in little cigars.

Comparing the cigarette products (commercial and research), *B. subtilis, K. pneumoniae, Pseudomonas alcaligenes* and *P. oryzae* were at a lower relative abundance in research cigarettes compared to commercial cigarettes and *P. stutzeri, P. putida, P. aeruginosa, P. agglomerans, E. hormaechei,* and *Micrococcus luteus* were at a higher relative abundance in research cigarettes compared to commercial cigarettes (**Figure 3**). Unique (absent in other products above 5% relative abundance) bacterial communities in commercial cigarettes comprised *Ramlibacter tataouinensis, Bordetella genomosp*. 13, and *Acinetobacter baumannii*, and that in research cigarettes included *Corynebacterium ammoniagenes, A. urinaeequi, Enterococcus gilvus*, and *Bacillus amyloliquefaciens* (**Figure S1**). The rest of the 11 bacterial species that had a relative abundance of greater than 5% were shared between the products.

3.4.2 Core and shared microbiomes across products

Comparing across products, *B. subtilis, K. pneumoniae, P. alcaligenes, P. putida*, and *P. aeruginosa* were found to be present in all five tobacco products forming the core microbiome

across all products (**Figure 4**). While each of the tobacco products harbored two to three unique bacterial species, some bacterial species were shared between two to four products. Cigarillos had three unique species while hookah and little cigars had two unique species each. The cigarette products each had a single unique bacterial species. *M. thermoacetica, Bacillus amyloliquefaciens* and *A. urinaeequi* formed the core microbiome of cigarillo and little cigar products along with *M. thermoacetica* in hookah and *B. amyloliquefaciens* and *A. urinaeequi* in research cigarettes. Commercial and research cigarettes, along with cigarillos, shared a core microbiome of *S. saprophyticus* and *Micrococcus luteus*. *P. stutzeri, P. oryzae*, and *C. ammoniagenes* formed the core microbiome of little cigars and research cigarettes, with *P. stutzeri* and *C. ammoniagenes* also shared with hookah products and *P. oryzae* shared with commercial cigarettes. While *P. agglomerans* and *Bacillus clausii* were absent in cigarillos, *Enterobacter hormaechei* was absent in hookah products. *Bacillus megaterium, Dickeya fangzhongdai, B. genomosp. 13, A. baumannii, L. fermentum, B. formosus*, and *C. botulinum* were each shared by two products. While cigarillo products shared *B. megaterium* with hookah products, *B. formosus* and *C. botulinum* were shared between cigarillos and little cigars. *B. genomosp. 13, A. baumannii*, and *L. fermentum* formed the core microbiome of commercial cigarettes and little cigars.

3.4.3 Effect of additives on the bacterial microbiome

To evaluate changes in the bacterial microbiome in response to additives (menthol and flavors) in the tobacco products, we compared the mentholated/flavored varieties to their non-mentholated/non-flavored counterparts (**Figure S2**). Bacterial genera with the highest relative abundance were *Staphylococcus, Pseudomonas* and *Bacillus* across all products. While the

addition of menthol in commercial cigarettes had a significant effect on bacterial community composition (NMG vs. NNM pairwise ADONIS, $p = 0.001$; NMB2 vs. NNM pairwise ADONIS, $p = 0.001$; MRB vs. MMB pairwise ADONIS, $p = 0.037$), the addition of menthol in research cigarettes had a significant effect only between two pairs (NRC200 vs. NRC201 pairwise ADONIS, $p = 0.01$; NRC404 vs. NRC501 pairwise ADONIS, $p = 0.045$) (**Table S2**). Significant mentholation effects were also observed in little cigar products (CMB vs. CFF pairwise ADONIS, $p = 0.002$).

Comparing products with or without additives (menthol/flavors), a Random Forest algorithm was applied to construct decision trees to predict biomarker taxa that were best associated with the additives. Looking across bacterial genera, *Corynebacterium*, *Brachybacterium, Methylorubrum, Propionibacterium, Xanthomonas, Brucella, Spingomonas* and *Bordetella* were associated with mentholated cigarette products, and *Pantoea, Aerococcus, Klebsiella, Enterococcus*, *Chrysobacterium* and *Agrobacterium* were associated with non-mentholated cigarette products (**Figure 5**). Across all brands, the highest alpha diversity was observed among the non-menthol Marlboro cigarettes and non-flavored cigarillos, while the lowest alpha diversity was observed among the flavored products (little cigar SSC and all hookah products) (**Figure S3**). Comparing within brands, menthol Newport cigarettes (Shannon index average ± SD for NMB2, 2.23 ± 0.34 and NMG, 2.50 ± 0.13), mint flavors of Al Fakher (AFMF 1.81 ± 0.29) and Fumari (FMCC 1.94 ± 0.23) hookah, and menthol research cigarettes (2.24 ± 0.34) had higher alpha diversity compared to their non-menthol/mint counterparts (Shannon index average ± SD: 2.21 ± 0.17 (NNM); 1.74 ± 0.27 (AFWF); 1.63 ± 0.25 (AFTA); 2.0 ± 0.33 (FWGB); 1.66 ± 0.91 (FAMB), and 2.16 ± 0.39 for non-menthol research cigarettes); however, these differences were not statistically significant (Holm's adjusted Dunn's test $p > 0.05$). In

contrast, lower alpha diversity was observed among mentholated little cigars and Marlboro cigarettes (CMB 2.35 ± 0.40; MMB 2.64 ± 0.28) when compared to non-mentholated (CFF 2.42 ± 0.44; MRB 2.74 ± 0.22) varieties. Comparing the little cigars, there were no statistically significant differences in the Shannon diversity index between mentholated and flavored varieties, although flavored little cigars had lower average values. All flavored hookah products had lower alpha diversity compared to all other brands, with the highest alpha diversity values observed for the mint flavors (Al Fakher Mint and Fumari Mint Chocolate Chill).

Comparing the menthol and non-menthol commercial cigarettes, there were 24 non-menthol cigarette samples containing unique bacterial genera, with nine samples resembling the menthol samples. Among the research cigarettes, 11 menthol samples had unique genera, while 18 samples were associated with genera similar to that of the menthol research cigarettes. Twenty-seven non-menthol research cigarettes samples had unique genera and seven had similar taxa found in their menthol counterparts. Comparing the little cigars samples, 24 flavored little cigar samples had unique genera and 16 non-flavored little cigars samples had unique genera. All 99 flavored hookah samples had unique bacterial genera not found in any other tobacco product.

3.4.4 Core and shared microbiomes across brands and flavors

Across the little cigar products, *L. salivarius, L. fermentum, B. formosus*, and *S. saprophyticus* formed the core microbiome of all products (**Figure 6a**). Meanwhile, bacterial species that were unique to the Cheyenne products were *P. alcaligenes, P. oryzae, A. urinaeequi* and *M. thermoacetica*, and the only species that was unique to the Swisher Sweets products was *C. ammoniagenes*. The shared bacterial species among the two Cheyenne products were *B.*

subtilis, C. botulinum, P. agglomerans and *E. hormaechei*, which were absent in the Swisher Sweets.

Comparing the different hookah products, only *B. subtilis* formed the core microbiome across the four flavors, while *B. megaterium, B. clausii* and *P. aeruginosa* were shared between three flavors (**Figure 6b**). The Al Fakher flavor, two apple, did not share any bacterial species with any of the other products. Two other flavors, Fumari mint chocolate chill, and the Al Fakher mint flavor, did not have any bacterial species identified at a relative abundance higher than 1%, and hence, are not included in the figure.

Among the research cigarettes, *K. pneumoniae, P. agglomerans, M. luteus, P. putida, P. aeruginosa* and *S. saprophyticus* comprised the core microbiome of both menthol and nonmenthol research cigarettes (**Figure 6c)**. There were no core bacterial species (>1% relative abundance) that were identified among the commercial cigarette brands. *B. subtilis* and *P. putida* were shared between 4 brands of commercial cigarettes except for Newport Non-menthol (**Figure 6d**).

3.4.5 Metabolically-active bacterial communities across products

Comparing our subset of samples tested for total (NOTRT) and metabolically-active (BRDU) bacterial communities, alpha diversity was statistically significantly ($p < 0.001$) lower in BrdU-treated samples with regard to both the observed number of species and Shannon diversity index metrics (**Figure S4a**). Beta diversity was also statistically significantly different (PERMANOVA $R^2 = 0.5005$; $p < 0.001$) with more than 50% of the variation explained by BrdU treatment (**Figure S4b**). Metabolically-active bacterial genera among the tobacco brands included *Pseudomonas* (highest relative abundance), followed by *Terribacillus, Lactobacillus*,

and *Gardenella* (**Figure 7**). *Oceanobacillus* was only identified among the BrdU-treated samples. Other taxa that were only identified in the BrdU-treated samples included Uncl. *Clostridiales* and Uncl. *Aeromonadaceae*. While the above-mentioned bacterial genera were present at a relative abundance of >10%, there were several bacterial taxa identified at the species level with a relative abundance of <10% (**Table S3**).

3.5 Discussion

In this study, we characterized total and metabolically-active (live) bacterial communities across diverse tobacco products (cigarettes, hookah, little cigars and cigarillos) that are widely used in the U.S. While cigarillos were characterized by the highest diversity in bacterial communities (measured by the Shannon diversity index), hookah products had the lowest diversity compared to all other products. Bacterial community composition present within the tobacco products was significantly different across product types, with each product type harboring a unique microbiome signature. In addition, the majority of the metabolically-active bacteria across all products belonged to the genus *Pseudomonas,* followed by several genera within the Firmicutes phylum (*Bacillus, Terribacillus*, and *Oceanobacillus*).

While the most abundant genera among all tobacco products were *Pseudomonas, Staphylococcus,* and *Bacillus,* all five tobacco products harbored a core microbiome that included the following bacterial species: *Pseudomonas aeruginosa, P. putida, P. alcaligenes, Bacillus subtilis* and *Klebsiella pneumoniae*. Previous work from our group and others has demonstrated the presence of the above-mentioned species as members of the "core microbiome" of cigarettes, little cigars, and cigarillo products with noted differences in diversity and abundance across brands, lots, and flavors [7–10,12,60,63,65,169]. These organisms being a part of the

core microbiome of all tested tobacco products is of concern since several of them are pathogenic and have been associated with adverse health effects. For example, *P. aeruginosa* causes multiple infections (e.g., chronic lung infections and cystic fibrosis among smokers) and often expresses antibiotic resistance, leading to mortalities in some cases [195–198]. In addition, although considered a non-pathogenic probiotic, *B. subtilis* strain G7 isolates can be characterized by unique genetic features which are potentially lethal to vertebrates [199]. Lastly, one of the most common nosocomial pathogens, *K. pneumoniae,* is known to express antibiotic resistance and virulence factors that aid the bacteria in spreading through the respiratory system, causing pneumonia, bloodstream infections and meningitis [200,201].

In addition to the shared microbiome across all products, we also observed that each type of product harbored a unique microbiome signature. For instance, a significantly higher relative abundance of *Staphylococcus* and *Bacillus* was observed in cigarillos and a higher relative abundance of *Lactobacillus* and *Brevibacillus* was observed in little cigars. Previous studies from our group also had similar findings [9,63]. Interestingly, we also observed the lowest alpha diversity (measured by the Shannon diversity index) in hookah products compared to all other tested products. This could be due to the higher levels of glycerol and honey that constitute about 70% of hookah tobacco [202,203]. Humectants, such as glycerol and propylene glycol, are usually added to tobacco filler to maintain moisture, dilute nicotine to a viscous consistency, and act as a carrier solvent. Glycerol (> 5% solutions) also has bactericidal activity, inhibiting bacterial growth and cellulolytic activity [204,205]. Interestingly, bacterial species within a few genera (*Lactobacillus, Klebsiella, Enterobacter, Citrobacter,* and *Clostridium)* can metabolize glycerol to produce the antimicrobial reuterin, which also possesses antimicrobial properties against commensal bacterial

populations [206,207]. Similarly, honey—which is one of the oldest known antimicrobials—also exhibits bactericidal activity against a variety of bacteria [208,209].

Furthermore, in our comparison between products, we observed significantly different microbiome signatures between mentholated versus non-mentholated products. Comparing within products, mentholated brands had lower alpha diversity compared to their non-mentholated varieties. Previous research has demonstrated that bacterial diversity is different between menthol and non-mentholated cigarettes and little cigars [8,9,9,63], and Chopyk et al. (2017) found that mentholated products harbor taxa that are known to survive harsh conditions such as *Anoxybacillus* and *Schlegella* [65].

In addition to evaluating the effects of mentholation, we found that all flavored varieties of tested tobacco products (Swisher Sweets Cherry and all hookah products) had the lowest alpha diversity compared to all other products. Even though we could not do a direct comparison between a flavored product with a non-flavored counterpart for the same type of tobacco product, our data potentially indicate that additives such as fruit flavorings may have a greater impact on bacterial diversity compared to other additives such as menthol or mint. Among the flavored little cigar and hookah products, we also found differences in the relative abundance of bacterial genera across products; however, we were unable to make comparisons against non-flavored counterparts. The observed differences in the relative abundance of bacterial taxa may be due to different curing techniques during tobacco processing and manufacturing. Given the limited data on bacterial community differences between mentholated/flavored tobacco products and non-flavored products, more in-depth studies are needed, particularly with regard to cigarettes, little cigars, and hookah products.

While we observed significant differences in the relative abundance of different bacterial species across the five tobacco products, we also identified known or emerging pathogens that could be important in terms of users' health. For example, commercial and research cigarettes harbored *P. putida,* and *P. agglomerans*, both opportunistic human pathogens [210,211]. Two more pathogenic species identified among the research cigarettes were *Aerococcus urinaeequi* and *Enterococcus gilvus,* which have previously been found to be sensitive to antimicrobials [212,213]. Our data also identified the bacterial pathogens *M. luteus* and *E. hormaechei* in four of the five tobacco products. Even though it is a part of the normal oral microflora, *M. luteus* can be an opportunistic pathogen [214] and *E. hormaechei* is a well-known causative agent of nosocomial infections, exhibiting resistance to multiple antibiotics [215–217].

Furthermore, we found that both cigarillos and little cigars harbored the spore-forming bacteria *C. botulinum,* and cigarillos alone harbored *L. sphaericus*. While *C. botulinum* is a well-known anaerobic species producing the botulinum neurotoxin [218], *L. sphaericus* produces insecticidal proteins leading to cytopathological effects in insect larvae [219]. A recent case report also described *L. sphaericus* as a causative agent of severe sepsis [220]. Another unique species identified in cigarillos was *Saccharomonospora viridis*. This thermophilic species is usually found in hay and compost, and prolonged exposure to its spores is known to cause farmer's lung disease, bagassosis (a type of interstitial lung disease), and humidifier fever [221].

Given the plethora of bacterial species that 1) were present in the tested tobacco products and 2) have the potential to be transmitted via aerosols to users' upper respiratory tracts, it was important to evaluate their viability. By coupling BrdU-labeling and sequencing, we were able to identify the metabolically-active fraction of the total bacterial communities identified across the tested tobacco products. Alpha diversity in BrdU-treated samples was lower than in non-BrdU-

treated samples, which was consistent with previous studies utilizing this coupled approach to detect metabolically-active bacterial communities in water samples [174]. Interestingly, we identified *Pseudomonas*—the bacterial genera detected as one of the most abundant overall in the tobacco products—to be metabolically-active. Other metabolically-active bacterial genera identified across the majority of tested products included *Bacillus* and *Paenibacillus*, while metabolically-active bacterial species included *Atopobium vaginae, Alcaligenes faecalis, Bacillus cereus, Haemophilus parainfluenzae, Rothia mucilaginosa, Streptococcus agalactiae*, and *Propionibacterium acnes*. These data are supported by previous findings from our group where we identified viable *Bacillus* and *Paenibacillus* in mainstream cigarette smoke extract using culture-dependent techniques [16]. While previous studies have identified a few of the above-mentioned species as pathogenic (*A. faecalis, B. cereus, Pseudomonas viridiflava, Rothia aeria, R. mucilaginosa, S. agalactiae*) [222–228], some species (*Methylobacterium adhaesivum, Acinetobacter johnsonii, A. lwoffii*) [229,230] are emerging animal/human pathogens, while others (*P. acnes, H. parainfluenzae*) are known to be associated with multiple diseases (e.g., inflammatory infections, bone and joint infections) [231,232]. It is important to note that two of the genera, *Pseudomonas* and *Bacillus,* that incorporated BrdU, are dominant members of the tobacco microbiome, and a few of the above-mentioned pathogenic species within these genera were also viable/metabolically-active. Hence, these organisms have the potential to be transferred to users during tobacco use, colonize the upper respiratory tract and potentially cause adverse health effects.

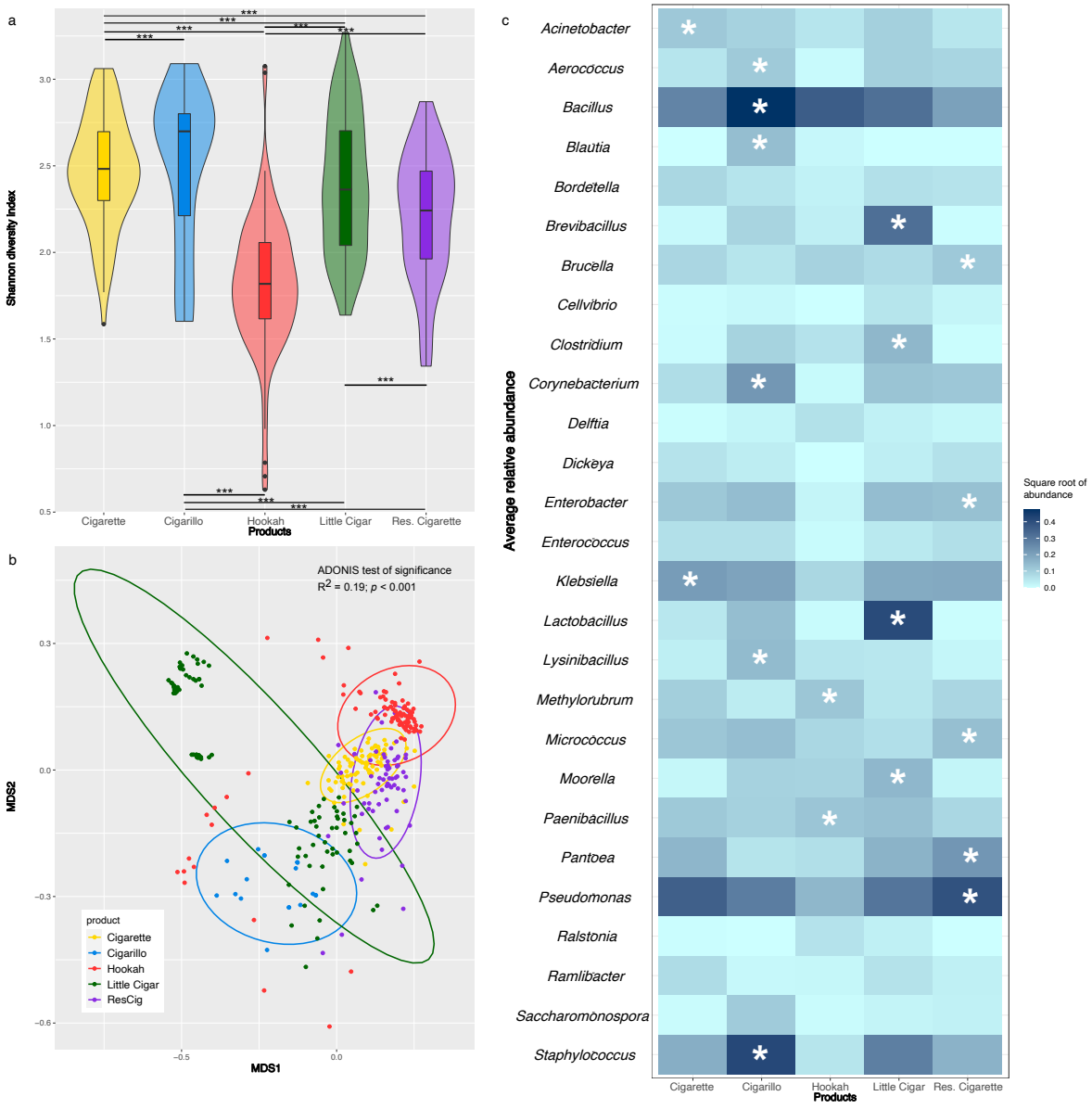
Strengths of this study include the sample size, the inclusion of multiple tobacco products, and the incorporation of a coupled BrdU-labeling/sequencing technique. However, like other sequencing-based studies, there were multiple limitations, including challenges with regard

to species-level taxonomic assignments. Here, we used Kraken to perform sequence classification and estimated species abundance using Bracken [190,191,233]. The kmer matching technique used by Kraken has been shown to supersede those pipelines using gene markers in terms of sensitivity and specificity [234,235], reducing runtime and memory usage [236] and reducing sensitivity to structural variations (e.g., inversions) within reads [237]. However, as with other classifiers, Kraken 2 also misclassifies reads at the species level, specifically with regard to genera or species sharing a high genomic identity [236]. In addition, there are only a few studies that have utilized the coupled BrdU-labeling/sequencing technique used here. This technique and other widely used approaches such as the incorporation of radiolabeled precursors (like thymidine and leucine) can have methodological limitations, including contrasting uptake of these compounds by different bacterial phyla [238–240]. Finally, since this labeling technique is limited to bacterial taxa that are capable of incorporating exogenous nucleotide precursors into replicating DNA [189], additional culture-dependent and independent studies are required to better characterize the capacity of BrdU incorporation across diverse bacterial communities in environmental samples.

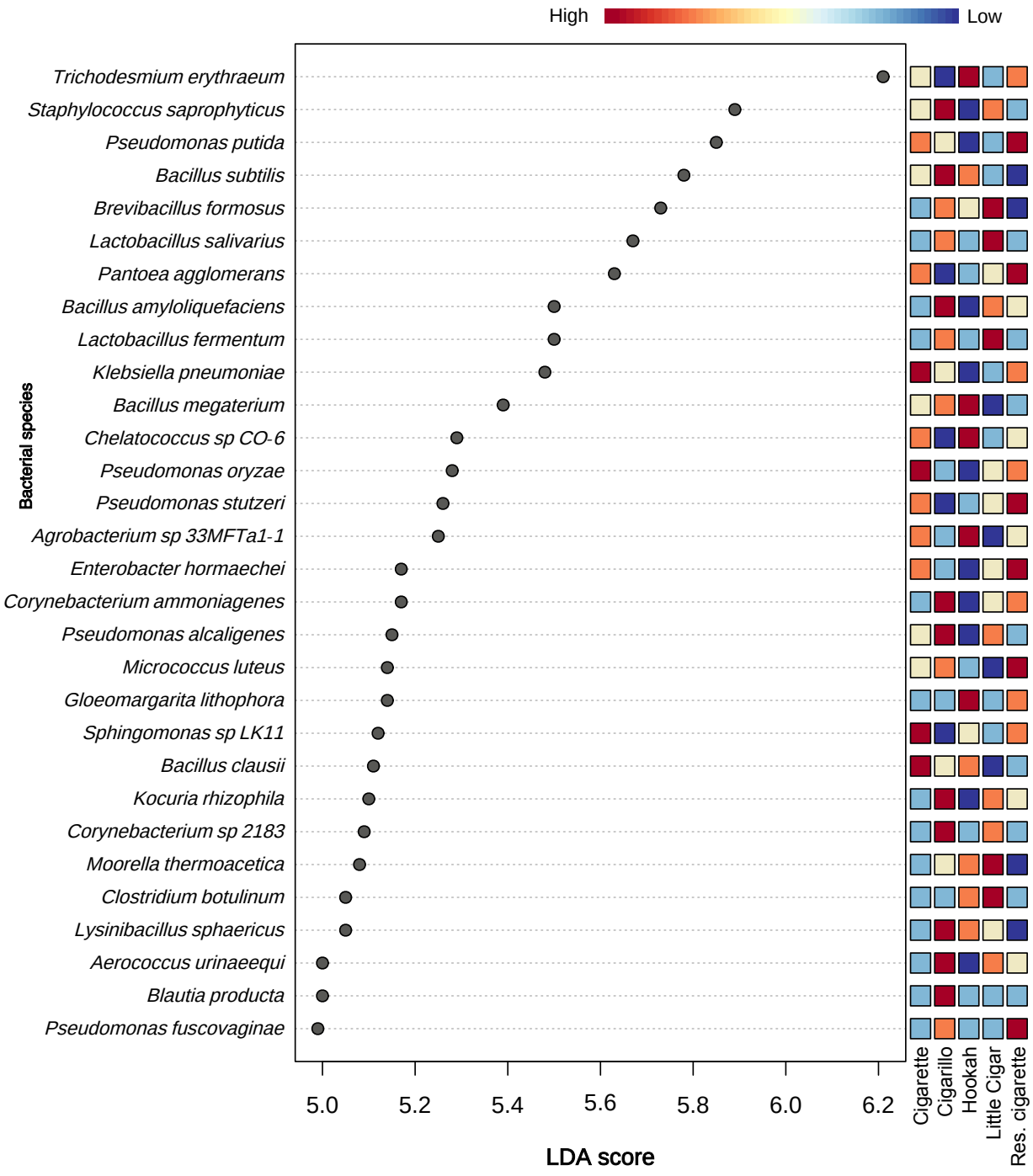
In closing, smoking tobacco products is known to impact the user's oral microbiome, shifting the normal microflora towards a pathogen prevalent community. To better understand the association between members of the tobacco microbiome and the oral microbiome, we need in-depth comparative characterizations of the microbiome of diverse tobacco products. Here, we not only characterized the unique/signature bacterial taxa in each tested tobacco product but also identified the core bacterial communities and metabolically-active bacteria that were present across all products. Future work should evaluate the role of the identified tobacco bacterial species in the potential shifting of smokers' oral and lung bacterial community compositions,

which can ultimately lead to disease development in the oral cavity, overall respiratory tract and other body systems.

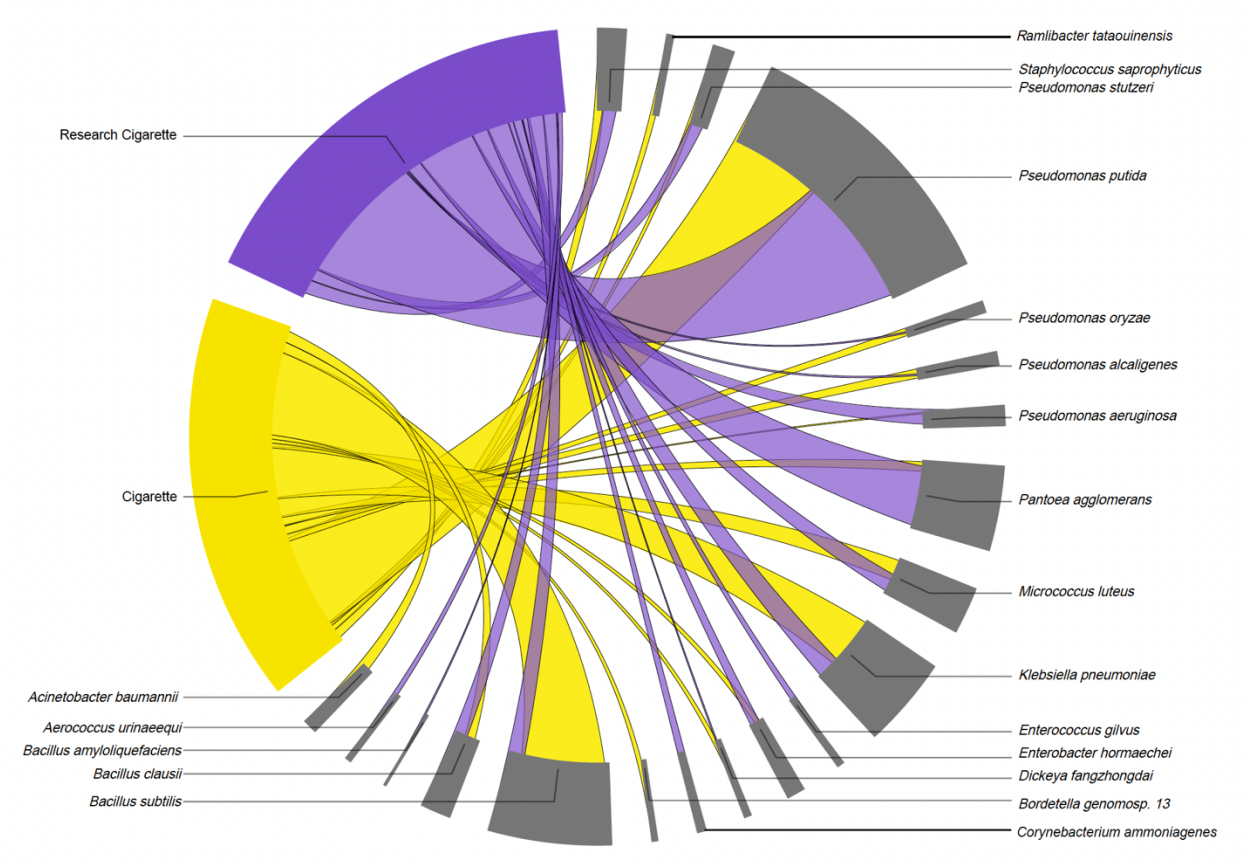
3.6 Figures



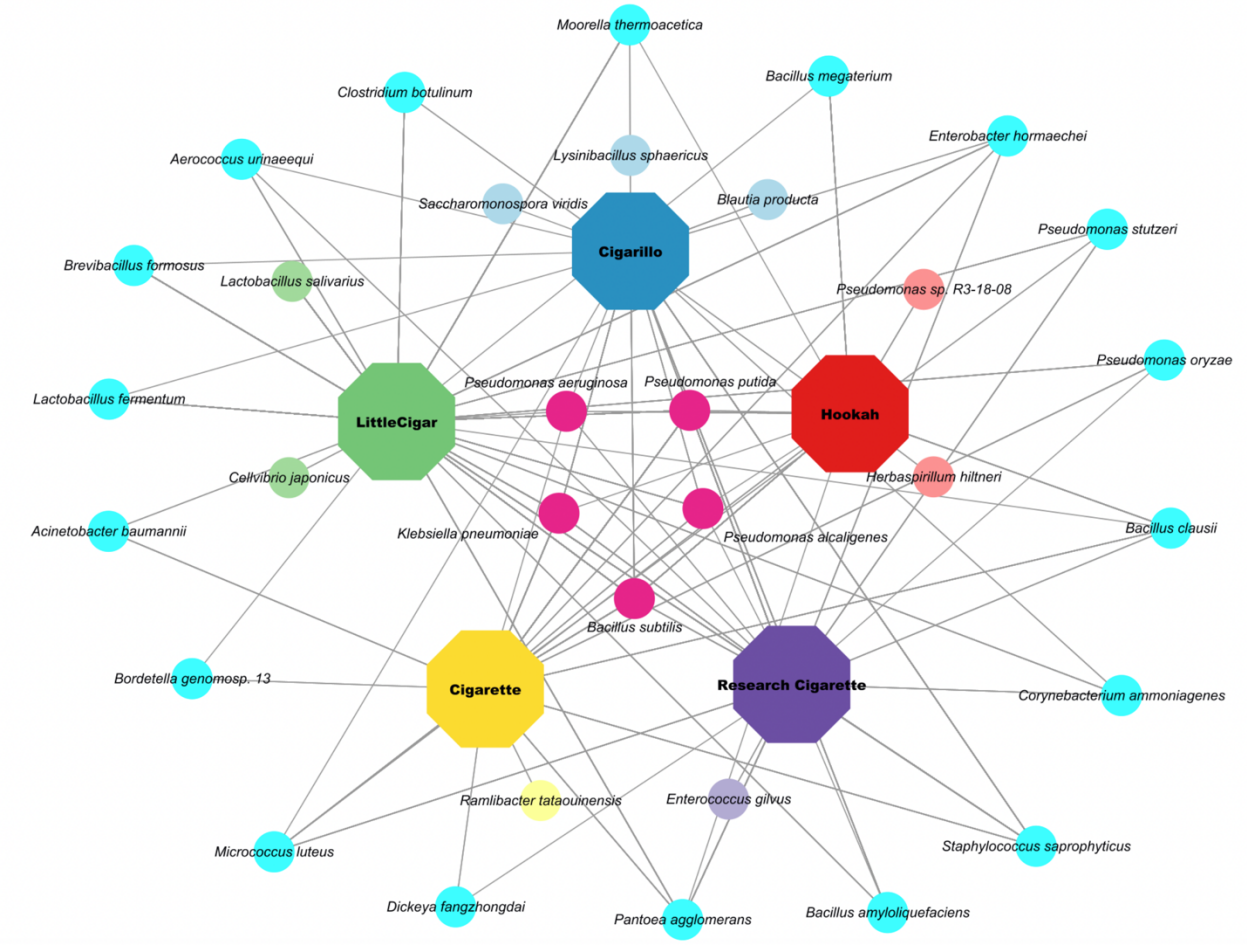
1. Figure 3.1 Diversity and abundance of bacterial communities across all tobacco products. (a) Alpha diversity across the five tobacco products measured using the Shannon diversity index. Statistical significance between products was determined using the Kruskal-Wallis test followed by a *post hoc* Dunn's test with Holm's adjusted *p*-values reported. $P < 0.001$ (***). (b) Principal coordinate analysis plot of Bray-Curtis computed distances across products. Ellipses are drawn at 95% confidence intervals. Differences in beta diversity between products were measured using ADONIS. (c) Heatmap showing the average relative abundance of the top bacterial genera present across tobacco products. Significantly higher differentially abundant genera were determined using a LEfSe analysis. $P < 0.05$ (*). Res. cigarette = Research cigarette.



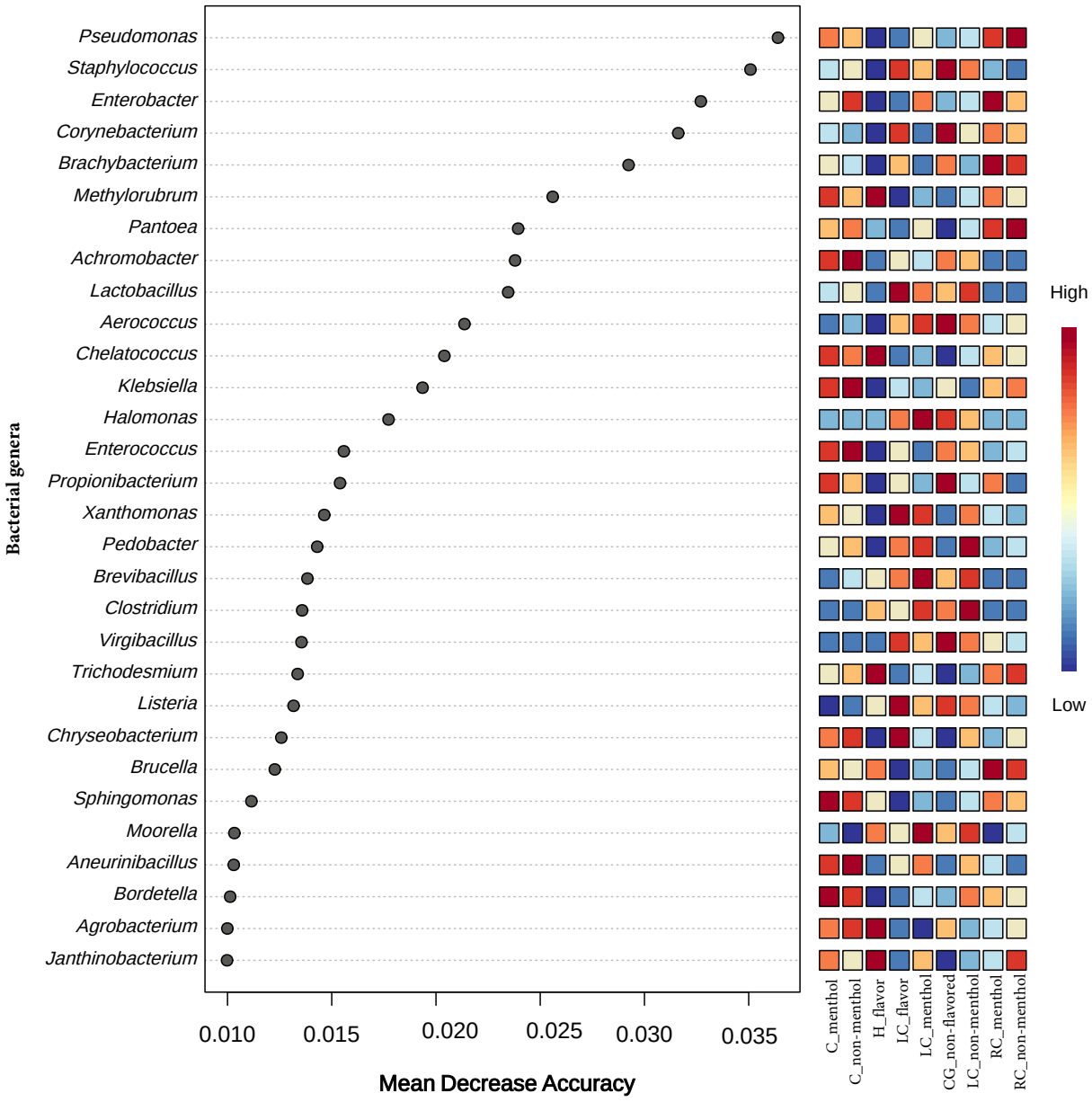
2. Figure 3.2: LEfSe analysis on the differential abundance of bacterial species across tobacco products. The color gradient on top shows the differential abundance of the taxa from low (blue) to high (red). Res. cigarette= Research cigarette.



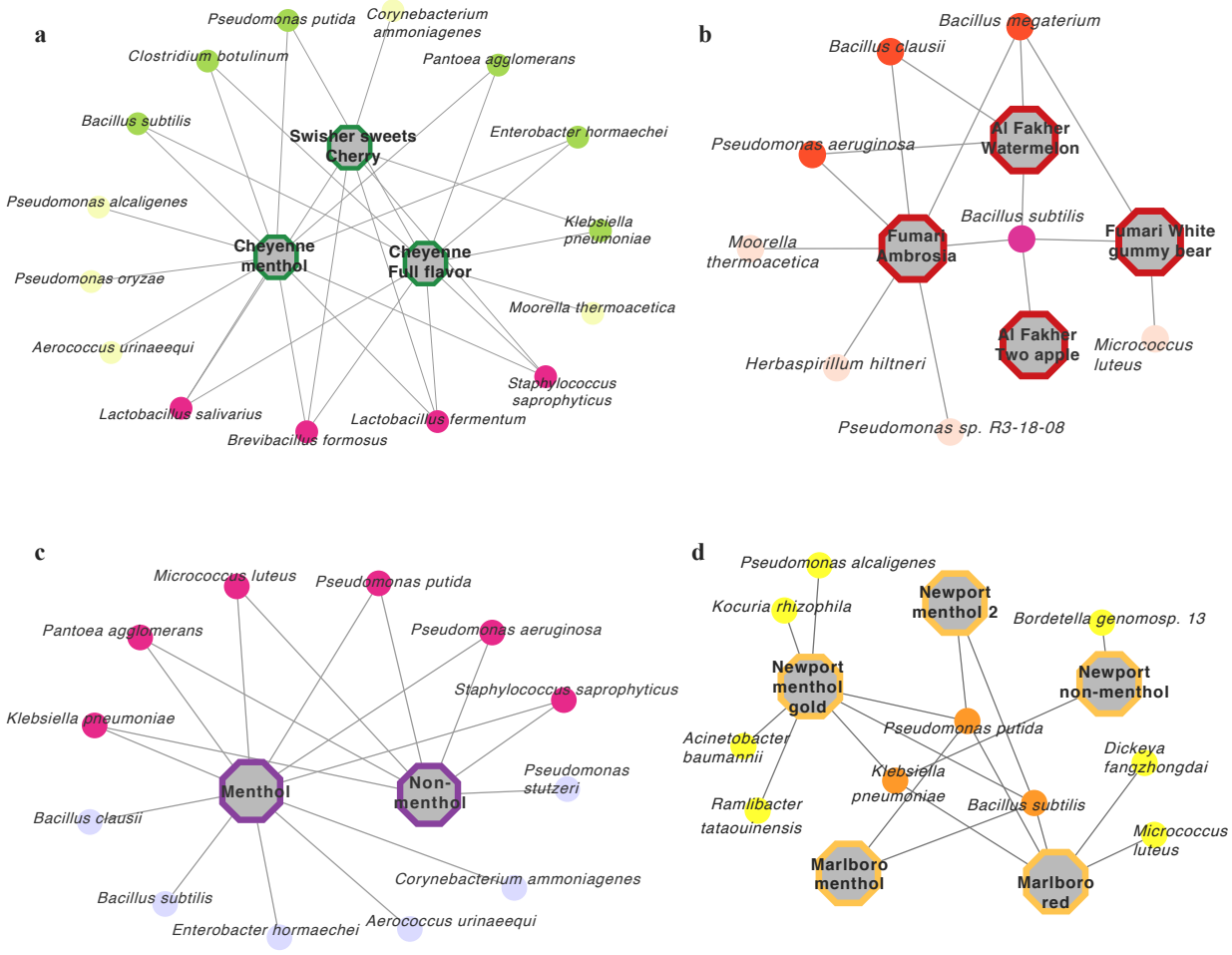
3. Figure 3.3: Chord plot showing the average relative abundance (above 5%) of bacterial species between commercial (yellow) and research (purple) cigarettes.



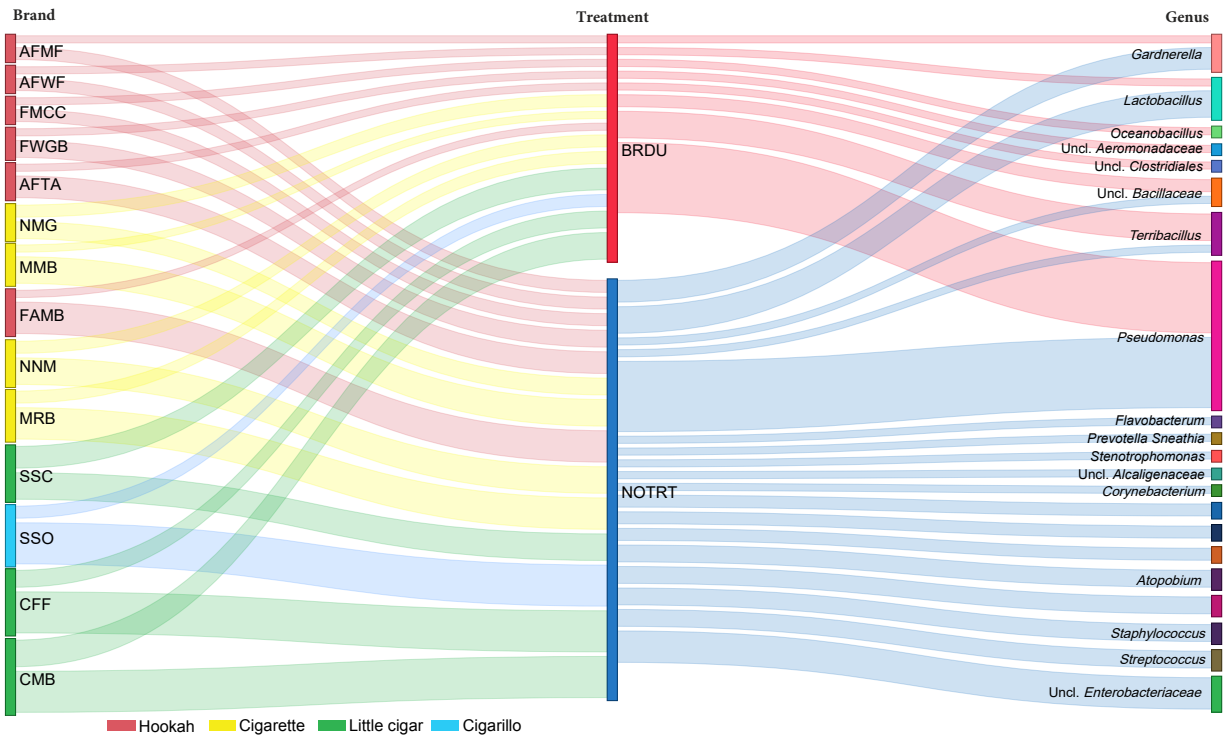
4. Figure 3.4: Network analysis showing shared and unique bacterial species across all tobacco products. The circles represent each bacterial species, and the hexagons represent the tobacco products. The nodes joining the shapes indicate the identification of the taxa within the product.



5. Figure 3.5: Predicted association between the top 30 bacterial genera and specific tobacco products with/without additives. The color gradient shows the degree of association from low (blue) to high (red). C: cigarette, LC: little cigar, CG: cigarillo, H: hookah, and RC: research cigarette.

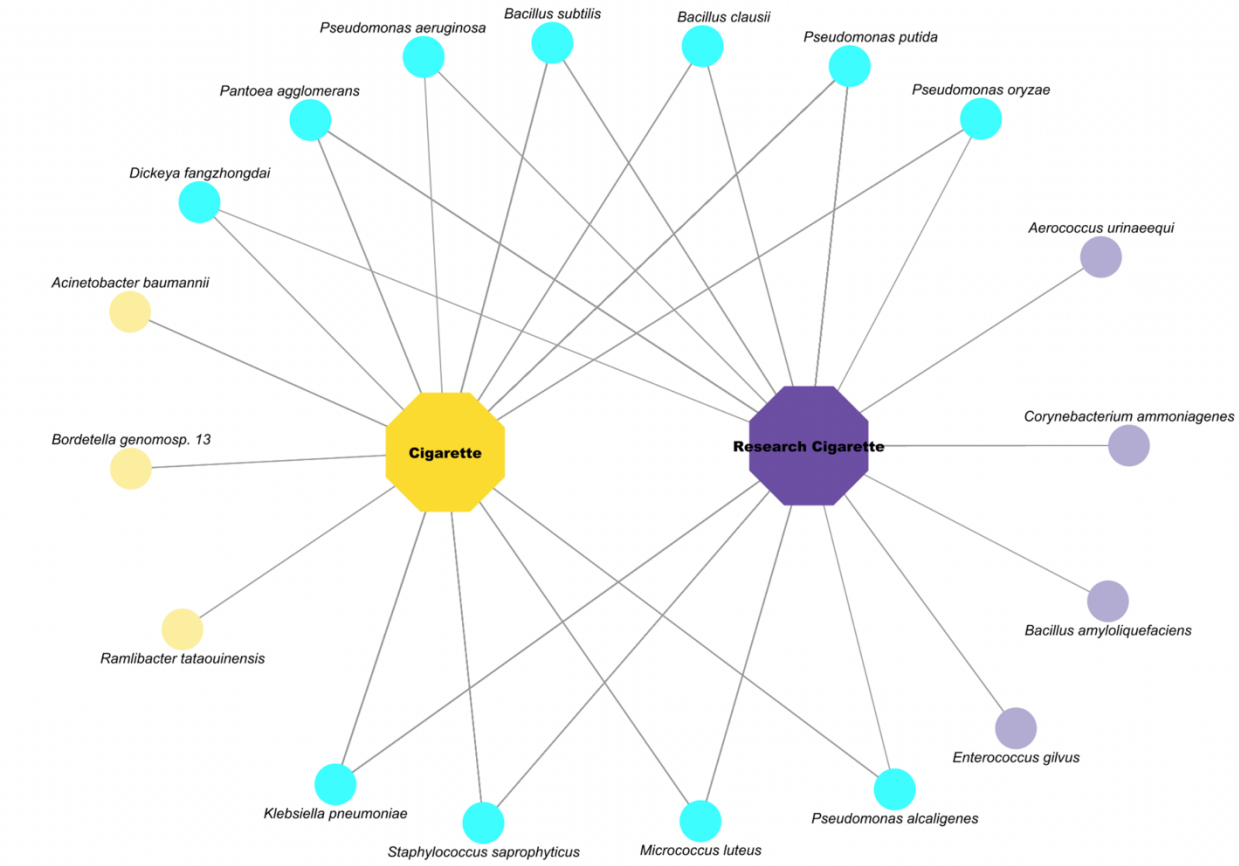


6. Figure 3.6: Network plots showing the shared and unique bacterial species within (a) hookah, (b) little cigars, (c) cigarettes, and (d) research cigarettes. The circles represent each bacterial species, and the hexagons represent the tobacco products. The nodes joining the shapes depict the identified taxa within the products.

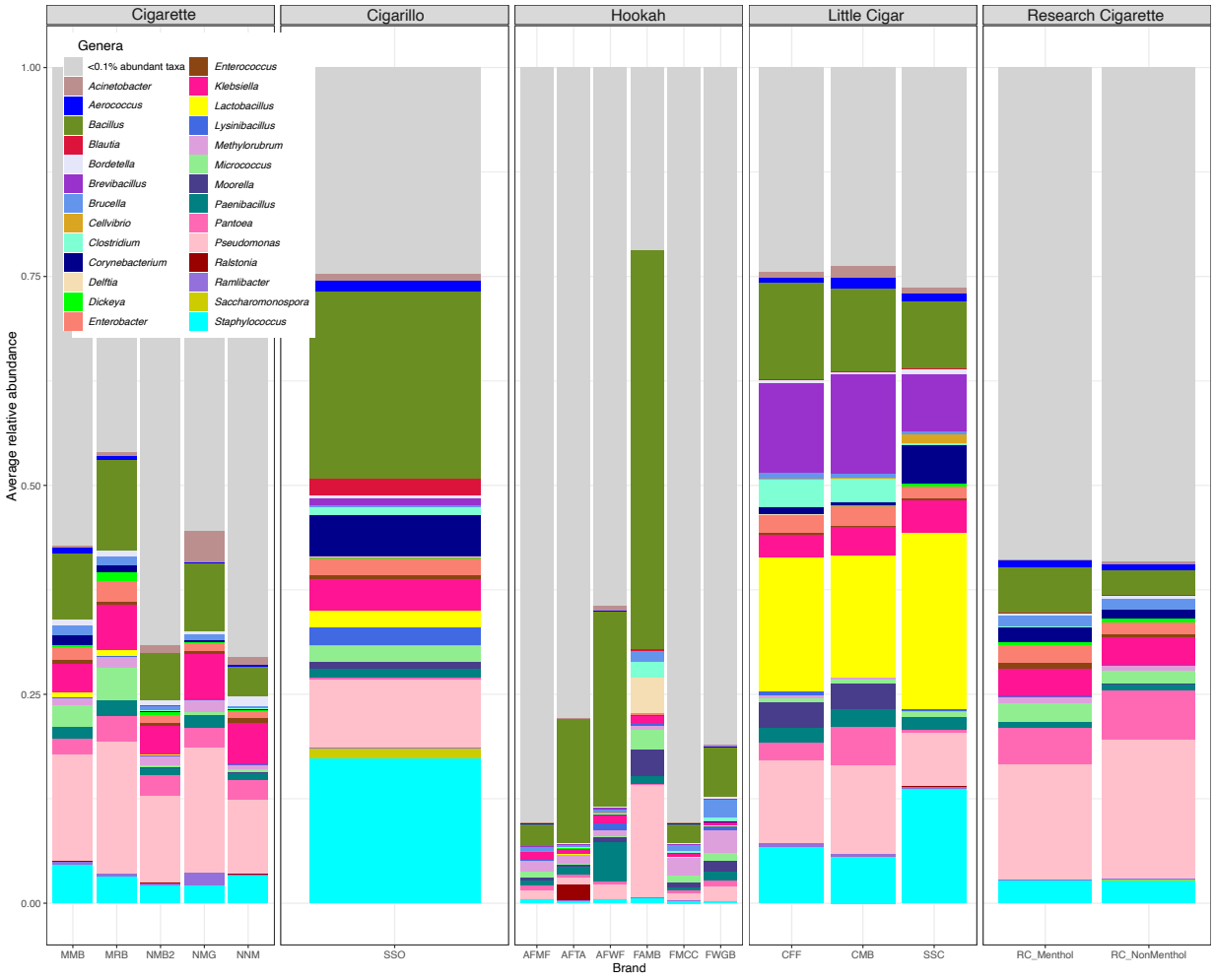


7. Figure 3.7: Sankey plot showing the metabolically-active (BrdU) bacterial species identified within the total bacterial community (NOTRT) across all tobacco brands and flavors. AFMF: Al Fakher mint, AFWF: Al Fakher watermelon, FMCC: Fumari mint chocolate chill, FWGB: Fumari white gummy bear, AFTA: Al Fakher two apple, NMG: Newport menthol gold, MMB: Marlboro menthol box, FAMB: Fumari Ambrosia, NNM: Newport non-menthol, MRB: Marlboro red, SSC: Swisher sweets cherry, SSO: Swisher sweets original, CFF: Cheyenne full flavor, CMB: Cheyenne menthol box.

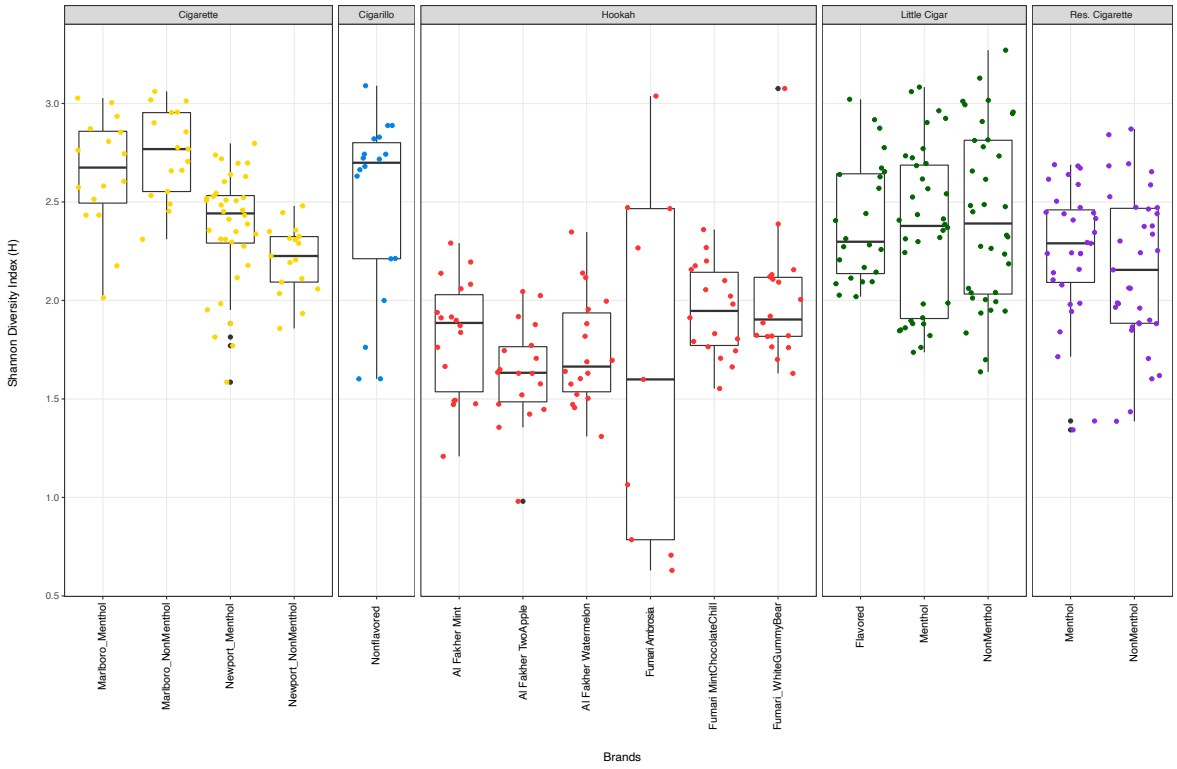
3.7 Supplementary figures



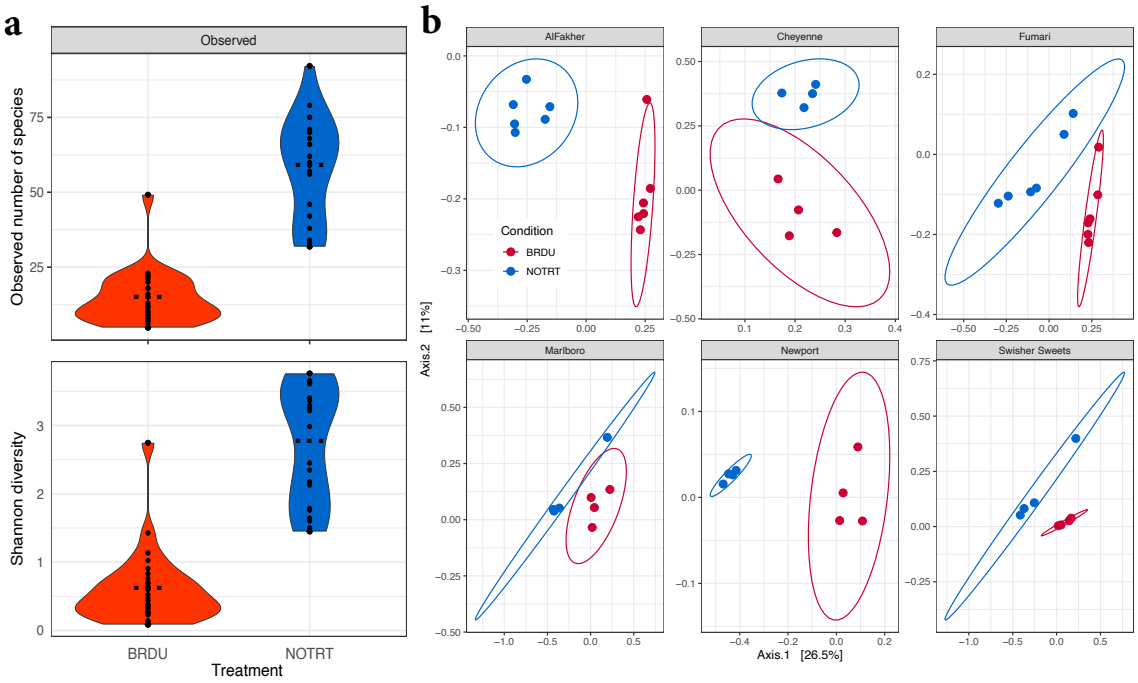
8. Figure S 3.1: Network plot showing the unique and shared bacterial species between commercial and research cigarettes.



9. Figure S 3.2: Average relative abundance of bacterial genera across all tobacco products and brands.



10. Figure S 3.3: Bacterial alpha diversity by tobacco product, brand and flavor.



11. Figure S 3.4: Bacterial a) alpha and b) beta diversity between BrdU-treated (BRDU) and non-treated (NOTRT) tobacco samples.

3.8 Tables

1. Table 3.1: Tobacco products included in this study.

Product	Brand	Additives	
		Mentholation	**Flavoring**
Commercial cigarettes (*n=90*)	Marlboro red (MRB)	Non menthol	
	Marlboro menthol (MMB)	Menthol	
	Newport non-menthol (NNM)	Non menthol	
	Newport menthol (NMB2)	Menthol	
	Newport menthol gold (NMG)	Menthol	
Hookah (*n=108*)	Al Fakher two apple (AFTA)		Two apple
	Al Fakher mint (AFMF)		Mint
	Al Fakher watermelon (AFWF)		Watermelon
	Fumari ambrosia (FAMB)		Ambrosia
	Fumari mint chocolate chill (FMCC)		Mint chocolate
	Fumari white gummi bear (FQGB)		White gummi bear
Little cigars (*n=108*)	Cheyenne menthol (CMB)	Menthol	
	Cheyenne full flavor (CFF)	Non menthol	
	Swisher sweets cherry (SSC)		Cherry
Cigarillos (*n=36*)	Swisher sweets original (SSO)		Non flavored
Research cigarettes (*n=72*)	NRC 102	Non menthol	
	NRC 200	Non menthol	
	NRC 300	Non menthol	
	NRC 400	Non menthol	
	NRC 404	Non menthol	
	NRC 600	Non menthol	
	NRC 103	Menthol	
	NRC 201	Menthol	
	NRC 301	Menthol	
	NRC 401	Menthol	
	NRC 501	Menthol	
	NRC 601	Menthol	

3.9 Supplementary tables

2. Table S 3.1: Pairwise ADONIS test between tobacco products.

Comparison	Degrees of freedom	Sums of squares	F test statistic	R^2	*p*-value	Adjusted *p*-value
Cigarette vs Hookah	1	4.098884353	22.63174856	0.110059603	0.001	0.01
Cigarette vs Little Cigar	1	6.112197504	31.92760815	0.149945836	0.001	0.01
Cigarette vs Cigarillo	1	2.916232342	15.12345883	0.130236035	0.001	0.01
Cigarette vs Research Cigarette	1	8.453616937	48.50868832	0.244365568	0.001	0.01
Hookah vs Little Cigar	1	1.551455574	9.116792071	0.044884482	0.001	0.01
Hookah vs Cigarillo	1	1.380816024	8.823981099	0.071842494	0.001	0.01
Hookah vs Research Cigarette	1	3.954489038	26.30507086	0.138955976	0.001	0.01
Little Cigar vs Cigarillo	1	0.518131614	2.999591622	0.026083498	0.001	0.01
Little Cigar vs Research Cigarette	1	1.53230773	9.48448284	0.055632528	0.001	0.01
Cigarillo vs Research Cigarette	1	0.495949977	3.707187598	0.043764735	0.001	0.01

3. Table S 3.2: Pairwise ADONIS test between mentholated/flavored and non-mentholated/non-flavored tobacco products.

Comparison	Degrees of freedom	Sums of squares	F test statistic	R^2	*p*-value	Adjusted *p*-value
Marlboro menthol vs Marlboro red	**1**	**0.508**	**2.810**	**0.083**	**0.037**	1
Marlboro menthol vs Newport menthol	1	0.116	0.617	0.019	0.693	1
Marlboro menthol vs Newport menthol gold	1	0.235	1.187	0.036	0.251	1
Marlboro menthol vs Newport nonmenthol	1	1.989	14.303	0.316	0.001	0.253
Marlboro menthol vs Al Fakher	1	0.350	1.744	0.025	0.071	1
Marlboro menthol vs Fumari	1	1.313	10.988	0.157	0.001	0.253
Marlboro menthol vs Cheyenne Full Flavor	1	1.138	5.513	0.099	0.001	0.253
Marlboro menthol vs Cheyenne Menthol Box	1	1.269	7.333	0.128	0.001	0.253
Marlboro menthol vs Swisher Sweets Cherry	1	0.974	6.511	0.146	0.001	0.253
Marlboro menthol vs Swisher Sweets Original	1	1.364	8.353	0.207	0.001	0.253
Marlboro menthol vs NRC102	1	0.503	2.824	0.124	0.023	1
Marlboro menthol vs NRC103	1	0.566	3.122	0.141	0.015	1
Marlboro menthol vs NRC200	1	0.563	3.070	0.139	0.007	1
Marlboro menthol vs NRC201	1	1.101	6.366	0.241	0.001	0.253
Marlboro menthol vs NRC300	1	1.219	7.562	0.274	0.001	0.253
Marlboro menthol vs NRC301	1	1.214	7.384	0.270	0.001	0.253
Marlboro menthol vs NRC400	1	1.090	6.058	0.232	0.001	0.253
Marlboro menthol vs NRC401	1	1.288	7.539	0.274	0.001	0.253
Marlboro menthol vs NRC404	1	1.177	6.818	0.254	0.001	0.253
Marlboro menthol vs NRC501	1	1.235	7.330	0.268	0.001	0.253
Marlboro menthol vs NRC600	1	1.297	7.829	0.281	0.001	0.253
Marlboro menthol vs NRC601	1	0.559	2.956	0.156	0.031	1
Marlboro red vs Newport menthol	1	0.812	4.727	0.125	0.009	1
Marlboro red vs Newport menthol gold	1	0.475	2.618	0.074	0.051	1
Marlboro red vs Newport nonmenthol	1	1.281	10.350	0.244	0.001	0.253

Marlboro red vs Al Fakher	1	1.319	6.846	0.090	0.001	0.253
Marlboro red vs Fumari	1	2.599	23.267	0.279	0.001	0.253
Marlboro red vs Cheyenne Full Flavor	1	2.156	11.024	0.178	0.001	0.253
Marlboro red vs Cheyenne Menthol Box	1	2.362	14.513	0.222	0.001	0.253
Marlboro red vs Swisher Sweets Cherry	1	2.213	16.176	0.293	0.001	0.253
Marlboro red vs Swisher Sweets Original	1	2.153	14.580	0.306	0.001	0.253
Marlboro red vs NRC102	1	1.038	6.781	0.244	0.001	0.253
Marlboro red vs NRC103	1	1.065	6.881	0.256	0.001	0.253
Marlboro red vs NRC200	1	0.948	6.046	0.232	0.001	0.253
Marlboro red vs NRC201	1	1.375	9.286	0.307	0.001	0.253
Marlboro red vs NRC300	1	1.559	11.391	0.352	0.001	0.253
Marlboro red vs NRC301	1	1.580	11.292	0.350	0.001	0.253
Marlboro red vs NRC400	1	1.473	9.521	0.312	0.001	0.253
Marlboro red vs NRC401	1	1.703	11.655	0.357	0.001	0.253
Marlboro red vs NRC404	1	1.619	10.955	0.343	0.001	0.253
Marlboro red vs NRC501	1	1.575	10.954	0.343	0.001	0.253
Marlboro red vs NRC600	1	1.628	11.538	0.355	0.001	0.253
Marlboro red vs NRC601	1	0.687	4.359	0.204	0.006	1
Newport menthol vs Newport menthol gold	1	0.423	2.244	0.062	0.066	1
Newport menthol vs Newport nonmenthol	**1**	**2.674**	**20.124**	**0.379**	**0.001**	0.253
Newport menthol vs Al Fakher	1	0.360	1.837	0.026	0.053	1
Newport menthol vs Fumari	1	1.272	10.890	0.151	0.001	0.253
Newport menthol vs Cheyenne Full Flavor	1	1.214	6.070	0.105	0.001	0.253
Newport menthol vs Cheyenne Menthol Box	1	1.364	8.133	0.135	0.001	0.253
Newport menthol vs Swisher Sweets Cherry	1	0.950	6.600	0.142	0.001	0.253
Newport menthol vs Swisher Sweets Original	1	1.392	8.933	0.208	0.001	0.253
Newport menthol vs NRC102	1	0.474	2.865	0.115	0.011	1
Newport menthol vs NRC103	1	0.548	3.268	0.135	0.005	1
Newport menthol vs NRC200	1	0.540	3.187	0.132	0.008	1

Newport menthol vs NRC201	1	1.157	7.206	0.247	0.001	0.253
Newport menthol vs NRC300	1	1.192	7.952	0.265	0.001	0.253
Newport menthol vs NRC301	1	1.194	7.813	0.262	0.001	0.253
Newport menthol vs NRC400	1	1.085	6.504	0.228	0.001	0.253
Newport menthol vs NRC401	1	1.291	8.132	0.270	0.001	0.253
Newport menthol vs NRC404	1	1.182	7.372	0.251	0.001	0.253
Newport menthol vs NRC501	1	1.221	7.799	0.262	0.001	0.253
Newport menthol vs NRC600	1	1.265	8.216	0.272	0.001	0.253
Newport menthol vs NRC601	1	0.543	3.151	0.149	0.018	1
Newport menthol gold vs Newport nonmenthol	**1**	**1.615**	**11.356**	**0.256**	**0.001**	0.253
Newport menthol gold vs Al Fakher	1	0.787	3.924	0.053	0.001	0.253
Newport menthol gold vs Fumari	1	2.138	17.539	0.223	0.001	0.253
Newport menthol gold vs Cheyenne Full Flavor	1	1.874	9.101	0.149	0.001	0.253
Newport menthol gold vs Cheyenne Menthol Box	1	2.070	11.918	0.186	0.001	0.253
Newport menthol gold vs Swisher Sweets Cherry	1	1.657	10.926	0.215	0.001	0.253
Newport menthol gold vs Swisher Sweets Original	1	1.963	11.904	0.259	0.001	0.253
Newport menthol gold vs NRC102	1	0.769	4.285	0.163	0.006	1
Newport menthol gold vs NRC103	1	0.799	4.383	0.173	0.003	0.759
Newport menthol gold vs NRC200	1	0.781	4.243	0.168	0.002	0.506
Newport menthol gold vs NRC201	1	1.430	8.188	0.271	0.002	0.506
Newport menthol gold vs NRC300	1	1.570	9.582	0.303	0.001	0.253
Newport menthol gold vs NRC301	1	1.567	9.393	0.299	0.001	0.253
Newport menthol gold vs NRC400	1	1.386	7.660	0.258	0.001	0.253
Newport menthol gold vs NRC401	1	1.650	9.553	0.303	0.001	0.253
Newport menthol gold vs NRC404	1	1.506	8.639	0.282	0.001	0.253
Newport menthol gold vs NRC501	1	1.546	9.066	0.292	0.001	0.253
Newport menthol gold vs NRC600	1	1.621	9.648	0.305	0.001	0.253
Newport menthol gold vs NRC601	1	0.686	3.618	0.167	0.011	1
Newport nonmenthol vs Al Fakher	1	3.619	20.791	0.232	0.001	0.253

Newport nonmenthol vs Fumari	1	5.350	59.286	0.497	0.001	0.253
Newport nonmenthol vs Cheyenne Full Flavor	1	4.301	25.252	0.331	0.001	0.253
Newport nonmenthol vs Cheyenne Menthol Box	1	4.690	34.103	0.401	0.001	0.253
Newport nonmenthol vs Swisher Sweets Cherry	1	4.456	42.930	0.524	0.001	0.253
Newport nonmenthol vs Swisher Sweets Original	1	3.992	36.719	0.527	0.001	0.253
Newport nonmenthol vs NRC102	1	2.267	24.695	0.540	0.001	0.253
Newport nonmenthol vs NRC103	1	1.956	21.625	0.520	0.001	0.253
Newport nonmenthol vs NRC200	1	1.791	19.392	0.492	0.001	0.253
Newport nonmenthol vs NRC201	1	2.204	25.387	0.547	0.001	0.253
Newport nonmenthol vs NRC300	1	2.503	33.141	0.612	0.001	0.253
Newport nonmenthol vs NRC301	1	2.556	32.510	0.608	0.001	0.253
Newport nonmenthol vs NRC400	1	2.347	25.128	0.545	0.001	0.253
Newport nonmenthol vs NRC401	1	2.546	30.021	0.588	0.001	0.253
Newport nonmenthol vs NRC404	1	2.522	29.164	0.581	0.001	0.253
Newport nonmenthol vs NRC501	1	2.580	31.269	0.598	0.001	0.253
Newport nonmenthol vs NRC600	1	2.619	32.819	0.610	0.001	0.253
Newport nonmenthol vs NRC601	1	1.099	13.429	0.441	0.007	1
Al Fakher vs Fumari	1	1.002	6.592	0.064	0.001	0.253
Al Fakher vs Cheyenne Full Flavor	1	1.060	5.178	0.056	0.001	0.253
Al Fakher vs Cheyenne Menthol Box	1	1.606	8.646	0.089	0.001	0.253
Al Fakher vs Swisher Sweets Cherry	1	1.004	5.699	0.070	0.001	0.253
Al Fakher vs Swisher Sweets Original	1	1.483	8.031	0.103	0.001	0.253
Al Fakher vs NRC102	1	0.431	2.218	0.037	0.017	1
Al Fakher vs NRC103	1	0.445	2.276	0.038	0.022	1
Al Fakher vs NRC200	1	0.431	2.196	0.037	0.024	1
Al Fakher vs NRC201	1	1.055	5.484	0.086	0.001	0.253
Al Fakher vs NRC300	1	1.106	5.874	0.092	0.001	0.253
Al Fakher vs NRC301	1	1.131	5.969	0.093	0.001	0.253
Al Fakher vs NRC400	1	1.072	5.504	0.087	0.001	0.253

Al Fakher vs NRC401	1	1.303	6.800	0.105	0.001	0.253
Al Fakher vs NRC404	1	1.109	5.767	0.090	0.001	0.253
Al Fakher vs NRC501	1	1.190	6.235	0.097	0.001	0.253
Al Fakher vs NRC600	1	1.256	6.614	0.102	0.001	0.253
Al Fakher vs NRC601	1	0.478	2.405	0.043	0.024	1
Fumari vs Cheyenne Full Flavor	1	0.766	5.299	0.063	0.001	0.253
Fumari vs Cheyenne Menthol Box	1	1.243	10.063	0.113	0.001	0.253
Fumari vs Swisher Sweets Cherry	1	0.830	8.193	0.109	0.001	0.253
Fumari vs Swisher Sweets Original	1	1.080	10.412	0.146	0.001	0.253
Fumari vs NRC102	1	0.396	4.157	0.078	0.001	0.253
Fumari vs NRC103	1	0.350	3.690	0.071	0.001	0.253
Fumari vs NRC200	1	0.300	3.139	0.061	0.003	0.759
Fumari vs NRC201	1	0.795	8.537	0.148	0.001	0.253
Fumari vs NRC300	1	0.795	9.006	0.155	0.001	0.253
Fumari vs NRC301	1	0.825	9.204	0.158	0.001	0.253
Fumari vs NRC400	1	0.912	9.508	0.163	0.001	0.253
Fumari vs NRC401	1	1.119	12.132	0.198	0.001	0.253
Fumari vs NRC404	1	0.859	9.237	0.159	0.001	0.253
Fumari vs NRC501	1	0.920	10.080	0.171	0.001	0.253
Fumari vs NRC600	1	0.964	10.700	0.179	0.001	0.253
Fumari vs NRC601	1	0.369	4.022	0.082	0.003	0.759
Cheyenne Full Flavor vs Cheyenne Menthol Box	**1**	**0.534**	**2.872**	**0.039**	**0.002**	0.506
Cheyenne Full Flavor vs Swisher Sweets Cherry	1	0.846	4.877	0.078	0.001	0.253
Cheyenne Full Flavor vs Swisher Sweets Original	1	0.546	2.959	0.054	0.003	0.759
Cheyenne Full Flavor vs NRC102	1	0.442	2.228	0.053	0.015	1
Cheyenne Full Flavor vs NRC103	1	0.405	2.018	0.049	0.047	1
Cheyenne Full Flavor vs NRC200	1	0.288	1.429	0.035	0.138	1
Cheyenne Full Flavor vs NRC201	1	0.467	2.383	0.056	0.019	1
Cheyenne Full Flavor vs NRC300	1	0.523	2.753	0.064	0.02	1

Cheyenne Full Flavor vs NRC301	1	0.517	2.701	0.063	0.014	1
Cheyenne Full Flavor vs NRC400	1	0.521	2.612	0.061	0.011	1
Cheyenne Full Flavor vs NRC401	1	0.675	3.462	0.080	0.004	1
Cheyenne Full Flavor vs NRC404	1	0.501	2.561	0.060	0.015	1
Cheyenne Full Flavor vs NRC501	1	0.570	2.944	0.069	0.011	1
Cheyenne Full Flavor vs NRC600	1	0.614	3.197	0.074	0.009	1
Cheyenne Full Flavor vs NRC601	1	0.249	1.211	0.033	0.188	1
Cheyenne Menthol Box vs Swisher Sweets Cherry	1	0.754	5.213	0.082	0.001	0.253
Cheyenne Menthol Box vs Swisher Sweets Original	1	0.324	2.127	0.039	0.024	1
Cheyenne Menthol Box vs NRC102	1	0.424	2.706	0.063	0.009	1
Cheyenne Menthol Box vs NRC103	1	0.394	2.499	0.060	0.018	1
Cheyenne Menthol Box vs NRC200	1	0.217	1.366	0.034	0.166	1
Cheyenne Menthol Box vs NRC201	1	0.343	2.224	0.053	0.021	1
Cheyenne Menthol Box vs NRC300	1	0.436	2.940	0.068	0.007	1
Cheyenne Menthol Box vs NRC301	1	0.468	3.122	0.072	0.005	1
Cheyenne Menthol Box vs NRC400	1	0.477	3.031	0.070	0.009	1
Cheyenne Menthol Box vs NRC401	1	0.659	4.308	0.097	0.002	0.506
Cheyenne Menthol Box vs NRC404	1	0.465	3.020	0.070	0.013	1
Cheyenne Menthol Box vs NRC501	1	0.503	3.313	0.076	0.003	0.759
Cheyenne Menthol Box vs NRC600	1	0.576	3.828	0.087	0.001	0.253
Cheyenne Menthol Box vs NRC601	1	0.242	1.521	0.041	0.072	1
Swisher Sweets Cherry vs Swisher Sweets Original	1	0.775	6.248	0.135	0.001	0.253
Swisher Sweets Cherry vs NRC102	1	0.131	1.112	0.038	0.302	1
Swisher Sweets Cherry vs NRC103	1	0.166	1.408	0.050	0.108	1
Swisher Sweets Cherry vs NRC200	1	0.140	1.174	0.042	0.258	1
Swisher Sweets Cherry vs NRC201	1	0.844	7.391	0.209	0.001	0.253
Swisher Sweets Cherry vs NRC300	1	0.859	8.127	0.225	0.001	0.253
Swisher Sweets Cherry vs NRC301	1	0.890	8.236	0.227	0.001	0.253
Swisher Sweets Cherry vs NRC400	1	0.876	7.351	0.208	0.001	0.253

Swisher Sweets Cherry vs NRC401	1	1.087	9.649	0.256	0.001	0.253
Swisher Sweets Cherry vs NRC404	1	0.831	7.293	0.207	0.001	0.253
Swisher Sweets Cherry vs NRC501	1	0.981	8.838	0.240	0.001	0.253
Swisher Sweets Cherry vs NRC600	1	0.980	8.996	0.243	0.001	0.253
Swisher Sweets Cherry vs NRC601	1	0.408	3.540	0.129	0.003	0.759
Swisher Sweets Original vs NRC102	1	0.487	3.771	0.146	0.003	0.759
Swisher Sweets Original vs NRC103	1	0.434	3.346	0.137	0.005	1
Swisher Sweets Original vs NRC200	1	0.291	2.211	0.095	0.017	1
Swisher Sweets Original vs NRC201	1	0.305	2.449	0.100	0.009	1
Swisher Sweets Original vs NRC300	1	0.296	2.604	0.106	0.013	1
Swisher Sweets Original vs NRC301	1	0.316	2.710	0.110	0.005	1
Swisher Sweets Original vs NRC400	1	0.464	3.556	0.139	0.002	0.506
Swisher Sweets Original vs NRC401	1	0.607	4.954	0.184	0.001	0.253
Swisher Sweets Original vs NRC404	1	0.385	3.100	0.124	0.001	0.253
Swisher Sweets Original vs NRC501	1	0.425	3.537	0.139	0.003	0.759
Swisher Sweets Original vs NRC600	1	0.478	4.067	0.156	0.001	0.253
Swisher Sweets Original vs NRC601	1	0.241	1.882	0.095	0.044	1
NRC102 vs NRC103	**1**	**0.125**	**1.058**	**0.105**	**0.432**	1
NRC102 vs NRC200	1	0.169	1.380	0.133	0.093	1
NRC102 vs NRC201	1	0.654	6.073	0.378	0.003	0.759
NRC102 vs NRC300	1	0.673	8.010	0.445	0.002	0.506
NRC102 vs NRC301	1	0.662	7.325	0.423	0.004	1
NRC102 vs NRC400	1	0.623	5.127	0.339	0.005	1
NRC102 vs NRC401	1	0.778	7.515	0.429	0.002	0.506
NRC102 vs NRC404	1	0.611	5.711	0.364	0.002	0.506
NRC102 vs NRC501	1	0.702	7.121	0.416	0.004	1
NRC102 vs NRC600	1	0.750	8.068	0.447	0.004	1
NRC102 vs NRC601	1	0.389	3.620	0.376	0.04	1
NRC103 vs NRC200	1	0.147	1.197	0.130	0.243	1

NRC103 vs NRC201	1	0.586	5.508	0.380	0.005	1
NRC103 vs NRC300	1	0.575	7.179	0.444	0.005	1
NRC103 vs NRC301	1	0.616	7.056	0.439	0.003	0.759
NRC103 vs NRC400	1	0.633	5.198	0.366	0.006	1
NRC103 vs NRC401	1	0.756	7.427	0.452	0.004	1
NRC103 vs NRC404	1	0.599	5.665	0.386	0.003	0.759
NRC103 vs NRC501	1	0.641	6.652	0.425	0.002	0.506
NRC103 vs NRC600	1	0.674	7.479	0.454	0.004	1
NRC103 vs NRC601	1	0.364	3.457	0.409	0.049	1
NRC200 vs NRC201	**1**	**0.379**	**3.421**	**0.275**	**0.01**	1
NRC200 vs NRC300	1	0.382	4.528	0.335	0.003	0.759
NRC200 vs NRC301	1	0.430	4.700	0.343	0.004	1
NRC200 vs NRC400	1	0.462	3.668	0.290	0.004	1
NRC200 vs NRC401	1	0.570	5.373	0.374	0.001	0.253
NRC200 vs NRC404	1	0.413	3.757	0.294	0.003	0.759
NRC200 vs NRC501	1	0.469	4.657	0.341	0.007	1
NRC200 vs NRC600	1	0.491	5.211	0.367	0.003	0.759
NRC200 vs NRC601	1	0.261	2.314	0.316	0.097	1
NRC201 vs NRC300	1	0.198	2.696	0.212	0.007	1
NRC201 vs NRC301	1	0.213	2.667	0.211	0.021	1
NRC201 vs NRC400	1	0.353	3.180	0.241	0.002	0.506
NRC201 vs NRC401	1	0.380	4.088	0.290	0.007	1
NRC201 vs NRC404	1	0.289	2.992	0.230	0.011	1
NRC201 vs NRC501	1	0.288	3.264	0.246	0.003	0.759
NRC201 vs NRC600	1	0.275	3.340	0.250	0.004	1
NRC201 vs NRC601	1	0.151	1.683	0.219	0.101	1
NRC300 vs NRC301	**1**	**0.062**	**1.100**	**0.099**	**0.39**	1
NRC300 vs NRC400	1	0.372	4.268	0.299	0.003	0.759
NRC300 vs NRC401	1	0.390	5.632	0.360	0.005	1

NRC300 vs NRC404	1	0.241	3.311	0.249	0.013	1
NRC300 vs NRC501	1	0.177	2.744	0.215	0.007	1
NRC300 vs NRC600	1	0.210	3.578	0.264	0.003	0.759
NRC300 vs NRC601	1	0.128	2.527	0.296	0.073	1
NRC301 vs NRC400	1	0.263	2.803	0.219	0.011	1
NRC301 vs NRC401	1	0.275	3.635	0.267	0.008	1
NRC301 vs NRC404	1	0.167	2.110	0.174	0.089	1
NRC301 vs NRC501	1	0.120	1.696	0.145	0.119	1
NRC301 vs NRC600	1	0.157	2.415	0.195	0.013	1
NRC301 vs NRC601	1	0.090	1.474	0.197	0.14	1
NRC400 vs NRC401	**1**	**0.087**	**0.818**	**0.076**	**0.691**	1
NRC400 vs NRC404	1	0.126	1.140	0.102	0.273	1
NRC400 vs NRC501	1	0.132	1.294	0.115	0.196	1
NRC400 vs NRC600	1	0.223	2.314	0.188	0.002	0.506
NRC400 vs NRC601	1	0.102	0.903	0.131	0.382	1
NRC401 vs NRC404	1	0.140	1.521	0.132	0.18	1
NRC401 vs NRC501	1	0.154	1.834	0.155	0.057	1
NRC401 vs NRC600	1	0.223	2.845	0.222	0.013	1
NRC401 vs NRC601	1	0.111	1.334	0.182	0.195	1
NRC404 vs NRC501	**1**	**0.163**	**1.865**	**0.157**	**0.045**	1
NRC404 vs NRC600	1	0.172	2.109	0.174	0.022	1
NRC404 vs NRC601	1	0.095	1.075	0.152	0.373	1
NRC501 vs NRC600	1	0.073	1.000	0.091	0.443	1
NRC501 vs NRC601	1	0.061	0.817	0.120	0.517	1
NRC600 vs NRC601	**1**	**0.054**	**0.827**	**0.121**	**0.684**	1

4. Table S 3.3: Relative abundance of identified bacterial species (<10%) present in BrdU treated tobacco samples.

Bacterial species (OTU ID)	Tobacco brand	Average relative abundance	SD
Acinetobacter johnsonii (125)	MR	0.000190949	0.000270043
Acinetobacter johnsonii (125)	SSC	0.000564975	0.00074316
Acinetobacter johnsonii (125)	AFMF	0.001597052	0.002258572
Acinetobacter lwoffii (131)	SSC	6.06E-05	8.57E-05
Acinetobacter lwoffii (131)	AFMF	0.00036855	0.000521209
Acinetobacter rhizosphaerae (204)	NNG	3.07E-05	4.34E-05
Aggregatibacter segnis (1260)	NNG	7.71E-05	0.000109088
Aggregatibacter segnis (346)	AFWF	9.56E-05	0.000135215
Aggregatibacter segnis (346)	FAMB	0.00010005	0.000141492
Aggregatibacter segnis (346)	NNG	0.000809935	0.001145421
Alcaligenes faecalis (90)	MMG	0.000131415	0.000185848
Arthrobacter psychrolactophilus (989)	SSO	6.96E-05	9.85E-05
Atopobium vaginae (88)	MMG	9.43E-05	6.00E-06
Atopobium vaginae (88)	SSO	0.000111861	3.87E-05
Atopobium vaginae (88)	SSC	0.000179028	8.18E-05
Atopobium vaginae (88)	NNG	0.000215604	0.000131441
Atopobium vaginae (88)	FMCC	0.000215816	3.56E-05
Atopobium vaginae (88)	AFWF	0.000309401	0.000167129
Atopobium vaginae (88)	CFF	0.000348128	2.76E-05
Atopobium vaginae (88)	FAMB	0.000389524	0.000126394
Atopobium vaginae (88)	AFMF	0.000419203	0.000102103
Atopobium vaginae (88)	AFTA	0.000492247	0.000696143
Atopobium vaginae (88)	MR	0.001424378	0.001744332
Atopobium vaginae (88)	FWGB	0.001693002	0.002394267
Atopobium vaginae (88)	CMB	0.014110647	0.019741755

Bacillus badius (147)	CFF	2.05E-05	2.90E-05
Bacillus badius (147)	AFWF	0.000143417	0.000202822
Bacillus badius (147)	AFMF	0.000208203	0.000294444
Bacillus badius (147)	AFTA	0.000253635	0.000358695
Bacillus cereus (66)	CFF	8.22E-05	0.000116181
Bacillus cereus (66)	FAMB	0.000460062	0.00048131
Bacillus cereus (66)	FMCC	0.000471083	5.28E-05
Bacillus cereus (66)	NMB	0.000523834	0.000740814
Bacillus cereus (66)	AFWF	0.001141992	0.000803731
Bacillus cereus (66)	FWGB	0.002056504	0.000514069
Bacillus cereus (66)	AFTA	0.002153103	0.001131908
Bacillus cereus (66)	AFMF	0.002309811	0.001598051
Bacillus cereus (66)	CMB	0.030202357	0.041644015
Bacillus clausii (16)	MR	4.77E-05	6.75E-05
Bacillus clausii (16)	CMB	5.04E-05	7.12E-05
Bacillus clausii (16)	SSC	9.87E-05	0.00013959
Bacillus clausii (16)	NNG	9.99E-05	3.22E-05
Bacillus clausii (16)	CFF	0.00011245	0.000100938
Bacillus clausii (16)	SSO	0.00017407	0.000246173
Bacillus clausii (16)	MMG	0.000379107	0.000164441
Bacillus clausii (16)	NMB	0.000868251	0.000980954
Bacillus coagulans (39)	MMG	0.000235511	0.000240138
Bacillus coagulans (39)	MR	0.000570912	0.000132285
Bacillus endophyticus (626)	SSC	0.000177669	0.000251262
Bacillus flexus (31)	MMG	1.64E-05	2.32E-05
Bacillus flexus (31)	AFMF	0.000595502	0.000547723
Bacillus flexus (31)	AFTA	0.001514301	0.001445402
Brevibacterium aureum (20)	NNG	3.86E-05	5.45E-05
Brevibacterium aureum (20)	AFWF	5.34E-05	7.56E-05

Brevibacterium aureum (20)	SSC	6.01E-05	2.92E-05
Brevibacterium aureum (20)	CMB	0.000176305	0.000249332
Brevibacterium aureum (20)	SSO	0.0002534	0.000358361
Brevibacterium aureum (20)	MMG	0.000278701	0.00011537
Brevundimonas diminuta (1184)	MMG	2.25E-05	3.18E-05
Brevundimonas diminuta (1184)	MR	0.000381898	0.000540085
Brevundimonas diminuta (91)	MMG	0.000292727	0.000413978
Clostridium perfringens (871)	AFMF	0.00012285	0.000173736
Corynebacterium durum (1040)	FAMB	5.99E-05	8.47E-05
Corynebacterium kroppenstedtii (281)	FMCC	9.64E-05	0.000136323
Corynebacterium kroppenstedtii (281)	SSO	0.000278513	0.000393876
Corynebacterium kroppenstedtii (281)	MR	0.000332226	0.000469838
Corynebacterium kroppenstedtii (281)	AFTA	0.000369185	0.000522107
Corynebacterium stationis (22)	CFF	4.60E-05	6.50E-05
Corynebacterium stationis (22)	SSC	6.06E-05	8.57E-05
Corynebacterium stationis (22)	MR	0.000332226	0.000469838
Erwinia dispersa (23)	FAMB	5.00E-05	7.07E-05
Erwinia dispersa (23)	CMB	7.56E-05	0.000106857
Erwinia dispersa (23)	MMG	0.000219639	0.000246928
Erwinia dispersa (23)	CFF	0.000251337	0.000225461
Erwinia dispersa (23)	SSC	0.000259803	3.24E-05
Erwinia dispersa (23)	SSO	0.000265956	1.78E-05
Erwinia dispersa (23)	NNG	0.000500442	0.000274057
Erwinia dispersa (23)	MR	0.002575877	0.002703163
Erwinia dispersa (23)	NMB	0.002630184	0.001250262
Flavobacterium succinicans (551)	AFMF	0.00012285	0.000173736
Flavobacterium succinicans (551)	NMB	0.000523834	0.000740814
Flavobacterium succinicans (653)	MMG	9.01E-05	0.000127378
Gracilibacillus dipsosauri (95)	SSC	1.97E-05	2.79E-05

Haemophilus parainfluenzae (115)	FMCC	2.41E-05	3.41E-05
Haemophilus parainfluenzae (115)	MMG	4.50E-05	6.37E-05
Haemophilus parainfluenzae (115)	AFMF	6.94E-05	9.81E-05
Haemophilus parainfluenzae (115)	NMB	8.73E-05	0.000123469
Haemophilus parainfluenzae (115)	FAMB	0.000129981	9.92E-05
Haemophilus parainfluenzae (115)	SSO	0.000146676	8.80E-05
Haemophilus parainfluenzae (115)	SSC	0.000632165	0.000836899
Haemophilus parainfluenzae (115)	NNG	0.001694502	0.0022873
Haemophilus parainfluenzae (115)	CMB	0.001754386	0.002481076
Haererehalobacter salaria (341)	SSC	0.000178122	0.000194785
Jeotgalicoccus psychrophilus (111)	SSC	0.000201939	0.000285584
Lactobacillus agilis (518)	MMG	4.93E-05	6.97E-05
Lactobacillus coleohominis (1196)	CFF	2.05E-05	2.90E-05
Lactobacillus iners (26)	MMG	0.000327981	4.57E-05
Lactobacillus iners (26)	NNG	0.000523205	0.000132779
Lactobacillus iners (26)	SSO	0.000524493	0.000144477
Lactobacillus iners (26)	CFF	0.000690358	0.000453498
Lactobacillus iners (26)	SSC	0.000757404	0.000157261
Lactobacillus iners (26)	FAMB	0.000929124	4.06E-05
Lactobacillus iners (26)	FMCC	0.000967336	0.000199694
Lactobacillus iners (26)	NMB	0.001433335	0.001286227
Lactobacillus iners (26)	AFWF	0.001726944	0.000278828
Lactobacillus iners (26)	AFMF	0.00178371	0.00095218
Lactobacillus iners (26)	AFTA	0.003037071	0.00022986
Lactobacillus iners (26)	MR	0.00408412	0.004560626
Lactobacillus iners (26)	FWGB	0.004448285	0.002488153
Lactobacillus iners (26)	CMB	0.028447971	0.039162939
Lactobacillus reuteri (417)	NNG	3.07E-05	4.34E-05
Lactobacillus reuteri (417)	SSO	4.22E-05	5.97E-05

Lactobacillus reuteri (417)	MMG	6.76E-05	9.55E-05
Lactobacillus reuteri (417)	AFMF	6.94E-05	9.81E-05
Lactobacillus reuteri (417)	FAMB	0.00010005	0.000141492
Lactobacillus reuteri (417)	FWGB	0.000268889	0.000380267
Lactobacillus reuteri (417)	NMB	0.000369848	2.92E-05
Lactobacillus salivarius (124)	SSO	4.22E-05	5.97E-05
Methylobacterium adhaesivum (46)	NNG	3.86E-05	5.45E-05
Methylobacterium adhaesivum (46)	FAMB	5.00E-05	7.07E-05
Methylobacterium adhaesivum (46)	FWGB	0.000134445	0.000190134
Methylobacterium adhaesivum (46)	AFMF	0.000242904	0.000343518
Methylotenera mobilis (1030)	CMB	2.52E-05	3.56E-05
Methylotenera mobilis (1030)	FAMB	5.00E-05	7.07E-05
Methylotenera mobilis (1030)	AFTA	0.000123062	0.000174036
Methylotenera mobilis (1030)	AFMF	0.0004914	0.000694945
Microbispora rosea (136)	SSO	3.48E-05	4.92E-05
Microbispora rosea (136)	CFF	4.60E-05	6.50E-05
Microbispora rosea (136)	FMCC	6.35E-05	8.99E-05
Morganella morganii (1032)	AFMF	3.47E-05	4.91E-05
Morganella morganii (1032)	CMB	0.003508772	0.004962153
Oceanobacillus caeni (721)	NMB	0.000195236	0.000276106
Oceanobacillus caeni (721)	CFF	0.001332721	0.001884752
Oceanobacillus caeni (721)	SSC	0.009789928	0.000891102
Paenibacillus chondroitinus (374)	SSO	3.48E-05	4.92E-05
Paenibacillus chondroitinus (374)	FMCC	4.82E-05	6.82E-05
Paenibacillus lautus (252)	CFF	4.11E-05	5.81E-05
Pediococcus acidilactici (133)	MMG	0.000202657	0.0002866
Peredibacter starrii (866)	FMCC	8.00E-05	2.32E-05
Peredibacter starrii (866)	FAMB	0.00010005	0.000141492
Peredibacter starrii (866)	AFMF	0.00012285	0.000173736

Peredibacter starrii (866)	AFTA	0.000123062	0.000174036
Prevotella melaninogenica (339)	MMG	2.25E-05	3.18E-05
Prevotella melaninogenica (339)	SSC	3.99E-05	6.40E-07
Prevotella melaninogenica (339)	CFF	4.11E-05	5.81E-05
Prevotella melaninogenica (339)	FWGB	0.000268889	0.000380267
Prevotella melaninogenica (339)	CMB	0.001754386	0.002481076
Prevotella melaninogenica (437)	NNG	3.86E-05	5.45E-05
Prevotella melaninogenica (437)	MMG	5.54E-05	1.46E-05
Prevotella melaninogenica (437)	SSO	6.96E-05	9.85E-05
Prevotella melaninogenica (437)	AFTA	0.000123062	0.000174036
Prevotella melaninogenica (437)	FWGB	0.000134445	0.000190134
Prevotella nanceiensis (992)	SSC	2.02E-05	2.86E-05
Prevotella nanceiensis (992)	MR	0.000332226	0.000469838
Prevotella nigrescens (573)	FAMB	0.000350175	0.000495222
Prevotella pallens (1087)	CFF	4.60E-05	6.50E-05
Propionibacterium acnes (24)	FAMB	0.000678999	0.000394281
Propionibacterium acnes (24)	CFF	0.000720655	0.000670617
Propionibacterium acnes (24)	SSC	0.001031513	0.000830495
Propionibacterium acnes (24)	AFTA	0.002329705	0.000186013
Propionibacterium acnes (24)	NNG	0.002352056	0.000490028
Propionibacterium acnes (24)	FMCC	0.00270493	0.000758084
Propionibacterium acnes (24)	SSO	0.002768569	0.000862805
Propionibacterium acnes (24)	MMG	0.00361424	0.003996216
Propionibacterium acnes (24)	FWGB	0.003654894	0.004408266
Propionibacterium acnes (24)	CMB	0.003685077	0.00471282
Propionibacterium acnes (24)	AFMF	0.006072594	0.008587944
Propionibacterium acnes (24)	MR	0.008430145	0.002525258
Propionibacterium acnes (24)	NMB	0.009128649	0.004760907
Propionibacterium acnes (24)	AFWF	0.011631634	0.009646878

Propionibacterium granulosum (256)	AFWF	9.56E-05	0.000135215
Propionibacterium granulosum (256)	MMG	0.000472866	0.000668734
Pseudomonas pseudoalcaligenes (116)	SSC	2.02E-05	2.86E-05
Pseudomonas pseudoalcaligenes (116)	FAMB	2.99E-05	4.23E-05
Pseudomonas pseudoalcaligenes (116)	AFMF	3.47E-05	4.91E-05
Pseudomonas pseudoalcaligenes (116)	MR	4.77E-05	6.75E-05
Pseudomonas pseudoalcaligenes (116)	NNG	6.13E-05	8.67E-05
Pseudomonas pseudoalcaligenes (116)	NMB	8.73E-05	0.000123469
Pseudomonas pseudoalcaligenes (116)	AFWF	0.000101253	7.98E-06
Pseudomonas pseudoalcaligenes (116)	AFTA	0.00016909	0.00023913
Pseudomonas pseudoalcaligenes (116)	FWGB	0.000282167	0.000399044
Pseudomonas stutzeri (68)	SSC	4.04E-05	5.71E-05
Pseudomonas stutzeri (68)	MMG	4.50E-05	6.37E-05
Pseudomonas viridiflava (81)	SSC	8.08E-05	0.000114234
Pseudomonas viridiflava (81)	NNG	0.000183993	0.000260205
Pseudomonas viridiflava (81)	NMB	0.000585709	0.000828317
Rothia aeria (294)	AFTA	0.00016909	0.00023913
Rothia aeria (294)	FAMB	0.000199681	0.000140899
Rothia aeria (294)	FWGB	0.000846501	0.001197133
Rothia aeria (294)	NNG	0.001048302	0.001135583
Rothia mucilaginosa (104)	FMCC	4.82E-05	6.82E-05
Rothia mucilaginosa (104)	AFWF	5.34E-05	7.56E-05
Rothia mucilaginosa (104)	AFTA	8.45E-05	0.000119565
Rothia mucilaginosa (104)	FAMB	8.98E-05	0.000126987
Rothia mucilaginosa (104)	NNG	0.000215604	0.000131441
Rothia mucilaginosa (104)	SSC	0.000221227	0.00020119
Rothia mucilaginosa (104)	MMG	0.000256183	8.35E-05
Rothia mucilaginosa (104)	CFF	0.000276755	0.000131424
Rothia mucilaginosa (104)	SSO	0.000278513	0.000393876

Rothia mucilaginosa (104)	AFMF	0.000280401	0.000298399
Rothia mucilaginosa (104)	FWGB	0.000416612	0.000208911
Rothia mucilaginosa (104)	NMB	0.000544459	0.00021777
Rothia mucilaginosa (104)	MR	0.000950875	0.000534613
Saccharibacillus kuerlensis (80)	NMB	0.001628571	0.001562333
Salana multivorans (103)	MMG	4.50E-05	6.37E-05
Sphingobacterium faecium (651)	MR	0.000190949	0.000270043
Sphingobacterium multivorum (1229)	MMG	1.64E-05	2.32E-05
Staphylococcus sciuri (722)	AFWF	5.34E-05	7.56E-05
Staphylococcus sciuri (722)	SSC	5.97E-05	2.73E-05
Streptococcus agalactiae (145)	CFF	2.05E-05	2.90E-05
Streptococcus agalactiae (145)	AFWF	5.34E-05	7.56E-05
Streptococcus agalactiae (145)	FAMB	5.99E-05	8.47E-05
Streptococcus agalactiae (145)	FMCC	6.35E-05	8.99E-05
Streptococcus agalactiae (145)	NNG	7.71E-05	0.000109088
Streptococcus agalactiae (145)	SSC	7.90E-05	0.000111672
Streptococcus agalactiae (145)	FWGB	0.000282167	0.000399044
Streptococcus agalactiae (145)	MR	0.000664452	0.000939677
Streptococcus anginosus (420)	SSC	1.97E-05	2.79E-05
Streptococcus anginosus (420)	AFMF	3.47E-05	4.91E-05
Streptococcus anginosus (420)	AFWF	0.000143417	0.000202822
Tetragenococcus halophilus (138)	SSC	0.000201939	0.000285584
Tetrathiobacter kashmirensis (549)	MMG	0.000164268	0.000232311
Tuberibacillus calidus (396)	CMB	2.52E-05	3.56E-05
Veillonella dispar (99)	AFWF	5.34E-05	7.56E-05
Veillonella dispar (99)	FMCC	6.35E-05	8.99E-05
Veillonella dispar (99)	CMB	7.56E-05	0.000106857
Veillonella dispar (99)	CFF	9.19E-05	0.000129983
Veillonella dispar (99)	NNG	9.20E-05	0.000130102

Veillonella dispar (99)	SSC	0.000159739	2.56E-06
Veillonella dispar (99)	AFTA	0.00016909	0.00023913
Veillonella dispar (99)	SSO	0.000208885	0.000295407
Veillonella dispar (99)	MMG	0.000233666	5.17E-05
Veillonella dispar (99)	AFMF	0.0002457	0.000347473
Veillonella dispar (99)	FWGB	0.000282167	0.000399044
Veillonella dispar (99)	NMB	0.000369848	2.92E-05
Veillonella dispar (99)	FAMB	0.000579368	0.000111889
Veillonella dispar (99)	MR	0.001804341	0.00214666

Chapter 4: Single use of a little cigar does not result in transient changes to the oral microbiome

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4.1 Abstract

Tobacco products are contaminated with a myriad of bacterial communities. Long-term tobacco use has been shown to alter the oral microbiome of the user, potentially leading to oral disease development. However, to our knowledge, there exists no data on the potential transfer of tobacco-related bacteria to the oral cavity or the transient impacts of using a specific tobacco product on a user's oral microbiome. To address these knowledge gaps, buccal swab and saliva samples were collected from forty cigarette smokers both before and after a single use of a little cigar product (flavored or non-flavored) on two separate visits. After total genomic DNA extractions of the samples (n=320), the V3V4 region of the 16S rRNA gene was amplified via PCR and sequenced on the Illumina HiSeq. Oral bacterial alpha and beta diversity were not significantly different between pre- and post-smoking samples among both buccal swab and saliva samples on both visits. However, post-smoking buccal swab and saliva samples were predicted to be associated with a higher relative abundance of the opportunistic pathogens *Delftia, Leptotrichia, Pseudomonas* and *Stenotrophomonas* when compared to the pre-smoking samples. Nevertheless, our overall data provide further evidence of temporal stability of the oral microbiome. In conclusion, our data demonstrate that a single use of a little cigar product among a group of cigarette smokers does not result in transient changes to the oral microbiome.

4.2 Introduction

Tobacco products have been shown to harbor a myriad of bacterial communities [7,8,11,12,63,169]. A recent study from our group also demonstrated for the first time that viable bacterial genera (e.g., *Bacillus, Paenibacillus* and *Terribacillus*) originating from cigarette tobacco can be aerosolized in mainstream cigarette smoke [16]. The oral cavity is the first to encounter this mainstream smoke and hence, has the greatest potential to be affected by it. While the long term use of tobacco products has been well established to cause dysbiosis (community disturbance) in the oral microbiome of users [18,19,78,107,114], the potential transfer of tobacco-related bacteria to the oral cavity or the immediate impacts of using a specific tobacco product on a user's oral microbiome have not been evaluated. This is of significant concern since changes in bacterial diversity and community composition of the oral microbiome can potentially initiate inflammation and subsequent disease development, including the growth of malignant lesions in the mouth [24,241,242].

These types of changes in the mouth can occur in users of diverse tobacco products from cigarettes to little cigars. Little cigars are comparable to traditional cigarettes in size and shape but contain more tobacco (100-200mg) and other chemical compounds [243,244]. In comparison to cigarettes, little cigars also have a greater puff volume and puff duration, delivering more carbon monoxide, tobacco-specific nitrosamines (TSNAs), and benzo(a)pyrene to users than cigarettes [39,244,245], consequently causing greater cytotoxicity and inflammation [243]. Similar to cigarettes, the tobacco of little cigars has been shown to harbor diverse bacterial communities [9,63]. For example, Smyth et al. (2019) demonstrated that the predominant bacterial genera within little cigar tobacco are *Pantoea, Pseudomonas* and *Staphylococcus*, including multiple bacterial pathogens such as *Staphylococcus sciuri* and *Pseudomonas pseudoalcaligenes* [9]. Nevertheless, to our knowledge

there are no data concerning whether or not the bacterial communities within little cigars could be transferred to the oral cavity of smokers and effect changes in the oral microbiome.

Oral microbiome dysbiosis from extrinsic perturbations such as tobacco smoking is dependent on the amount and frequency of products smoked/used per day [246]; however, the overall composition of the oral microbiome has been shown to remain relatively stable over time [83,137,138]. Yet, to date, studies evaluating temporal changes in the oral microbiome [135,247–249] have not employed the use of tobacco products by study subjects, and hence, data on potential transient oral microbiome shifts among smokers are limited.

Other studies characterizing oral microbiome dysbiosis associated with tobacco smoking have focused on the use of traditional and/or electronic cigarettes [78,132,150,246,247,250] and indicated that specific oral bacterial genera are impacted by exposures to smoke over a one week period [107]. For example, greater shifts in bacterial communities among smokers' plaque samples were demonstrated when compared to that of non-smokers over 7 days, with the enrichment of a pathogen-dominated bacterial community (e.g., *Fusobacterium nucleatum, Acinetobacter johnsonii, A. baumannii, Streptococcus mutans*) among smokers [107]. Moreover, while levels of predominant genera such as *Streptococcus, Neisseria*, and *Veillonella* were stable over seven days among non-smokers, less stability was observed among smokers during the same time period, while the colonization of pathogens associated with periodontitis occurred within 24 hours of biofilm development [107]. However, as noted above, to our knowledge, no study has evaluated the immediate changes in the oral microbiome after a single use of a little cigar. Therefore, the objective of our study was to evaluate the transient changes in the oral microbiome after cigarette users smoked a single little cigar product on two different occasions.

4.3 Methods

4.3.1 Product selection

Swisher Sweets little cigars were chosen for inclusion in the study because they are characterized by the highest market sales among little cigar brands in the U.S. (CDC 2015; Delnevo 2017). Two products (Swisher Sweets Original (SSORG) and Swisher Sweets Cherry (SSCHR)) were purchased in Columbus, OH and stored at 4°C in original packaging before being smoked by study participants.

4.3.2 Study population

Participants were recruited through word-of-mouth in Columbus, OH. Once an individual expressed interest in participating in the study, they completed a phone screening process to ensure that they met our inclusion criteria. All participants had to be healthy individuals that self-recognized as current smokers (smoked at least six tobacco products on a typical day for the previous three years). Inclusion criteria included generally good oral health, with no antibiotic use, no heart or lung problems and no diagnosis of pneumonia in the past six months. Exclusion criteria included untreated lesions or oral abscesses in the mouth, clinically diagnosed candidiasis or halitosis, a pregnancy or plans to become pregnant in the next six months. The study was approved by the Battelle, OH Institutional Review Board and all participants completed the informed consent process on their first visit.

4.3.3 Laboratory visits, smoking process, and sample collection

Upon recruitment, study participants visited the laboratory twice, with 24 hours to 35 days between visits. During their first visit, participants completed three questionnaires focused on demographics, tobacco product use, and oral health history. During each of the two visits, each participant provided pre-smoking (PRE) buccal swab and saliva samples then smoked one of two tobacco products (SSCHR or SSORG) and then provided post-smoking (POST) buccal swab and saliva samples.

For saliva samples, participants were asked to let saliva form in their mouth for at least one minute before collecting 2-5 ml in a 50 mL falcon tube. RNALater (3X volume) solution (Thermo Fisher, MA) was then added to the saliva sample, and the sample was vortexed and then incubated at 4°C for 24 hours. After incubation, all samples were frozen at -80°C until DNA extractions could be completed. Buccal swab samples were collected with four e-swabs (Copan, CA) from four oral sites: the tongue dorsum, the hard palate, and the left and right buccal mucosa. Surfaces were swabbed with e-swabs for 1 min, and all four e-swabs were added to a single 50 mL Falcon tube with 5 mL RNALater solution. Similar to the saliva samples, all of the buccal swab samples were vortexed and incubated at 4°C for 24 hours. Afterwards, samples were then frozen at -80°C until DNA extractions could be completed.

4.3.4 Total DNA extraction, 16S rRNA gene amplification and sequencing

All buccal swab and saliva samples were thawed on ice. To 500 µL of each saliva sample, 500 µL of ice-cold 1X molecular grade Phosphate Buffer Solution (PBS) was added. Both sample types (buccal swabs and saliva) were then centrifuged at 10,000 rpm for 30 mins.

Next, the supernatant was discarded and the pellet was resuspended in 1 mL of ice-cold 1X PBS, and then transferred into Lysing Matrix B tubes (MP Biomedicals, Solon, OH). DNA extraction was carried out following previously published protocols using enzymatic digestion and mechanical lysis of cells [6]. Briefly, samples were incubated twice in water baths with the addition of two enzymatic cocktails (Cocktail A: Mutanolysin, Lysostaphin, and Lysozyme; Cocktail B: Proteinase K and 10% sodium dodecyl sulfate). After mechanical lysis of cells using an MP Biomedical FastPrep 24 (Santa Ana, CA), DNA lysate was purified using a QIAmp DSP DNA mini kit (Qiagen, CA) following the manufacturer's protocol. PCR amplification of the V3V4 hypervariable region of the 16S rRNA gene was then carried out using 319F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) universal primers. Each primer was barcoded with a linker sequence and a 12-bp heterogeneity spacer index sequence. Amplicons were purified using the SequelPrep Normalization Kit (Invitrogen Inc. Carlsbad, CA), samples were pooled at a final concentration of 25 ng/amplicon and the pooled samples were sequenced on an Illumina HiSeq2500 (Illumina, San Diego, CA), using previously published protocols [187,188].

4.3.5 Sequence quality filtering and bioinformatic analysis

16S rRNA sequencing reads were screened for low quality and short length, assembled using PANDAseq, demultiplexed, and chimera trimmed using UCHIME. Quality reads were then incorporated into Quantitative Insights Into Microbial Ecology (QIIME v1.9.0) and clustered de-novo using VSEARCH. Taxonomies were then assigned using the Greengenes database (v. 132), using a 0.97 confidence threshold. The resulting operational taxonomic unit (OTU) table, reference sequences, and phylogenetic tree files were imported into R Statistical

computing software (v. 0.99.473) using the phyloseq R package (1.22.3) for downstream analysis.

The Phyloseq package [251] in R was used to calculate alpha diversity and results were tested for significance using ANOVA. Cumulative sum scaling (CSS) was used to normalize reads using the MetagenomeSeq (v. 1.16.0) package [252], and normalized reads were used to compute beta diversity using the vegan and Phyloseq packages in R and results were tested for significance using ANOSIM. Core microbiome profiling was performed at the genera level when the genus was prevalent in at least 20% of the samples at a relative abundance of 0.2 on Microbiome Analyst [192,193]. Decision trees were generated using the Random Forest algorithm to predict bacterial biomarker taxa associated with each time point (PRE and POST) for each sample type (buccal swabs and saliva).

4.3.6 Data availability

Data concerning the samples included in this study are deposited under the NCBI BioProject accession number PRJNA690810.

4.4 Results

4.4.1 Study participants

A total of forty participants were enrolled in the study with 40% identifying as female, and 47.5% identifying as Black (**Table 1**). The majority (55%) of the participants were single (or never married) and 58% were between 25 to 45 years old. All of the participants were currently cigarette smokers: 35.13% smoked Newport brands and 21.6% smoked Marlboro products, with

the majority (59.4%) smoking mentholated varieties of the cigarette brand that they used (**Table 2**). Over 90% of the participants had ever tried smoking a little cigar product, with 23.5% currently smoking a little cigar every day and 53% smoking little cigars on some days. Thirty-two percent of the subjects who had tried little cigars had smoked Swisher products, followed by 20.5% who had used a Black & Mild product.

4.4.2 Sequencing data

A total of 320 samples yielded 2,472,070 sequences, comprising 713 OTUs, with an average number of sequences per sample of 11,392.03 (+/- 8563.63 SD). After removing low-quality samples and samples with Good's coverage values ≤ 0.95, there were 2,469,617 sequences from 208 samples, with an average number of sequences per sample of 11873.16 (+/- 8421.07 SD), which were used for downstream analysis.

4.4.3 Transient changes in oral bacterial diversity

Alpha diversity was measured using the observed number of species metric and the Shannon index (**Figure 1**). There were no significant differences in alpha diversity ($p > 0.05$) between PRE and POST smoking samples for each of the tobacco products stratified by sample type (buccal swab and saliva) across all study participants. When we looked at participant-level alpha diversity, there were also no significant differences between PRE and POST samples for each participant during both visits (**Figure S1**).

To evaluate beta diversity between time points (PRE and POST), we performed Principal Coordinates Analysis (PCoA) on weighted UniFrac distance matrices of normalized OTU

relative abundances. (**Figure 2**). The first two principal axes explained 56.8% of the variation in bacterial community composition. Similar to the alpha diversity results, there were no statistically significant changes (ANOSIM $p > 0.05$) in bacterial diversity between PRE and POST buccal swab samples or PRE and POST saliva samples for both visits. Evaluating further at the participant level, we also compared the UniFrac distances for each subject for each sample. PRE and POST smoking samples (of each sample type) from a single participant were characterized by the lowest UniFrac distances and clustered closer together when compared to samples from other participants (data not shown).

4.4.4 Core microbiome and correlation between bacterial genera

The core oral microbiome in buccal swab and saliva samples was significantly dominated by five taxa: *Streptococcus, Veillonella, Rothia, Actinomyces*, *Granulicatella, Haemophilus* and *Prevotella* (**Figure S2**). Among all bacterial genera, *Streptococcus* was at the highest relative abundance in both buccal swab (49%) and saliva (38.2%) samples. While *Rothia* (16%) and *Veillonella* (12%) were the other two bacterial genera at >10% relative abundance in buccal swabs, *Veillonella* was at a 14.2% relative abundance in saliva samples. Other genera identified in both buccal swab and saliva samples included *Lactobacillus* (1.5% buccal swabs and 2.2% in saliva), *Pseudomonas* (1.2% in buccal swabs and 2.4% in saliva), *Stenotrophomonas* (1% in buccal swabs) and *Atopobium* (1.3% in saliva).

4.4.5 Biomarker bacterial taxa associated with a single smoking exposure

A Random Forest algorithm was applied to construct decision trees to predict the bacterial biomarker taxa best associated with PRE and POST samples for both buccal swab and saliva samples (**Figure 3)**. The POST buccal swab samples from both visits were enriched with four bacterial genera, *Delfia, Leptotrichia, Pseudomonas*, and *Stenotrophomonas*, when compared to the PRE samples (**Figure 3a and c**). The POST saliva samples were enriched with *Catonella* when compared to the PRE samples from both visits (**Figures 3b and d**). After a single exposure to SSCHR, both buccal swab and saliva POST samples were associated with a depletion of *Peptostreptococcus*, with enrichment of *Atopobium* (**Figures 3a and b**). After a single use of an SSORG product, the higher relative abundance of *Stenotrophomonas* and *Catonella* were associated with POST samples, compared to PRE samples for both buccal swab and saliva samples (**Figures 3c and d**).

4.4.6 Changes in the relative abundance of bacterial taxa with a single exposure to smoking

To evaluate the transient changes in the abundance of specific bacterial taxa, we compared the mean relative abundance of the top 20 bacterial OTUs between the PRE and POST smoking samples across buccal swab and saliva samples separately **(Figure 4)**. Three OTUs among the *Streptococcus* genera (OTU # 1, 102, and 2), *Veillonella dispar* (OTU# 3 and 25), *Rothia mucilaginosa* (OTU # 4 and 328), and *Prevotella melaninogenica* (OTU# 12 and 6) were identified among all samples. There were no statistically significant differences in the relative abundance of the top 20 bacterial taxa between PRE and POST samples when stratified by product smoked and sample type.

4.4.7 Differences in the oral microbiome within and between participants

To evaluate changes in the oral microbiome within participants (intra-individual variation), we compared the PRE samples per participant across sample types and time points (visits). There were no significant differences in bacterial community composition of PRE buccal swab and saliva samples across the two-time points for a single individual participant (**Table 3**). However, the UniFrac distances between bacterial community members within an individual were lower in the buccal swab samples compared to the saliva samples.

To evaluate bacterial diversity differences between individuals (inter-individual variation), we compared the PRE smoking samples from all 40 participants at each time point. Shannon diversity metrics among all participants were characterized by statistically significant inter-individual differences, among both buccal swab ($p = 0.0195$) and saliva samples ($p = 0.0004$) (**Figure S1**). Beta diversity was evaluated using weighted UniFrac distances between samples all pre-smoking samples (**Figure 5**). Across both sample types, while there were no significant differences on their first visit (buccal swabs ANOSIM R = -0.0799; $p > 0.05$; saliva ANOSIM R = 0.724; $p > 0.05$), there was significant variation in bacterial community composition across participants during their second visit (buccal swab ANOSIM R = 0.5004; $p < 0.05$; saliva ANOSIM R = 0.5003; $p < 0.05$). The inter-individual variation in bacterial genera was also different among the top ten most abundant bacterial genera present in the PRE smoking samples across all participants (**Table S1**).

4.5 Discussion

To our knowledge, this is the first study to show that the oral microbiome of cigarette smokers remains stable after exposure to the mainstream smoke of a single little cigar product. This may indicate that bacteria originating from little cigar products may not be immediately transferred to users' oral cavities or that, if these bacteria are transferred, they may be present at low levels (below our detection limit) immediately after smoking. Furthermore, our data provide a comprehensive characterization of the oral microbiome of cigarette smokers, as well as further evidence of the temporal stability of the oral microbiome.

Previous studies from our group that have evaluated bacterial communities present in the tobacco of little cigars have identified several bacterial genera (e.g., *Staphylococcus, Pseudomonas, Corynebacterium* and *Bacillus*) in Swisher sweets little cigars [9,63] . In the present study, the bacterial genera that were associated with our post-smoking samples were identified as *Delftia, Leptotrichia, Pseudomonas, Stenotrophomonas* and *Catonella*. While *Pseudomonas* was identified in little cigar tobacco products, the other above-mentioned bacterial species were not detected. This could suggest the transfer of a few bacterial species (and not others) from little cigar tobacco to the oral cavity but could also indicate that some bacteria (e.g., *Pseudomonas)* are present at a higher detectable abundance in oral samples, while other bacterial genera are not. While the quantity and duration of cigarette smoking has been shown to influence the periodontal pathogens found in smoker's oral cavity [253,254], relatively unstable colonization of biofilm occurs among smokers (when compared to non-smokers) at least within 24 hours, along with early colonization of periodontal pathogens (e.g., *Fusobacterium*, *Cardiobacterium* and *Selenomonas*) [107]. Therefore, in our study, a single exposure to little cigar smoke might not have been adequate to trigger these responses in smokers.

Looking into specific bacterial taxa, we found that the commensal *Streptococcus* was the most abundant genera among all participants, followed by *Rothia, Veillonella*, and *Actinomyces*. While the relative abundance of *Streptococcus, Veillonella* and *Actinomyces* were at a lower relative abundance in post-smoking samples (when compared to pre-smoking samples) during both visits, *Rothia* was characterized by a decrease in relative abundance only during visit 2. While a number of studies have pointed to the role of commensals in oral disease development [255,256], the identification of specific putative pathogens in oral disease initiation is still unclear [257]. A higher relative abundance of all of these genera has been shown previously in healthy smokers [22], defining the "core microbiome" of the oral community [91].

While we observed the above-mentioned commensals in all of our samples at a comparatively higher relative abundance (compared to other identified bacterial genera), we also identified a few opportunistic pathogens associated with the post-smoking samples (e.g., *Delftia, Leptotrichia, Stenotrophomonas, Pseudomonas and Atopobium*). Demonstrating antibiotic resistance to multiple groups of antibiotics, *Delftia* is an emerging member of the opportunistic healthcare-associated pathogens [258–260]. Even though species of *Delftia* are used as plant growth-promoters, a few species are also capable of transforming toxic selenium and chromium in polluted environments [261]. Another common oral cavity member, *Leptotrichia,* has been frequently implicated in periodontal diseases and tooth decay [262]. With production of potent endotoxin, *Leptotrichia* has been associated with a spectrum of human diseases [262,263]. *Stenotrophomonas* has been noted as a persistent colonizer of biofilms, but antibiotic resistance among some species of this bacteria enable them to play a major role in the development of infectious diseases, specifically among immunocompetent individuals [264,265]. Finally, *Atopobium* has been associated with periodontal diseases and gingival squamous cell carcinomas [266,267].

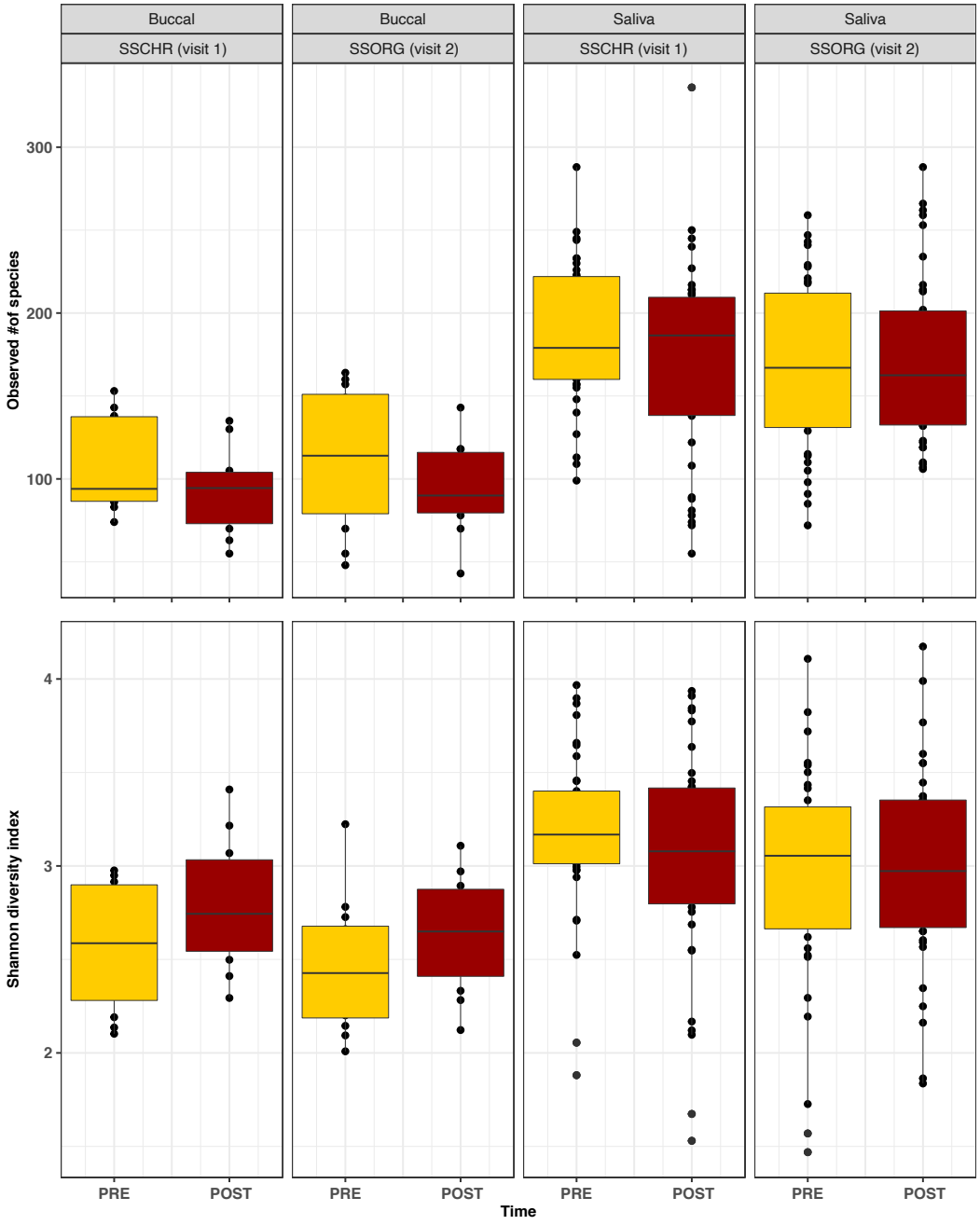
In addition to the commensal and pathogenic bacteria that were identified among the smokers' oral microbiomes, we also compared variations in bacterial community composition 1) across all subjects at one visit (inter-individual variation) and 2) within each subject across two visits (intra-individual variation). Comparing across individuals, we found significantly more variation in the oral microbiome in comparison to intra-individual variation. This was consistent with previous studies that show that oral microbiome composition is variable among participants, given the multiple intrinsic and extrinsic factors that can affect the oral microbiome of an individual [135,136,268,269]. We also found that the degree of temporal variability (measured with the Shannon diversity index per individual) in the composition of the bacterial communities was different across all individuals. This was consistent with previous microbiome studies of samples from other areas of the body such as the palm, gut, tongue and vagina [268,270]. In our study, although there was considerable variation between subjects, bacterial profiles within subjects (intra-individual) were stable over the study period. Previous studies have shown similar results when evaluating a range of time periods (24 hours to 10 months) [136,269].

Finally, there are multiple strengths to note in this study. To our knowledge, this study is the first of its kind to evaluate the possible transfer of tobacco-related bacteria to the oral cavity or the transient changes in the oral microbiome resulting from a single exposure to a little cigar. While previous studies have evaluated oral microbiome impacts from tobacco smoking [18,116,271,272], none involves specifically smoking a tobacco product during the study and evaluating the immediate impacts on the oral bacteria of the smoker. Our data also relied on high throughput 16S rRNA sequencing from a robust sample size, and sampling from two different oral cavity locations. Nevertheless, there were notable limitations to this study as well. First of all, we could not measure all of the possible factors that could contribute to individual-level

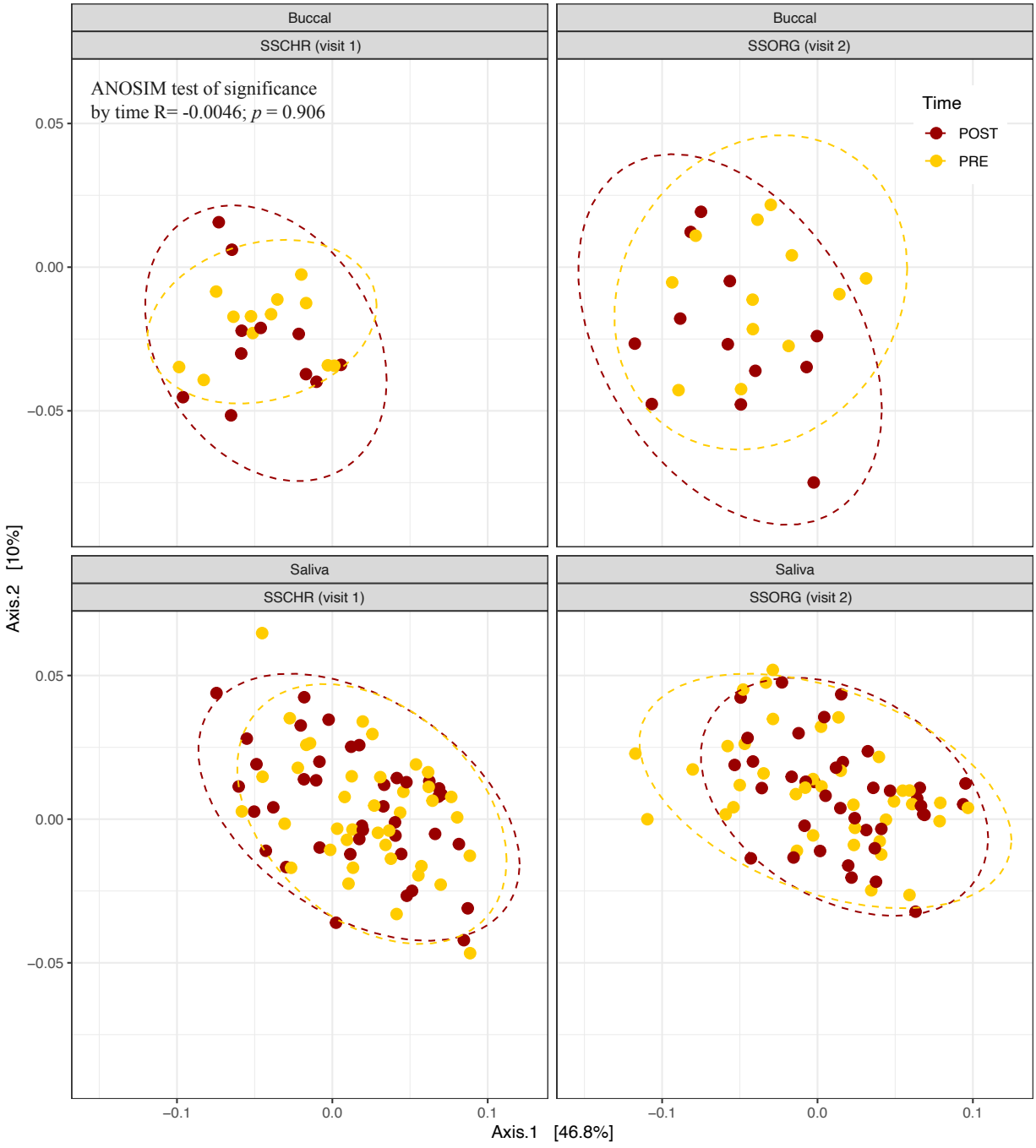
variations in the oral microbiome. Possible factors driving such variations could be an individual's genetic makeup, diet, or lifestyle/behavior [273,274]. Another limitation of our study was the inability to assess functional capabilities of the oral microbiome since we were not able to subject our samples to a metagenomic sequencing approach due to costs. Additionally, since our work only included data from 16S rRNA gene sequencing, this limited our ability to complete species-level taxonomic assignments.

In conclusion, we found that a single-use of a little cigar product did not result in transient changes to the oral microbiome of cigarette smokers. This may suggest that the heterogenous bacteria that are present in little cigars are not transferred immediately to a user's oral cavity or it could suggest that our approaches were not sensitive enough to detect this transfer. Bacteria that are potentially transferred from the mainstream smoke of little cigars to the oral cavity may require time to colonize mucosal surfaces before they can be detected. Future prospective studies that further evaluate these issues are necessary.

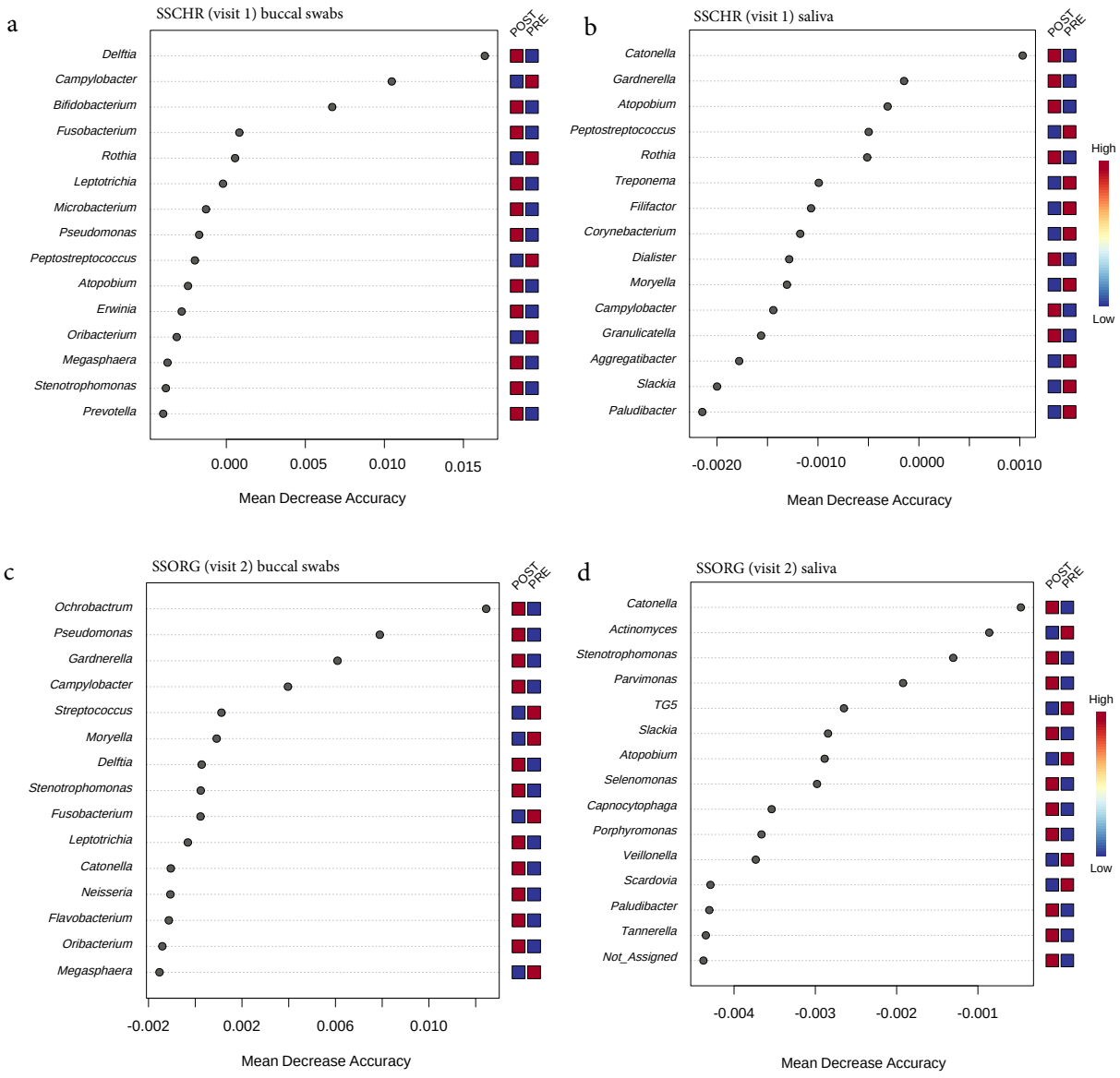
4.6 Figures



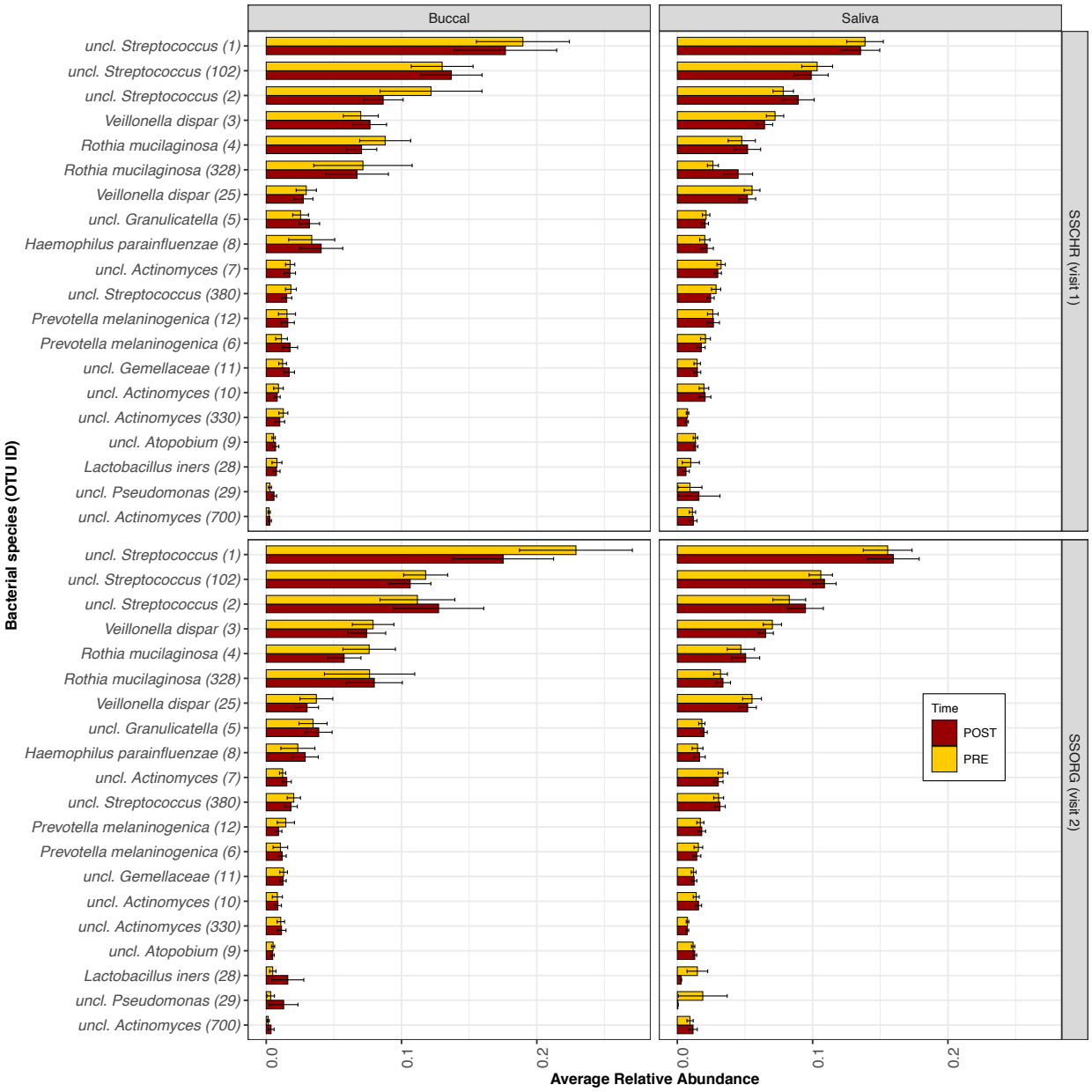
12. Figure 4.1: Alpha diversity analysis of buccal swab and saliva samples collected pre- and post-smoking of two little cigar products: Swisher sweets original (SSORG) and Swisher sweets cherry (SSCHR). Diversity was measured between pre-smoking (PRE) and post-smoking (POST) samples using ANOVA with Tukey's HSD post hoc test.



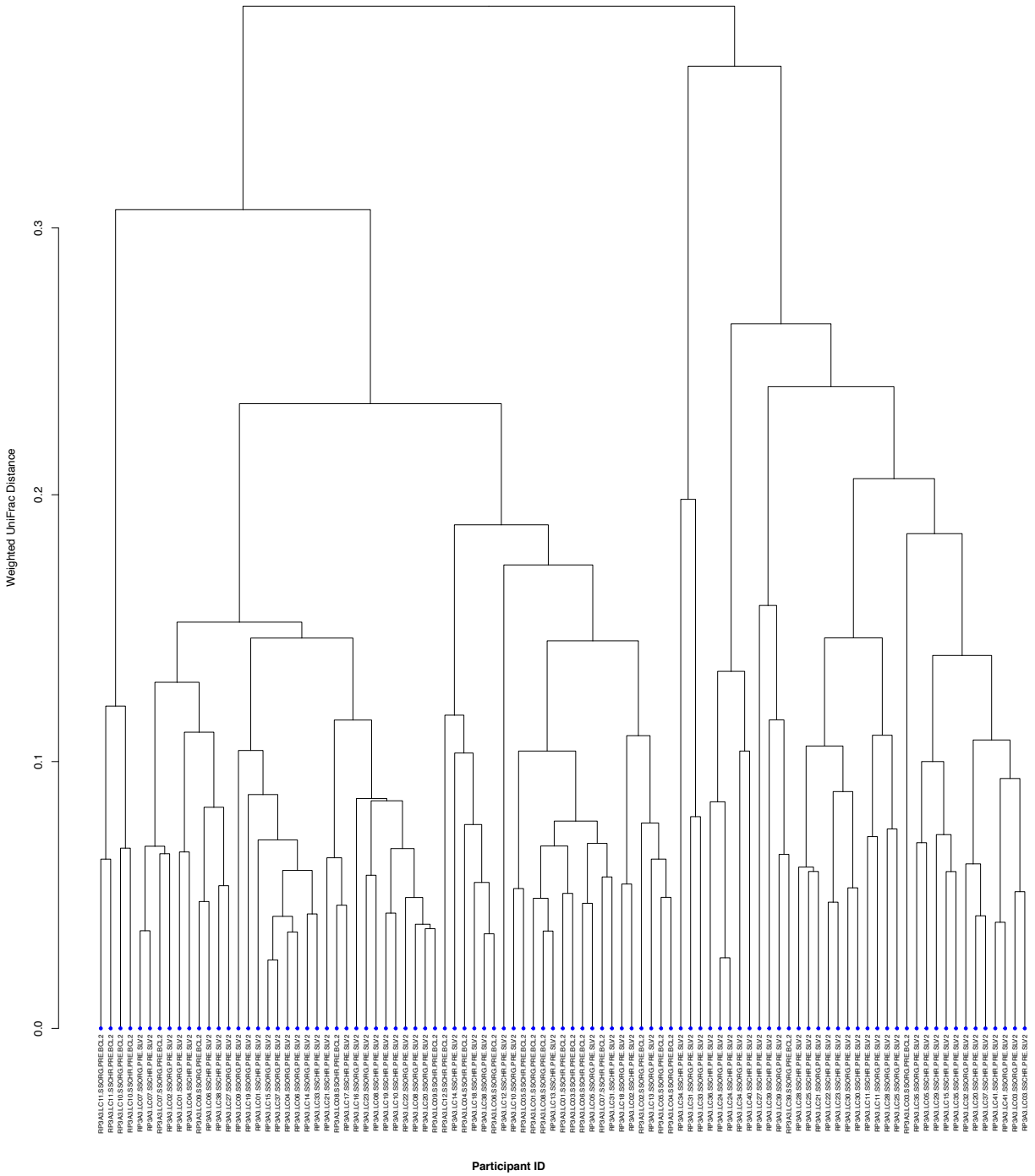
13. Figure 4.2: PCoA coordination plot measuring beta diversity between pre- and post-smoking samples from each visit. Ellipses are drawn at 95% confidence intervals. Statistical significance of beta diversity between time was measured by ANOSIM test of significance and *p*-values <0.05 were considered significant.



14. Figure 4.3: Predicted association of bacterial species between pre- (PRE) and post-smoking (POST) samples using Random Forest algorithm in (a) buccal swabs from visit 1; (b) saliva from visit 1; (c) buccal swabs from visit 2; and (d)saliva from visit2. The increasing abundance of each bacterial genera is represented by the color scale (blue to red).

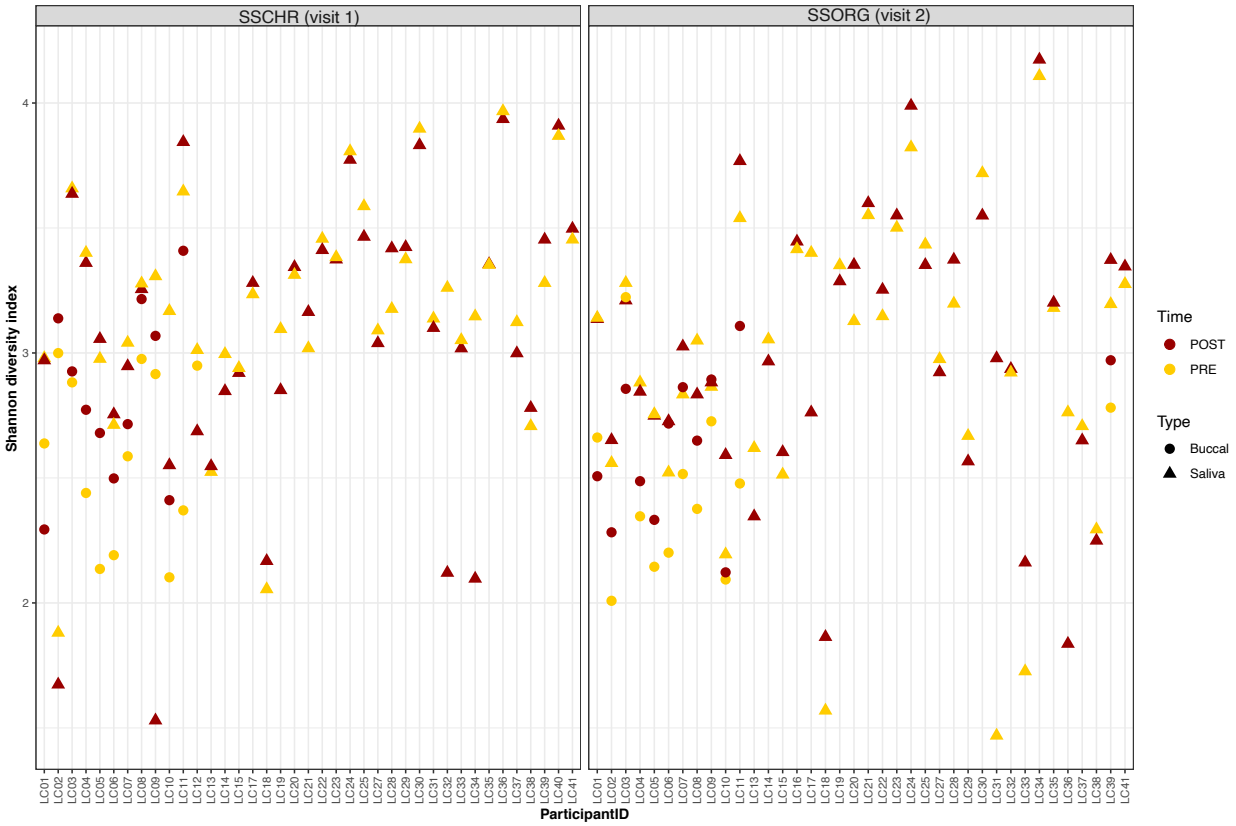


15. Figure 4.4: Average relative abundance (+/- SE) of the top 20 bacterial species (OTU ID) present in buccal swab and saliva samples from pre- and post-smoking samples in each visit.

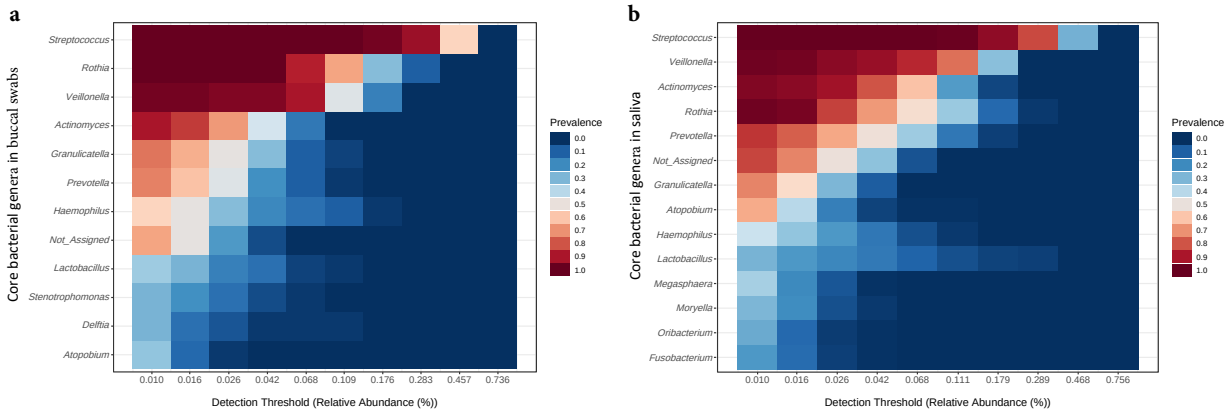


16. Figure 4.5: Histogram of weighted UniFrac distances of all pre-smoking samples across all participants.

4.7 Supplementary figures



17. Figure S 4.1: Alpha diversity analysis of oral microbiome per individual subject collected pre- (red) and post-smoking (yellow) of two little cigar products: Swisher sweets original (SSORG) and Swisher sweets cherry (SSCHR).



18. Figure S 4.2: Core bacterial genera present in (a) buccal swab samples and (b) saliva samples. The color gradient shows the prevalence (%) of each genus from zero (blue) to all (red) samples.

4.8 Tables

5. Table 4.1: Demographics of the study participants.

	n = 40	*%*
Gender		
Female	16	40
Male	21	52.5
Don't know/Refused	3	7.5
Age		
25-35	11	27.5
36-45	11	27.5
46-55	8	20
55+	7	17.5
Don't know/Refused	3	7.5
Marital Status		
Legally married	2	5
Living with Partner	4	10
Single/ never married	22	55
Divorced	7	17.5
Separated	2	5
Don't know/Refused	3	7.5
Race		
Black or African-American	19	47.5
White	18	45
Don't know/Refused	3	7.5
Employment		
Full-Time, 35+ hrs/week	13	32.5
Part-Time, irregular hrs/day work	7	17.5
Part-Time, regular hrs	5	12.5
Retired/Disabled	1	2.5
Homemaker	2	5
Unemployed	9	22.5
Don't know/Refused	3	7.5

6. Table 4.2: Cigarette and little cigar use of all study subjects.

Subject ID	Cigarette use						Tried little cigar	Little cigar use						
	Brand	Filter	Mentholation	Flavor	Size	Frequency of smoking		Frequency of smoking	# smoked	# of days per week smoked	# of days per month smoked	# of days smoked in past 30 days	Brand	Flavor
LC01	Newport	Filtered	Menthol	Full flavor	KINGS	Everyday	Yes	Some days	1	4	15	15	Swisher Sweets	Full Flavor
LC02	Pall Mall	Unfiltered	Non-menthol	Full flavor	100'S	Everyday	Yes	Some days	0	2	8	8	Don't know	Menthol
LC03	Mixed	Filtered	Non-menthol	Light	REGULAR	Everyday	Yes	Not at all	0	0	0	1	Winchester	Regular
LC04	Newport	Filtered	Menthol	Full flavor	KINGS	Everyday	Yes	Everyday	5	7	30	30	Garcia game	Grape/honey
LC05	Newport	Filtered	Menthol	Full flavor	100'S	Everyday	Yes	Not at all	0	0	0	1	Black & mild	Regular
LC06	Newport	Filtered	Menthol	Full flavor	100'S	Everyday	Yes	Not at all	0	0	0	0	Swisher	Grape
LC07	Newport	Filtered	Menthol	Full flavor	100'S	Everyday	Yes	Everyday	3	7	30	30	Black & mild	regular
LC08	Newport	Filtered	Menthol	Full flavor	100'S	Everyday	Yes	Not at all	0	0	0	0	NA	NA
LC09	Newport	Filtered	Menthol	Full flavor	100'S	Everyday	Yes	Some days	3	4	15	15	Swisher	sweets
LC10	Newport	Filtered	Menthol	Full flavor	KINGS	Everyday	Yes	Some days	10	5	20	30	Swisher	Regular, grape, sour apple
LC11	Newport	Filtered	Menthol	Full flavor	100'S	Everyday	Yes	Everyday	20	7	30	30	Seneca	Full Flavor
LC12	Marlboro	Filtered	Non-menthol	Full flavor	REGULAR	Everyday	Yes	Some days	2	4	10	10	Swisher Sweets	Regular
LC13	Marlboro	Filtered	Non-menthol	Light	100'S	Everyday	Yes	Some days	0	0	0	3	Swisher	Regular
LC14	Kool	Filtered	Menthol	Full flavor	KINGS	Everyday	Yes	Some days	4	6	24	24	Garcia vega game and swisher sweets	Cherry
LC15	Kool	Filtered	Menthol	Light	KINGS	Everyday	Yes	Everyday	8	7	30	30	Black & mild	Cigarillo
LC16	Camel	Filtered	Special/Mild	Light	REGULAR	Everyday	Yes	Not at all	0	0	0	0	NA	NA
LC17	Benson & Hedges	Filtered	Non-menthol	Light	100'S	Everyday	Yes	Everyday	2	7	31	31	Swisher and black & mild	Tropial
LC18	Benson & Hedges	Filtered	Non-menthol	Full flavor	REGULAR	Everyday	No	Not at all	0	0	0	0	NA	NA
LC19	Marlboro	Filtered	Non-menthol	Full flavor	KINGS	Everyday	Yes	Some days	5	3	10	7	Cigarillos	Cherry
LC20	Kool	Filtered	Menthol	Light	100'S	Everyday	Yes	Some days	2	1	3	3	Black & mild	Wine
LC21	Camel	Filtered	Non-menthol	Full flavor	REGULAR	Everyday	Yes	Not at all	0	0	0	1	Swisher	Regular
LC22	Kool	Filtered	Menthol	Full flavor	100'S	Everyday	Yes	Everyday	5	7	30	30	Swisher Sweets	Grape
LC23	Marlboro	Filtered	Non-menthol	Full flavor	REGULAR	Everyday	Yes	Not at all	NA	NA	2	1	Swisher	Regular
LC24	Newport	Filtered	Menthol	Full flavor	KINGS	Everyday	Yes	Some days	1	3	12	12	Black & mild	Regular
LC25	Newport	Filtered	Menthol	Full flavor	KINGS	Everyday	Yes	Some days	2	3	15	10	Swisher Sweet Cigarillos	Plain
LC27	GENERICS	Filtered	Menthol	Full flavor	100'S	Everyday	No	Not at all	NA	NA	0	0	NA	NA
LC28	Marlboro	Filtered	Non-menthol	Full flavor	REGULAR	Everyday	Yes	Everyday	10	7	30	30	cheyenne	Full flavor
LC29	Benson & Hedges	Filtered	Menthol	Full flavor	100'S	Everyday	Yes	Some days	0	1	4	2	Swisher Sweet Cigarillos	Peach
LC30	Benson & Hedges	Filtered	Menthol	Full flavor	100'S	Everyday	Yes	Everyday	12	7	30	30	Swisher Sweet Cigarillos	Sweet
LC31	Marlboro	Filtered	Non-menthol	Full flavor	KINGS	Everyday	No	Not at all	NA	NA	0	0	NA	NA
LC32	Mixed	Filtered	Non-menthol	Full flavor	REGULAR	Everyday	Yes	Some days	20	2	8	10	Seneca	Regular
LC33	Newport	Filtered	Menthol	Full flavor	KINGS	Everyday	Yes	Not at all	NA	NA	0	0	NA	NA
LC34	Kool	Filtered	Menthol	Full flavor	100'S	Everyday	Yes	Some days	20	100	300	150	show	Mango
LC35	Newport	Filtered	Menthol	Full flavor	REGULAR	Everyday	Yes	Some days	3	7	30	30	Black & mild	Wine
LC36	Marlboro	Filtered	Non-menthol	Light	REGULAR	Everyday	Yes	Some days	1	1	5	5	Djarum	Clove
LC37	Benson & Hedges	Filtered	Menthol	Full flavor	100'S	Everyday	Yes	Some days	10	4	16	14	Swisher	Regular
LC38	Marlboro	Filtered	Non-menthol	Full flavor	100'S	Everyday	Yes	Some days	1	2	8	5	Black & mild	Cherry blend
LC39	Marlboro	Filtered	Non-menthol	Light	REGULAR	Everyday	Yes	Not at all	0	0	0	0	NA	NA
LC40	Marlboro	Filtered	Non-menthol	Full flavor	REGULAR	Everyday	Yes	Some days	2	1	4	5	Any/Random	Cherry blend
LC41	Newport	Filtered	Menthol	Full flavor	100'S	Everyday	Yes	Some days	1	1	2	2	Black & mild	Regular

7. Table 4.3: Within and between individual variation in UniFrac distances.

Variation	Sample type	Product smoked visit #	Time	UniFrac distance	
				Average	SD
Intra-individual (within a subject)	Saliva	Visits 1 + 2	Pre	0.11	0.07
			Post	0.11	0.08
	Buccal	Visits 1 + 2	Pre	0.09	0.04
			Post	0.11	0.06
Inter-individual (between subjects)	Saliva	Visits 1 + 2	Pre	0.16	0.07
			Post	0.18	0.08
		SSCHR (visit 1)	Pre	0.16	0.06
			Post	0.17	0.07
		SSORG (visit 2)	Pre	0.17	0.08
			Post	0.18	0.09
	Buccal	Visits 1 + 2	Pre	0.15	0.06
			Post	0.15	0.06
		SSCHR (visit 1)	Pre	0.14	0.06
			Post	0.14	0.05
		SSORG (visit 2)	Pre	0.17	0.06
			Post	0.16	0.07

4.9 Supplementary tables

8. Table S 4.1: Relative abundance of top 10 bacteria in pre-smoking samples across all participants.

Subject ID	**Bacterial genera**									
	Actinomyces	*Atopobium*	*Granulicatella*	*Haemophilus*	*Lactobacillus*	*Prevotella*	*Pseudomonas*	*Rothia*	*Streptococcus*	*Veillonella*
LC01	0.136	0.037	0.003	0.000	0.155	0.059	0.007	0.119	0.956	0.307
SSCHR (visit 1)	0.100	0.020	0.001	0.000	0.058	0.027	0.005	0.050	0.505	0.112
SSORG (visit 2)	0.035	0.017	0.001	0.000	0.097	0.031	0.002	0.069	0.451	0.195
LC02	0.067	0.013	0.036	0.001	0.011	0.013	0.001	0.263	1.325	0.209
SSCHR (visit 1)	0.029	0.003	0.016	0.001	0.006	0.006	0.000	0.180	0.716	0.014
SSORG (visit 2)	0.038	0.010	0.020	0.001	0.005	0.007	0.001	0.083	0.609	0.195
LC03	0.159	0.025	0.052	0.011	0.041	0.079	0.039	0.243	0.760	0.204
SSCHR (visit 1)	0.084	0.012	0.023	0.007	0.014	0.035	0.004	0.120	0.448	0.094
SSORG (visit 2)	0.075	0.013	0.029	0.004	0.026	0.044	0.035	0.123	0.312	0.110
LC04	0.108	0.021	0.031	0.016	0.070	0.037	0.001	0.311	0.970	0.288
SSCHR (visit 1)	0.057	0.011	0.011	0.009	0.065	0.018	0.000	0.178	0.408	0.167
SSORG (visit 2)	0.051	0.010	0.020	0.007	0.006	0.019	0.000	0.133	0.562	0.121
LC05	0.090	0.012	0.062	0.112	0.008	0.048	0.002	0.208	1.115	0.232
SSCHR (visit 1)	0.032	0.004	0.039	0.088	0.005	0.028	0.001	0.113	0.528	0.098
SSORG (visit 2)	0.058	0.008	0.023	0.023	0.003	0.021	0.001	0.095	0.588	0.134
LC06	0.109	0.017	0.117	0.006	0.003	0.051	0.001	0.377	0.917	0.317
SSCHR (visit 1)	0.063	0.011	0.047	0.003	0.001	0.034	0.000	0.153	0.459	0.179
SSORG (visit 2)	0.046	0.006	0.070	0.003	0.002	0.017	0.000	0.224	0.458	0.138
LC07	0.057	0.027	0.037	0.004	0.082	0.100	0.002	0.180	0.948	0.391
SSCHR (visit 1)	0.029	0.013	0.026	0.003	0.041	0.040	0.002	0.128	0.473	0.158
SSORG (visit 2)	0.028	0.014	0.010	0.001	0.041	0.060	0.000	0.052	0.474	0.233
LC08	0.145	0.024	0.070	0.056	0.005	0.156	0.001	0.175	0.872	0.334
SSCHR (visit 1)	0.084	0.014	0.041	0.037	0.001	0.102	0.000	0.072	0.359	0.175
SSORG (visit 2)	0.061	0.010	0.029	0.019	0.004	0.054	0.000	0.102	0.514	0.159
LC09	0.150	0.012	0.093	0.040	0.004	0.078	0.077	0.208	0.825	0.362
SSCHR (visit 1)	0.075	0.004	0.064	0.019	0.001	0.058	0.001	0.102	0.414	0.178
SSORG (visit 2)	0.075	0.008	0.029	0.021	0.003	0.020	0.076	0.107	0.411	0.184
LC10	0.123	0.007	0.023	0.224	0.008	0.023	0.002	0.379	0.876	0.206
SSCHR (visit 1)	0.079	0.005	0.014	0.108	0.003	0.019	0.001	0.173	0.371	0.141
SSORG (visit 2)	0.043	0.002	0.009	0.116	0.005	0.004	0.001	0.205	0.505	0.065

LC11	0.231	0.018	0.016	0.071	0.003	0.099	0.002	0.471	0.639	0.169
SSCHR (visit 1)	0.107	0.007	0.007	0.048	0.002	0.040	0.002	0.238	0.308	0.070
SSORG (visit 2)	0.124	0.011	0.008	0.023	0.001	0.059	0.000	0.232	0.330	0.100
LC12	0.019	0.006	0.005	0.001	0.299	0.014	0.009	0.132	0.284	0.078
SSCHR (visit 1)	0.019	0.006	0.005	0.001	0.299	0.014	0.009	0.132	0.284	0.078
LC13	0.112	0.016	0.045	0.002	0.026	0.030	0.004	0.151	1.259	0.159
SSCHR (visit 1)	0.061	0.012	0.017	0.001	0.018	0.025	0.001	0.067	0.595	0.118
SSORG (visit 2)	0.051	0.003	0.028	0.000	0.008	0.006	0.003	0.085	0.664	0.041
LC14	0.222	0.005	0.122	0.056	0.010	0.020	0.000	0.234	0.923	0.159
SSCHR (visit 1)	0.100	0.001	0.071	0.034	0.007	0.005	0.000	0.151	0.466	0.015
SSORG (visit 2)	0.121	0.004	0.050	0.023	0.003	0.015	0.000	0.083	0.457	0.144
LC15	0.145	0.006	0.107	0.072	0.013	0.094	0.000	0.264	0.854	0.295
SSCHR (visit 1)	0.076	0.003	0.067	0.066	0.004	0.070	0.000	0.099	0.377	0.127
SSORG (visit 2)	0.069	0.003	0.040	0.006	0.009	0.025	0.000	0.164	0.477	0.168
LC16	0.073	0.020	0.014	0.006	0.004	0.061	0.000	0.008	0.442	0.154
SSORG (visit 2)	0.073	0.020	0.014	0.006	0.004	0.061	0.000	0.008	0.442	0.154
LC17	0.105	0.011	0.027	0.019	0.001	0.106	0.000	0.037	0.389	0.194
SSCHR (visit 1)	0.105	0.011	0.027	0.019	0.001	0.106	0.000	0.037	0.389	0.194
LC18	0.011	0.004	0.002	0.003	0.114	0.007	0.001	0.617	1.172	0.040
SSCHR (visit 1)	0.009	0.002	0.002	0.002	0.096	0.005	0.000	0.343	0.507	0.017
SSORG (visit 2)	0.002	0.002	0.000	0.001	0.018	0.002	0.001	0.274	0.664	0.023
LC19	0.198	0.010	0.010	0.035	0.008	0.050	0.000	0.131	0.906	0.292
SSCHR (visit 1)	0.101	0.006	0.007	0.024	0.003	0.035	0.000	0.055	0.438	0.183
SSORG (visit 2)	0.098	0.004	0.003	0.011	0.006	0.014	0.000	0.075	0.468	0.109
LC20	0.132	0.045	0.072	0.043	0.015	0.167	0.000	0.236	0.778	0.327
SSCHR (visit 1)	0.066	0.026	0.029	0.037	0.007	0.093	0.000	0.132	0.362	0.140
SSORG (visit 2)	0.066	0.019	0.043	0.006	0.008	0.075	0.000	0.104	0.417	0.187

LC21	0.223	0.022	0.032	0.014	0.007	0.270	0.000	0.048	0.722	0.326
SSCHR (visit 1)	0.070	0.011	0.016	0.006	0.004	0.138	0.000	0.024	0.430	0.199
SSORG (visit 2)	0.154	0.011	0.017	0.008	0.002	0.132	0.000	0.024	0.292	0.126
LC22	0.166	0.038	0.028	0.054	0.004	0.186	0.000	0.080	0.770	0.398
SSCHR (visit 1)	0.090	0.029	0.007	0.010	0.004	0.115	0.000	0.017	0.357	0.197
SSORG (visit 2)	0.076	0.008	0.022	0.043	0.001	0.071	0.000	0.063	0.413	0.201
LC23	0.193	0.038	0.007	0.005	0.006	0.183	0.001	0.039	0.711	0.375
SSCHR (visit 1)	0.083	0.019	0.003	0.003	0.005	0.119	0.000	0.016	0.333	0.208
SSORG (visit 2)	0.109	0.019	0.004	0.003	0.001	0.064	0.000	0.023	0.378	0.167
LC24	0.193	0.053	0.026	0.015	0.003	0.331	0.000	0.054	0.412	0.366
SSCHR (visit 1)	0.102	0.028	0.013	0.007	0.002	0.159	0.000	0.026	0.217	0.180
SSORG (visit 2)	0.090	0.024	0.013	0.008	0.001	0.172	0.000	0.028	0.195	0.186
LC25	0.347	0.031	0.023	0.014	0.005	0.153	0.001	0.037	0.649	0.352
SSCHR (visit 1)	0.155	0.015	0.014	0.013	0.002	0.102	0.000	0.019	0.317	0.164
SSORG (visit 2)	0.191	0.016	0.009	0.001	0.003	0.050	0.000	0.018	0.332	0.188
LC27	0.262	0.083	0.027	0.000	0.183	0.106	0.001	0.134	0.545	0.471
SSCHR (visit 1)	0.189	0.048	0.013	0.000	0.053	0.043	0.001	0.039	0.203	0.269
SSORG (visit 2)	0.073	0.034	0.014	0.000	0.130	0.063	0.000	0.095	0.342	0.201
LC28	0.299	0.047	0.021	0.006	0.012	0.201	0.001	0.054	0.615	0.459
SSCHR (visit 1)	0.133	0.025	0.013	0.003	0.003	0.139	0.001	0.024	0.343	0.175
SSORG (visit 2)	0.166	0.022	0.008	0.004	0.010	0.062	0.001	0.030	0.272	0.284
LC29	0.134	0.011	0.005	0.030	0.006	0.055	0.000	0.044	0.456	0.109
SSCHR (visit 1)	0.134	0.011	0.005	0.030	0.006	0.055	0.000	0.044	0.456	0.109
LC30	0.172	0.030	0.030	0.039	0.004	0.145	0.001	0.068	0.693	0.300
SSCHR (visit 1)	0.061	0.013	0.017	0.027	0.001	0.075	0.000	0.046	0.332	0.142
SSORG (visit 2)	0.111	0.016	0.013	0.012	0.003	0.070	0.000	0.022	0.361	0.158
LC31	0.039	0.013	0.051	0.004	0.066	0.043	0.690	0.126	0.668	0.163
SSCHR (visit 1)	0.033	0.011	0.049	0.003	0.050	0.040	0.000	0.106	0.460	0.157
SSORG (visit 2)	0.006	0.002	0.001	0.001	0.016	0.003	0.690	0.020	0.208	0.006

LC32	0.086	0.008	0.029	0.051	0.007	0.082	0.000	0.186	0.322	0.130
SSORG (visit 2)	0.086	0.008	0.029	0.051	0.007	0.082	0.000	0.186	0.322	0.130
LC33	0.072	0.009	0.026	0.027	0.004	0.028	0.538	0.135	0.675	0.191
SSCHR (visit 1)	0.062	0.006	0.023	0.024	0.002	0.027	0.001	0.126	0.442	0.158
SSORG (visit 2)	0.010	0.003	0.003	0.003	0.001	0.001	0.537	0.009	0.233	0.033
LC34	0.139	0.047	0.024	0.028	0.008	0.148	0.346	0.020	0.419	0.203
SSCHR (visit 1)	0.068	0.033	0.009	0.006	0.004	0.035	0.345	0.004	0.154	0.109
SSORG (visit 2)	0.071	0.014	0.015	0.022	0.004	0.113	0.001	0.016	0.265	0.094
LC35	0.154	0.015	0.047	0.191	0.008	0.069	0.000	0.143	0.786	0.198
SSCHR (visit 1)	0.066	0.007	0.025	0.070	0.005	0.059	0.000	0.063	0.406	0.097
SSORG (visit 2)	0.088	0.008	0.023	0.121	0.002	0.011	0.000	0.080	0.380	0.101
LC36	0.112	0.031	0.014	0.030	0.003	0.147	0.004	0.021	0.149	0.154
SSCHR (visit 1)	0.112	0.031	0.014	0.030	0.003	0.147	0.004	0.021	0.149	0.154
LC37	0.102	0.015	0.074	0.066	0.011	0.099	0.000	0.340	0.806	0.324
SSCHR (visit 1)	0.068	0.009	0.051	0.066	0.003	0.081	0.000	0.152	0.328	0.153
SSORG (visit 2)	0.034	0.006	0.023	0.001	0.008	0.018	0.000	0.187	0.478	0.171
LC38	0.116	0.016	0.086	0.000	0.046	0.100	0.000	0.399	0.926	0.244
SSCHR (visit 1)	0.080	0.011	0.045	0.000	0.028	0.094	0.000	0.131	0.374	0.194
SSORG (visit 2)	0.036	0.005	0.040	0.000	0.019	0.006	0.000	0.268	0.552	0.050
LC39	0.206	0.024	0.110	0.017	0.012	0.286	0.032	0.057	0.472	0.508
SSCHR (visit 1)	0.091	0.013	0.028	0.004	0.011	0.176	0.001	0.016	0.214	0.262
SSORG (visit 2)	0.115	0.011	0.082	0.013	0.001	0.110	0.031	0.041	0.258	0.246
LC40	0.073	0.008	0.023	0.039	0.003	0.113	0.000	0.011	0.210	0.126
SSCHR (visit 1)	0.073	0.008	0.023	0.039	0.003	0.113	0.000	0.011	0.210	0.126
LC41	0.218	0.028	0.045	0.108	0.012	0.102	0.001	0.257	0.579	0.365
SSCHR (visit 1)	0.108	0.013	0.020	0.046	0.005	0.063	0.001	0.121	0.293	0.174
SSORG (visit 2)	0.110	0.015	0.025	0.062	0.007	0.039	0.000	0.136	0.287	0.191

Chapter 5: Dysbiosis of the oral microbiome varies between cigarette smokers, smokeless tobacco users and non-users

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5.1 Abstract

Tobacco use adversely impacts every organ of the human body and causes significant changes in the oral microbiome. However, less is known about how specific tobacco products may impact the oral microbiome over time. To address this knowledge gap, we characterized the oral microbiome of cigarette users, smokeless tobacco users, and non-users over four months. Buccal swab and saliva samples (n=611) were collected, DNA extracted, PCR-amplified targeting the V3V4 region of the 16S rRNA gene, and sequenced. Cigarette users and smokeless tobacco users had more diverse oral bacterial communities, a higher relative abundance of *Firmicutes* and a lower relative abundance of *Proteobacteria*, compared to non-users. Non-users had a higher relative abundance of *Actinomyces, Granulicatella, Haemophilus, Neisseria, Oribacterium, Prevotella, Pseudomonas, Rothia*, and *Veillonella* in buccal swab samples, compared to tobacco users (cigarette and smokeless tobacco). While the most abundant bacterial genera were relatively constant over time, some species demonstrated significant shifts in relative abundance between the first and last time points. In addition, some opportunistic pathogens were detected among tobacco users including *Neisseria subflava, Bulleidia moorei* and *Porphyromonas endodontalis*. Overall, our results provide a more holistic understanding of the structure of oral bacterial communities in tobacco users compared to non-users.

5.2 Introduction

Over 700 bacterial species are known to inhabit the oral cavity, collectively known as the oral microbiome [69,275]. It is one of the most diverse microbial communities in the human body and studies over the past two decades have characterized these communities in great depth [275,276]. Given a normal body temperature of 37°C and optimal nutrients, the oral cavity provides a stable environment for a variety of bacterial species to survive in their specific niches [277]. These different niches or surfaces in the oral cavity comprise either hard (e.g., teeth, palates, gingival sulcus) or soft tissues (e.g., tongue, cheeks) [275]. Specific bacteria preferentially colonize these different surfaces due to variable surface adhesins and oral receptors [278]. For instance, the microbiome of saliva is more similar to that of the tongue, while the microbiome of soft tissues is largely dissimilar from that of hard surfaces like the teeth [279]. Nevertheless, results from previous oral microbiome studies are difficult to compare given the variability in sampling methods and sampling sites within the oral cavity [18,22,109,246,272].

Previous studies have decisively demonstrated, however, that one of the most significant factors influencing the oral microbiome is tobacco use [18,19,109] . Chemical constituents and bacterial communities from tobacco products and smoke heavily influence the oral microbiome of tobacco users [19,108,109,131,280], causing shifts from eubiosis to dysbiosis. Dysbiosis is characterized by three scenarios: 1) loss of microbial diversity; 2) loss of beneficial bacteria; and 3) increases in pathogenic bacteria[281]. Multiple factors related to smoking and smokeless tobacco use affect oral microbial homeostasis. For example, the use of smokeless tobacco has been shown to be associated with a depletion of beneficial bacterial genera such as *Lactobacillus* and *Haemophilus* in the oral cavity [108]. Similarly, cigarette smoking has been shown to reduce the

diversity of oral Gram-positive bacterial populations [282,283], and the duration of cigarette smoking has been shown to affect the prevalence of pathogenic bacteria in the oral cavity[253].

However, less is known about how the use of specific tobacco products (e.g., cigarettes versus smokeless tobacco) may result in different changes in the human oral microbiome over time. These changes are important because they could result in adverse health impacts among users. The oral microbiome is closely linked to the physiological state of the body, especially with regard to changes in the immune system. These changes can potentially lead to substantial shifts in the symbiotic balance between host and microbiome, as well as pathogen colonization, leaving individuals susceptible to disease development [284–286]. For example, a shift from Gram-positive aerobes to Gram-negative anaerobes in the oral cavity has been linked to the development of periodontal disease [287]. Therefore, studying the temporal variability of oral bacterial communities is useful in understanding the role of these communities in both disease development and the maintenance of a healthy mouth [288]. To improve our understanding of the effects of specific tobacco product use on oral microbiome dysbiosis over time, we employed next-generation sequencing approaches to perform a comprehensive comparison of temporal changes in the oral microbiome of cigarette smokers, smokeless tobacco users, and non-users over four months.

5.3 Methods

5.3.1 Study population

Convenience sampling was conducted in and around the University of Maryland, College Park, MD to recruit participants. Specifically, participants were recruited through word-of-

mouth, physical advertisements posted in and around the University of Maryland, and digital advertisements posted through email, campus websites, and social networking sites. To ensure the inclusion of racially diverse participants, we also partnered with the University of Maryland Center for Health Equity and recruited through their existing partnerships with black barbershops in our area.

Subjects were all healthy individuals, 18-55 years of age. Exclusion criteria included the use of antibiotics in the last six months, dependence on alcohol, diagnosis of pneumonia in the last six months, heart or lung problems in the last six months, or a diagnosis of emphysema, cancer, hepatitis B virus, hepatitis C virus or HIV. Participants also had to be in good oral health. Exclusion criteria included a diagnosis of dry mouth, untreated cavitated carious lesions, oral abscesses, precancerous or cancerous oral lesions, oral candidiasis, or clinically meaningful halitosis. Participants who had more than 8 missing teeth, had any major dental or oral surgery in the past six months, had taken any of the listed drugs within the last 6 months (e.g., systemic antibiotics, antifungals, antivirals, or antiparasitics; oral, intravenous, intramuscular, nasal, or inhaled corticosteroids; cytokines; methotrexate or immunosuppressive cytotoxic agents; or large doses of commercial probiotics) or were pregnant, breastfeeding or planning on becoming pregnant within the next six months were also excluded from the study.

Once a potential participant expressed interest in the study, a phone screen was first conducted to ensure that the participant met eligibility criteria and fell into one of two study groups: cigarette users (CG) or smokeless tobacco users (ST). After the recruitment of tobacco users, non-tobacco users (NU) were recruited similarly and matched to each tobacco user participant by age (+/- 3 years), race, and gender. All cigarette users had smoked more than six cigarettes on a typical day for the previous three years and all smokeless tobacco users had used

tobacco at least one time per week for the previous whole year. Non-tobacco users had smoked less than 20 cigarettes or used smokeless tobacco less than 20 times in their lifetime.

Upon enrollment, participants completed three baseline questionnaires (described below). In addition, we obtained buccal swab and saliva samples from participants once every 30 (+/-2) days for four consecutive months (T1-T4) (described in detail below). Participants refrained from using any tobacco products or ingesting food, water, or caffeine for at least two hours prior to sample collection. Participants also refrained from ingesting alcohol for at least 24 hours before sample collection. All participants were provided with overall project goals and engaged in the informed consent process. All protocols were approved by the Institutional Review Board of the University of Maryland.

5.3.2 Baseline questionnaires

Three baseline self-administered questionnaires were completed by each participant: a demographics questionnaire; an oral health and hygiene questionnaire, and a tobacco use questionnaire. The oral health and hygiene questionnaire included questions related to factors that might affect the oral microbiome (e.g., alcohol use, recent dental work, recent upper respiratory and gastrointestinal infections). Questions on the tobacco use questionnaire focused on self-reported measures of tobacco exposure (e.g., brand and type of tobacco used/smoked, amount and frequency of each product used/smoked, use of other types of tobacco (e.g., cigars, little cigars, and electronic cigarettes) and pulmonary history.

5.3.3 Saliva and buccal swab collection

To obtain saliva samples, participants were asked to let saliva collect in the mouth for at least one minute, and then expel 5 mL of saliva into a labeled 50 mL Falcon tube. 8 mL of RNALater solution (Thermo Fisher, MA) was added to the 50 mL Falcon tube, vortexed, and incubated at 4°C for 24 hours. After incubation, all samples were stored at -80°C until DNA extraction. Buccal swabs were collected using four E-swabs (Copan, CA) from four sites inside the oral cavity: the tongue dorsum, the hard palate, and the left and right buccal mucosa. Using the first e-swab, 1cm^2 of the center of the tongue was swabbed vigorously for 60 seconds and the swab was placed in a 50 mL Falcon tube containing 5 mL of RNALater solution. The second e-swab was then used to swab the entire hard palate vigorously for 60 seconds and the swab was placed in the same 50 mL Falcon tube. The third and fourth e-swabs were then used to swab the left and right buccal mucosa for 60 seconds each, taking care not to touch the teeth, and the swabs were then placed in the same 50 mL Falcon tube. The 50 mL Falcon tube with the four swabs was then vortexed for 30 seconds and incubated at 4°C for 24 hours. After initial incubation, all samples were stored at -80°C until DNA extraction.

5.3.4 Nicotine and cotinine analysis

Nicotine and cotinine analyses were performed to validate tobacco exposures reported on the tobacco use questionnaire. For each participant,1 mL of saliva was spiked with internal standards (Nicotine-d 4 and Cotinine-d 3) and cleaned using solid-phase extraction (SPE) methods. All samples were analyzed for nicotine and cotinine using positive electrospray ionization (ESI+) isotope dilution liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS) methods on an Applied Biosystems ABI3000 coupled with Shimadzu HPLC systems.

Quantitation was performed based on selective reaction monitoring (SRM) transitions for the analytes (163 → 130 and 177 → 80 for nicotine and cotinine, respectively) as well as their internal standards (167 → 121 and 180 → 80 for Nicotine-d 4 and Cotinine-d 3, respectively). All results were adjusted for recovery rates and laboratory blanks. Samples with concentrations below the limit of detection (LOD) were assigned a value equivalent to ½ the limit of detection (LOD) following an established practice.

5.3.5 DNA extraction and 16S rRNA gene sequencing

500 µL of ice-cold 1X Phosphate-buffered saline (PBS; Thermo Fisher, MA) was added to 500 µL of each saliva sample and briefly vortexed. These saliva sample tubes, along with the buccal swab sample tubes, containing 1000 µL of the buccal swab solution, were centrifuged at 10,000 rpm for 30 mins. The supernatant was discarded and 1 mL of ice-cold 1X PBS was then added to the cells left in the tubes. Total genomic DNA was then extracted from both saliva and buccal swab samples using previously published protocols (using both enzymatic and mechanical lysis approaches) followed by DNA purification with the Qiagen DSP DNA extraction kit (Qiagen, MD) per the manufacturer's protocol[6]. DNA quality checks were then performed using Nanodrop spectrophotometric measurements and gel electrophoresis. PCR amplification of the V3-V4 hypervariable region of the 16S rRNA gene was then performed using the 319 F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) universal primers and resulting amplicons were sequenced on an Illumina MiSeq (Illumina, San Diego, CA) using a duel-indexing technique developed and validated at the Institute for Genomic Sciences[6,187].

5.3.6 Sequence quality filtering

16S rRNA gene sequencing reads were screened for low quality and short length and assembled using PANDAseq[289], demultiplexed, and chimera trimmed using UCHIME[290]. Quality reads were then incorporated into QIIME v1.9[291] and clustered de-novo using VSEARCH and taxonomies were assigned using the Greengenes database, using a 0.97 confidence threshold. The resulting operational taxonomic unit (OTU) table, reference sequences, and phylogenetic tree files were then imported into R Statistical computing software (v. 0.99.473) using the Phyloseq R package (1.22.3)[251].

5.3.7 Statistical analysis

Alpha diversity was estimated using the *phyloseq* package (v. 1.19.1) with Shannon indices and observed number of species metrics after rarefaction at a minimum depth of 2,301 sequences for all samples. Reads were visualized using the ggplot2 package (v. 2.2.1). Statistical analyses were carried out using analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) *post hoc* test at a 95% confidence level to measure variation among the samples within each group: *p*-values less than 0.05 were considered statistically significant. Cumulative sum scaling (CSS) was carried out to normalize reads using the *MetagenomeSeq* (v. 1.16.0) package [252]. Beta diversity was estimated using *vegan* v. 2.4.5 and *phyloseq* packages. Beta diversity was calculated using principal coordinate analysis (PCoA) and Bray-Curtis dissimilarity. Distances were tested for significance using analysis of similarities tests (ANOSIM) on 999 permutations between groups of samples. Relative abundances of bacterial taxa were compared across the sample types and user groups using the nonparametric one-way ANOVA test. Statistical differences ($p < 0.05$) among bacterial OTUs relative abundances

between samples were calculated using the DESeq2 package (at $\alpha = 0.001$) on OTUs present at greater than 0.1% relative abundance [292]. Data were visualized with RStudio (v. 1.1.383) and the R package *ggplot2* (v. 2.2.1).

5.4 Results

5.4.1 Study participants

A total of 85 (24 CG, 18 ST, and 43 NU) participants were included in the study (**Table 1**). The majority of the participants were male and single (never married) (**Table 1**). 7%, 33% and 55% of all participants were Asian, Black and White, respectively (**Table 1**). The majority of the participants were employed full time, with greater than 12 years of formal education across all participants (**Table 1**).

The majority of cigarette users smoked 6-10 cigarettes per day and smoked filtered, menthol, and full flavor cigarettes (**Table 2**). The majority of the smokeless tobacco users used tobacco for 20 – 30 days in the past month. While Newports were the most popular brand of cigarettes, followed by Marlboros, among the cigarette users, Copenhagen, Grizzley, and Skoal were the most popular brands among the smokeless tobacco users. Seventeen (70.83%) cigarette users, 16 (89.89%) smokeless tobacco users, and 12 (27.91%) non-users had tried smoking a cigar, cigarillo, or little cigar previously. Eighteen (75%) cigarette users, ten (55.5%) smokeless tobacco users, and three (7%) non-users had used electronic cigarettes previously. Our nicotine and cotinine data (**Figure S1**) validated our tobacco use questionnaire data, demonstrating statistically significantly lower levels of both cotinine and nicotine concentrations in the saliva of the NU group compared to the CG and ST groups across all time points.

5.4.2 Sequencing data

A total of 32,276,329 sequencing reads were obtained from 611 samples, with a mean of 52,898.36 sequences per sample (SD +/- 29608.81). To ensure appropriate sequence coverage across samples, Good's estimate of coverage was calculated for each sample, and samples with Good's values ≤ 0.95 were removed from further downstream analysis. After quality filtering, a total of 32,265,093 reads were obtained from 556 samples for downstream analysis, with a maximum of 178,599 and a minimum of 1,085 (average 21872.57; SD 26020.04) reads/sample. The total number of sequences from 250 buccal swab samples was 14,636,962 and that from 306 saliva samples was 17,639,367. Overall, sequences were clustered into 6,092 OTUs.

5.4.3 Bacterial diversity and differentially abundant bacterial species across time points

Alpha diversity within buccal swab and saliva samples across the four-time points was calculated using the observed number of species and the Shannon diversity index (**Figure 1**). Looking across the four sampling events, we observed no significant ($p > 0.05$) differences in alpha diversity among buccal swab or saliva samples within any of the user groups. Computing beta diversity on Bray-Curtis distances, our data did not show any significant effect ($p > 0.05$) of time on the bacterial community composition of both buccal swab and saliva samples (**Figure S2**).

Moreover, there were no significant temporal differences observed among the dominant six bacterial genera in buccal swab or saliva samples within any of the user groups over the four time points (**Figure S3**). However, at the bacterial OTU level, we observed statistically significant ($p < 0.05$) differential abundance shifts in OTUs in both buccal and saliva samples across the three user groups between T1 and T4 (**Figure 2**). Within the buccal swab samples, the

majority of the OTUs that were at a statistically significantly (p<0.05) higher relative abundance at T4 (16 OTUs) belonged to the CG group, while six OTUs were in the NU group and only one OTU was in the ST group (**Figure 2a**). In contrast, only 5 OTUs in the NU group (*Lactobacillus* (OTU#29), *Blautia* (OTU#1380), *Staphylococcus* (OTU#321), *Enterobacter cloacae* (OTU#527) and *Streptococcus luteciae* (OTU#453)) were at a statistically significantly higher relative abundance at T1 compared to T4, and only one OTU (*Pseudomonas* (OTU#17)) in the CG group was at a significantly higher relative abundance at T1 compared to T4.

Within the saliva samples, comparing across the T1 and T4 time points, two OTUs (*Rhodococcus* (OTU#169), *Pseudomonas* (OTU#17)) in CG samples were at a statistically significantly higher relative abundance at T1, while one OTU (*Achromobacter* (OTU#239) in CG samples was at a higher relative abundance at T4 **(Figure 2b)**. OTU#145 (*Mycoplasmataceae)* from ST samples was at a statistically significantly higher relative abundance at T4 compared to T1. There were no OTUs in NU saliva samples that were at a statistically significantly different relative abundance (p > 0.05) between T1 and T4.

5.4.4 Bacterial community diversity between user groups

Since there was no significant effect of time on the alpha diversity indices, we merged the four-time points for each participant for other downstream analyses. Across all user groups, alpha diversity, measured using the Shannon diversity index, was significantly lower in buccal swab samples (average 3.44 (+/- 0.46 SD)) (p < 0.05) compared to saliva samples (average 3.81+/- 0.32) across all user groups (**Figure 3a**). Comparing alpha diversity in buccal swab samples between the three groups (CG, NU, and ST), the NU group was characterized by the lowest Shannon diversity index (average 3.36 +/- 0.40). The Shannon diversity indices among

buccal swab samples for the CG (average 3.55 +/- 0.52) and ST (average 3.56 +/- 0.52) groups were comparable. Similar to the buccal swab samples, Shannon diversity among the saliva samples in the NU group was also found to be the lowest (average 3.76 +/- 0.30) compared to the CG (average 3.94 +/- 0.29) and ST (average 3.80 +/- 0.38) groups.

To evaluate the effects of tobacco use on bacterial community composition, we computed beta diversity on Bray-Curtis distances and performed a PCoA analysis. Within the buccal swab samples, 9% of the variation in the bacterial community composition was explained by user groups (CG, ST, and NU) (ANOSIM R: 0.09, $p < 0.001$) (**Figure 3b)**. However, this difference in beta diversity was not found when saliva samples were compared between the three groups. Constructing a biplot of bacterial species on the PCoA plot demonstrated that the smaller cluster of buccal swab samples was potentially being driven by *Lactobacillus mucosae* and *L. reuteri* (**Figure 3c**).

5.4.5 Diversity and composition of bacterial communities across race and gender

Beta diversity on Bray-Curtis dissimilarity distances was computed by gender and race across all participants and their samples. 4% of the variation in bacterial community composition was significantly explained by race (**Figure S4**). Comparing bacterial genera, the buccal swab samples of tobacco users (cigarette and smokeless tobacco) had a higher relative abundance of *Veillonella* among Asian participants compared to other races, while this was not observed among the non-users (**Figure S5**). While *Streptococcus* and *Rothia* were at a lower relative abundance in all white participants' buccal swab samples when compared with that from Black participants, *Neisseria* and *Haemophilus* were at a lower relative abundance in buccal swabs from white tobacco users. *Lactobacillus* and *Prevotella* were at a higher relative abundance in

buccal swabs from white tobacco users when compared to that from Black participants. Within the saliva samples from smokeless tobacco users, the highest relative abundance of *Streptococcus* was observed among Asians, and that of *Veillonella* and *Prevotella* among mixed-race participants when compared to other races.

Comparing genders, 9% of the variation in bacterial community composition was explained by gender (**Figure S6**). Across both genders, *Streptococcus* was at the highest relative abundance in all samples (**Figure S7**). Within the buccal swab samples, the top five bacterial species (*Streptococcus, Veillonella, Prevotella, Rothia* and *Actinomyces*) were higher in relative abundance in males across tobacco users (cigarette and smokeless tobacco) when compared to females of the same user group. Within the non-users, the above-mentioned genera showed a similar trend in males except for *Prevotella* and *Actinomyces* which were at similar average relative abundances between males and females.

Within the saliva samples from females, *Streptococcus, Rothia* and *Granulicatella* were at a higher relative abundance when compared to that from males. *Prevotella* and *Neisseria* were observed to be at a higher relative abundance among male tobacco users when compared to female tobacco users.

5.4.6 Relative abundance of bacterial communities between user groups

The top five bacterial phyla identified in all samples belonged to *Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Fusobacteria* (**Figure S8)**. Comparing across buccal swabs from the three user groups, the relative abundance of *Firmicutes* was the lowest in the NU group (52%), compared to the CG (63.1%) and ST (63.1%) groups, while the relative abundance of *Proteobacteria* was highest in the NU group (16%) when compared to the CG

(9%) and ST (12.5%) groups. Similar to the buccal swab samples, saliva samples from the NU group also had the lowest relative abundance of *Firmicutes* (44%) (CG (47%) and ST (46%)) and the highest relative abundance of *Proteobacteria* (14%) (CG (8.4%) and ST (12%)). All three groups were characterized by a similar relative abundance of *Bacteroidetes*, *Fusobacteria* and *Actinobacteria.*

Comparing the relative abundance of bacterial genera within buccal swab samples across user groups, *Actinomyces, Granulicatella, Leptotrichia, Prevotella* and *Oribacterium* were at statistically significantly different relative abundances in the NU group compared to either the CG or ST groups (**Figure 4**). Among the above-mentioned five bacterial genera, while the highest relative abundance of *Leptotrichia* was among the CG group, the other four genera (*Actinomyces, Granulicatella, Prevotella*, and *Oribacterium*) were at a higher relative abundance in the NU group. In the buccal swab samples, the relative abundance of *Neisseria* and *Veillonella* was significantly higher in the NU group compared to the CG group. Comparing the relative abundance of bacterial genera in buccal swabs from the ST and CG groups, the relative abundance of *Leptotrichia* was significantly lower and *Pseudomonas* was significantly higher in the ST group compared to the CG group. The buccal swabs of the ST group also had the lowest relative abundance of *Actinomyces* and *Veillonella* compared to the other user groups. Buccal swab samples had a higher relative abundance of *Granulicatella, Haemophilus, Lactobacillus* and *Pseudomonas* compared to saliva samples across all participant groups (**Figure S9**).

Within the saliva samples, the relative abundance of *Fusobacterium, Haemophilus, Pseudomonas* and *Rothia* was statistically significantly higher in the NU group compared to the CG group (**Figure 4**). Relative abundances of *Fusobacterium*, *Granulicatella, Haemophilus, Leptotrichia, Neisseria, Oribacterium, Porphyromonas,* and *Rothia* were higher in the saliva

samples of the ST group compared to the CG group. *Actinomyces, Lactobacillus, Prevotella,* and *Veillonella* were at a lower relative abundance in the saliva samples of the ST group compared to the CG group. Finally, saliva samples had a higher relative abundance of *Actinomyces, Fusobacterium, Leptotrichia, Oribacterium, Porphyromonas,* and *Prevotella* compared to buccal swab samples across all participant groups (**Figure S9**).

5.4.7 Differentially abundant bacterial genera across user groups

In terms of statistically significantly different ($p < 0.05$) OTUs within buccal swab samples across the three user groups, three Gram-positive OTUs (*Streptococcus anginosus* (OTU#102), *Actinomyces* (OTU#62) and *Abiotrophia* (OTU#66)), and two Gram-negative OTUs (*Aggregatibacter* (OTU#110) and *Leptotrichiaceae* (OTU#73)) were at a statistically significantly higher relative abundance in the NU group compared to the CG group (**Figure 5a**). 19 OTUs (six Gram-positive and 13 Gram-negative) were at a statistically significantly higher relative abundance in the CG group compared to the NU group. 17 OTUs (five Gram-negative and 12 Gram-positive) were at a statistically significantly higher relative abundance in the NU group compared to the ST group, while only one Gram-positive bacteria (*Coprococcus* (OTU#229)) was at a higher relative abundance in the ST group compared to the NU group (**Figure 5b**).

Comparing bacterial OTUs in saliva samples, 18 Gram-negative and 26 Gram-positive OTUs were at a statistically significantly higher relative abundance in the CG group compared to the NU group, while 31 Gram-negative and 19 Gram-positive OTUs were at a statistically significantly higher relative abundance in the NU group compared to the CG group (**Figure 6a**).

Comparing among the NU and ST groups, 2 Gram-positive and 3 Gram-negative OTUs were at a statistically significantly higher relative abundance in the ST group (**Figure 6b)**.

5.5 Discussion

Cigarettes and smokeless tobacco products contain multiple chemical and microbiological constituents [58,293] that can alter users' oral microbiomes [18,19,108,109,131,280]. In this study, we found that using smokeless tobacco or smoking cigarettes played a role in dictating the changes in the oral microbiome of the user. While the majority of the oral microbiome composition remained relatively stable over time for individual tobacco users, the relative abundance of a few oral bacteria changed significantly over the four-month study period.

While previous studies evaluating the impacts of smoking on the oral microbiome have either categorized the smokers as light/heavy smokers or short/mild/long-term smokers based on their reported cigarette consumption (per/day, pack/year, or nicotine dependence levels) [246,294], no previous studies, to our knowledge, have followed the participants over time to evaluate the stability of the oral microbiome among tobacco users compared to non-users. Here, in our longitudinal study, interestingly, we did not observe any significant effect of time on the bacterial diversity of buccal swab and saliva samples from all three user groups (Figure 1; Figure S2). Previous oral microbiome studies reported from the Human Microbiome Project demonstrated temporal stability of the heterogeneous oral microbiome [134]. Salivary microbiome diversity remained relatively stable within individuals over a short-time period [135,269,295,296] up to a year [137], and this result did not change with the use of antibiotics [68,268]. Furthermore, adjusting for the use of antibiotics, the estimates of temporal stability did not show a significant change [136]. Nevertheless, even though the overall diversity and composition of the oral microbiome remains

relatively stable over time, comparing between our T1 and T4 time points, our data demonstrated significant changes in the differential abundance of a few bacterial species across tobacco users (CG and ST) and non-users (Figure 2).

Even though we provide evidence of temporal stability of the healthy oral microbiome, environmental perturbations like smoking tobacco significantly affects oral bacterial community diversity and composition [18,22,109,246]. Numerous bacteria provide benefits to the human host and dysbiosis of these microbiomes can be associated with a loss in bacterial diversity and potential detrimental health consequences [297]. But, not all human microbiomes follow the positive correlation of high bacterial diversity with better health (e.g., bacterial vaginosis, a state of inflammation, is associated with an increase in bacterial species diversity within the vagina) [242,298]. In our study, the buccal swabs and saliva of non-users had significantly lower alpha diversity when compared to tobacco users (CG or ST) (Figure 3). These findings corroborate previous studies that have found higher levels of bacterial diversity within the oral microbiomes of tobacco users compared to non-users [107,108,151]. From a disease development perspective, interestingly, the initiation and perpetuation of periodontal diseases has been associated with an increase in bacterial species diversity rather than a decrease [116,299]. Moreover, smokers have been characterized as harboring an oral microenvironment that potentially supports early colonization and enrichment of bacterial pathogens compared to non-smokers [107,300,301]. Subsequently, the abundance of pathogenic bacterial species in smokers' subgingival microbiomes has been linked to loss of resiliency and decreased resistance to future episodes of gingivitis and periodontitis [114,116].

While previous studies have revealed changes in diversity in the oral bacterial microbiome with tobacco use, these changes are not consistent across all sampling sites within the oral cavity. Here we identified that the majority of buccal swab and saliva samples were characterized by the presence of *Streptococcus, Veillonella, Prevotella, Rothia, Actinomyces*, *Haemophilus,* and *Neisseria*. However, the relative abundances of these and other bacteria changed by sample type across all three user groups (CG, ST, and NU) (Figure 4). For example, in comparison to tobacco users (CG and ST), non-users had a higher relative abundance of *Actinomyces, Granulicatella, Haemophilus, Neisseria, Oribacterium, Prevotella, Pseudomonas, Rothia*, and *Veillonella* in buccal swab samples. However, in the saliva samples, while *Haemophilus, Neisseria,* and *Pseudomonas* were also at a higher relative abundance in non-users, the other above-mentioned genera did not follow the same patterns observed in the buccal swab samples. Significant differences in bacterial genera between the two oral sampling sites (buccal swabs versus saliva) point towards distinct microbial niches, as shown previously in studies comparing the buccal mucosa, saliva, dental plaques, palates, and the tongue [109,134]. The differences in proportions of bacterial taxa in these different oral sites might be due to different site receptors on bacterial cell walls, specific species interactions, and specific surface properties that affect the bacteria's survival and growth [302].

Extensive studies have established that smoking causes dysbiosis in the oral microbiome, with concurrent enrichment of pathogens and depletion of commensals [19,107,303]. Furthermore, culture-based studies have shown that tobacco smoking inhibits the growth and reduces the diversity of Gram-positive bacteria (encompassing multiple pathogenic species) when compared to Gram-negative bacteria [13 283]. Consistent with previous findings, we identified statistically significantly lower differentially abundant Gram-positive species in the saliva from cigarette

users compared to that from non-users (Figure 4; Figure S9). Evaluating the relative abundances of bacterial genera in buccal swab samples, we identified a number of Gram-positive genera, such as *Actinomyces, Granulicatella, Oribacterium, Rothia*, and *Streptococcus,* that were at a lower relative abundance in cigarette smokers compared to non-users (Figure 4; Figure S9). In the saliva samples, similar trends were seen except with regard to *Actinomyces* and *Streptococcus*. Comparing the smokeless tobacco users to non-users, we found similar trends for the above-mentioned Gram-positive genera in the buccal swab samples. However, in the saliva samples, this was not true for *Granulicatella, Oribacterium, Rothia*, and *Streptococcus*. This difference between cigarette smokers and smokeless tobacco users might be due to the fact, that smokeless tobacco is not burned and inhaled as in cigarette smoking, which deposits toxicants from cigarette smoke, depleting oxygen and increasing salivary pH [18]. Recent studies have shown a significant association of higher relative abundance of genera such as *Rothia, Lactobacillus,* and *Streptococcus* in the oral microbiome of smokeless tobacco users compared to non-users [304]. Gram-positive *Rothia* is a usual member of the oral microbiome, but is more abundant in individuals with tongue leukoplakia lesions and periodontal diseases compared to healthy controls[305–307]. This genus is also known to produce high levels of acetaldehyde while lacking acetaldehyde dehydrogenases to detoxify acetyl aldehyde (a compound that can contribute to oral and gastrointestinal carcinogenesis) [308].

In addition to the Gram-positive species that were differentially abundant in the oral microbiome between tobacco user groups, a few of the Gram-negative species were also characterized by notable changes in relative abundance across user groups. One of the most abundant commensals and early colonizers of the oral cavity, *Neisseria,* was shown to be depleted in buccal swabs and the saliva of tobacco users (CG and ST) compared to that of non-

users. This was consistent with previous studies that found *Neisseria* (a Gram-negative member of the *Proteobacteria*) to be depleted in smokers' oral mucosas [309], lower respiratory tracts [110], nasopharynges, and oropharynges [310]. Usually considered obligate aerobes, both *Neisseria* and *Rothia* can also thrive in anaerobic biofilms and exhibit active denitrification [311,312], reducing nitrate to nitrite and eventually nitric oxide, a free radical with antimicrobial properties. Nitrite molecules can react with various tobacco alkaloids to generate carcinogenic tobacco-specific nitrosamines (TSNA). Therefore, depletion of *Neisseria* and *Rothia* species in tobacco users' oral microbiomes might potentially build an oral ecosystem conducive to producing TSNAs. Comparing the tobacco users (CG and ST), our data also showed a higher relative abundance of *Neisseria* in smokeless tobacco users compared to cigarette users. This might be due to the fact that cigarette smoking is known to increase the acidity of saliva [283], and *Neisseria* is sensitive to acidic conditions. While using smokeless tobacco is also known to lower the salivary pH [313], limited studies have compared the effects of changes in pH in the oral cavity on the oral microbiome.

While different constituents in tobacco products (cigarettes and smokeless tobacco) might have an inhibitory effect on the relative abundance of several bacterial species [170,314], smoking has also been linked with an increase in certain oral bacterial genera. Here, we found that the saliva from cigarette users had a higher relative abundance of *Prevotella* (a facultative anaerobe) and *Veillonella* (an obligate anaerobe). *Prevotella* is a dominant member of the gut microbiome but its higher abundance in the gut has also been linked to colon cancer and colitis susceptibility [315–318]. A pilot study demonstrated a significantly higher relative abundance of *Prevotella* in the gut of tobacco smokers [131], and another study noted an increase in the relative abundance of *P. bivia* and *V. dispar* in the oral microbiome of heavy smokers [246]. We also found a statistically

significantly higher relative abundance of *Veillonella* in the buccal swabs of non-users when compared to cigarette users. Commensal *Veillonella* can utilize lactic acid and convert it to weaker acids and in turn produce nitrite from nitrate [319]. With antimicrobial properties, nitrite has been shown to inhibit the growth and metabolism of oral pathogenic bacteria [320].

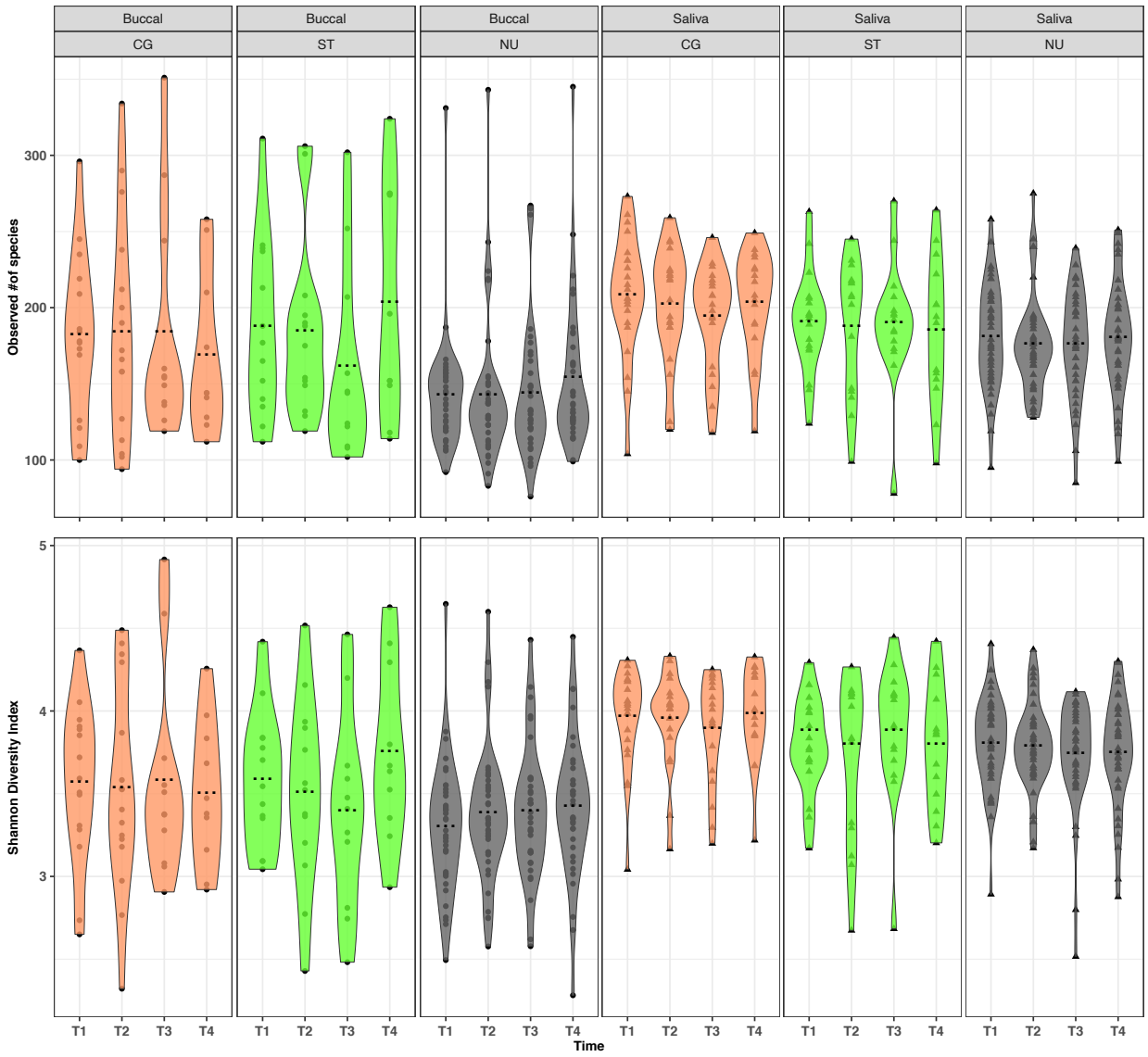
There are multiple strengths of this study. First of all, our study included a detailed comparison of the dysbiosis of the microbiome (both diversity and composition) that occurs among cigarette smokers versus smokeless tobacco users. There are limited studies with a comparable approach that have evaluated changes in the oral microbiome associated with variable tobacco use. Next, by following the participants over four months, we evaluated changes in the oral microbiome that might occur with tobacco use over time. Previous studies evaluating the impacts of tobacco use on the oral microbiome have either included only one type of tobacco product user, solely relied on culture-based techniques, or were limited with regard to longitudinal data [18,78,109,114,116,131,271,272,294,321,322]. Finally, our total number of samples was robust and user groups included comparable controls (non-users) matched by age, sex, and race with each of the tobacco users (cigarette smokers and smokeless tobacco users).

Although our study had multiple strengths, there are limitations to note as well. First of all, because we carried out 16S rRNA sequencing instead of metagenomic sequencing, we could not explore the functional attributes of the identified oral bacterial communities. In addition, even though we included 85 participants, the majority of our subjects were Black or White. Thus, while we identified significant differences in the oral microbiome across user groups, these results may not be generalizable to larger, more diverse populations.

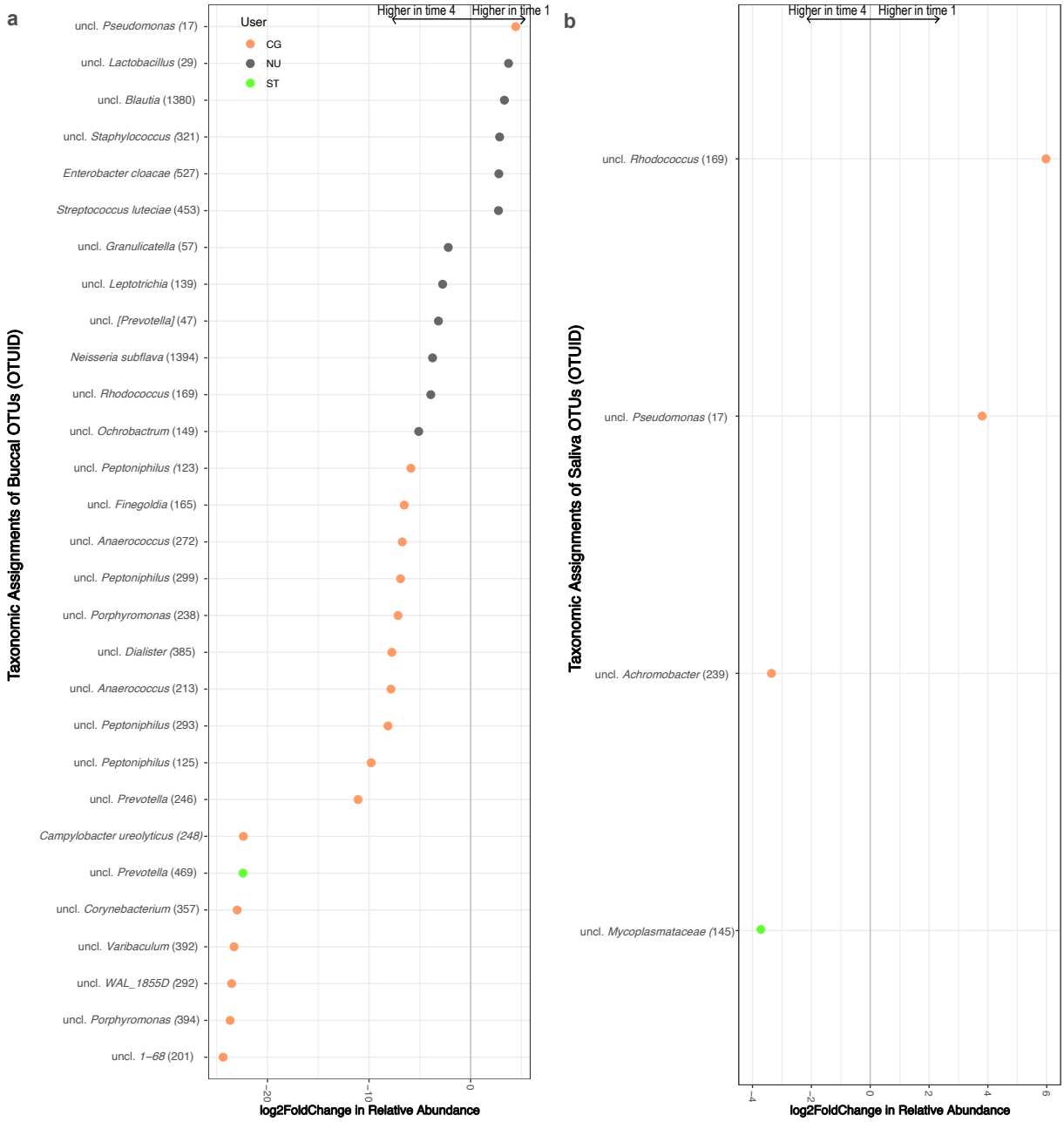
In summary, we observed that the composition of the oral microbiome is closely related to smoking status, and dysbiosis of the oral bacterial community is related to the type of tobacco

product used (e.g., cigarettes versus smokeless tobacco). Since the use of tobacco alters the oral microbiome [18,19,107,151,283], and perturbed balance in the oral microbiome has been linked to multiple oral and non-oral diseases [323,324], these microbiomes may well play a role in the development of malignant and carcinogenic conditions [77,277,315,325]. Therefore, characterizing dysbiosis in oral microbiomes associated with specific tobacco product use and studying the impacts of long-term tobacco use on these bacterial communities may provide insights into preventative and/or treatment approaches that can reduce the prevalence of these oral diseases.

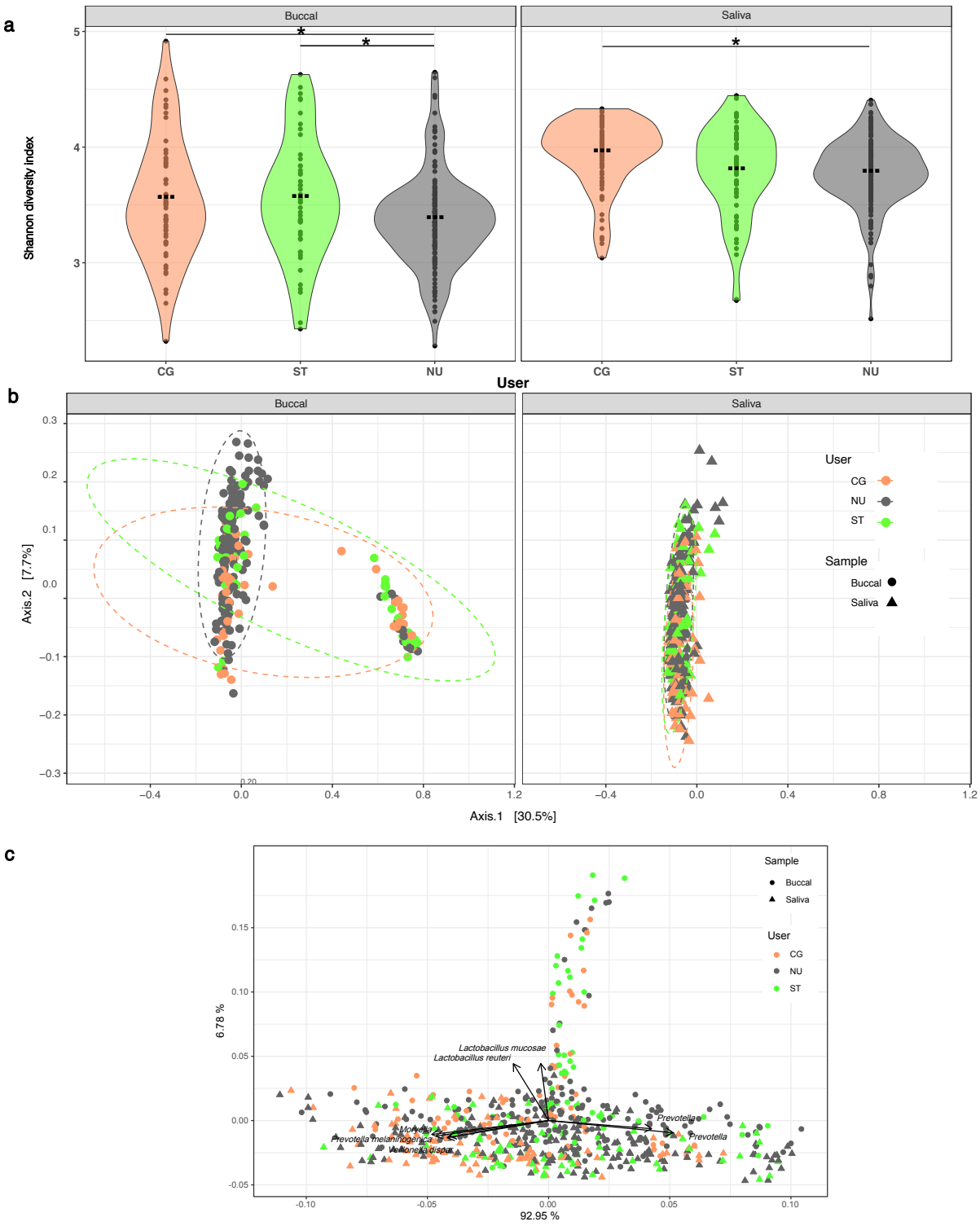
5.6 Figures



19. Figure 5.1: Alpha diversity analysis of buccal swab and saliva samples, by user group, across four-time points. Colors represent the user groups (cigarette user (CG), orange; smokeless tobacco user (ST), green; non-user (NU), grey). Alpha diversity was measured for time-points 1 through 4 (T1- T4) and compared using ANOVA with Tukey's HSD *post hoc* test.



20. Figure 5.2: Differential abundance of bacterial OTUs in (a) buccal swab and (b) saliva samples that were statistically significantly different ($p < 0.05$) between time-points 1 and 4. The OTUs are colored by user groups (cigarette user (CG), orange; smokeless tobacco user (ST), green; non-user (NU), grey). A positive log2-fold change value denotes an OTU that is significantly higher in time-point 1 samples, while a negative log2-fold change indicates an OTU that is significantly higher in time-point 4 samples. The grey line and arrows highlight the conversion in log2-fold change from negative to positive values.



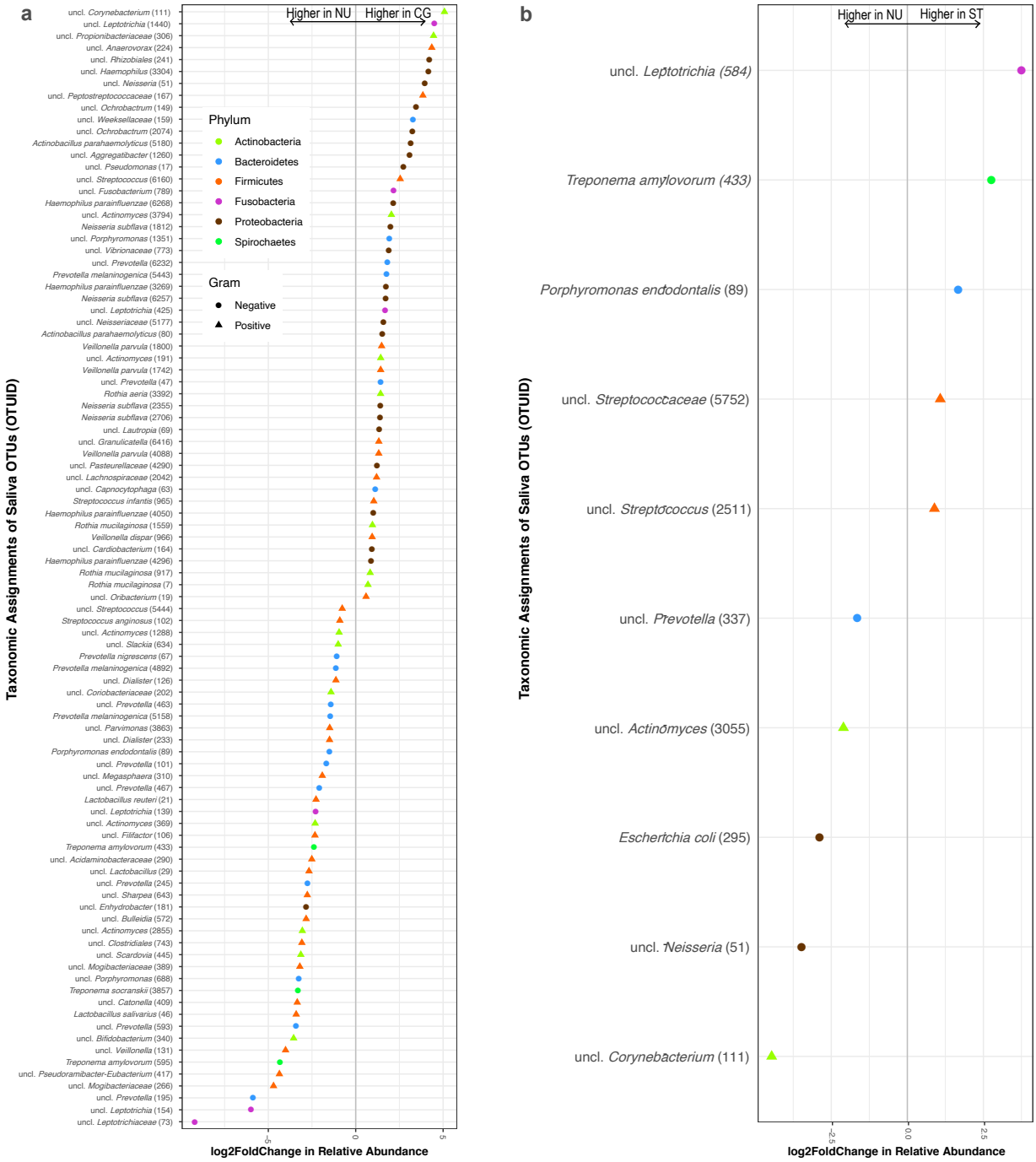
21. Figure 5.3: Bacterial diversity of buccal swab samples and saliva samples from cigarette users (CG), smokeless tobacco users (ST), and non-users (NU). **(a)** Alpha diversity violin plots were generated using the Shannon diversity index. Black lines represent significant changes between user groups and * represents statistically significant differences identified through

Tukey's HSD *posthoc* test ($\alpha = 0.05$). (**b**) Beta diversity was visualized through PCoA plots of Bray- Curtis computed distances among sample types. Ellipses are drawn at 95% confidence intervals. (**c**) PCoA bi-plot of beta diversity based on Bray-Curtis computed distances of buccal swab and saliva samples collected from cigarette users (CG), smokeless tobacco users (ST), and non-users (NU) showing significant correlations with specific bacterial taxa. The black arrows reflect the relationships between the bacterial taxa, with the direction of the point of the arrow showing increasing values of the taxa, and the cosine of the angle between the arrows reflecting the correlations between the taxa.

22. Figure 5.4: Box plots of relative abundance of the top 14 bacterial genera by user group: cigarette user (CG, orange), smokeless tobacco user (ST, green), and non-user (NU). (*) represents p-value < 0.05.

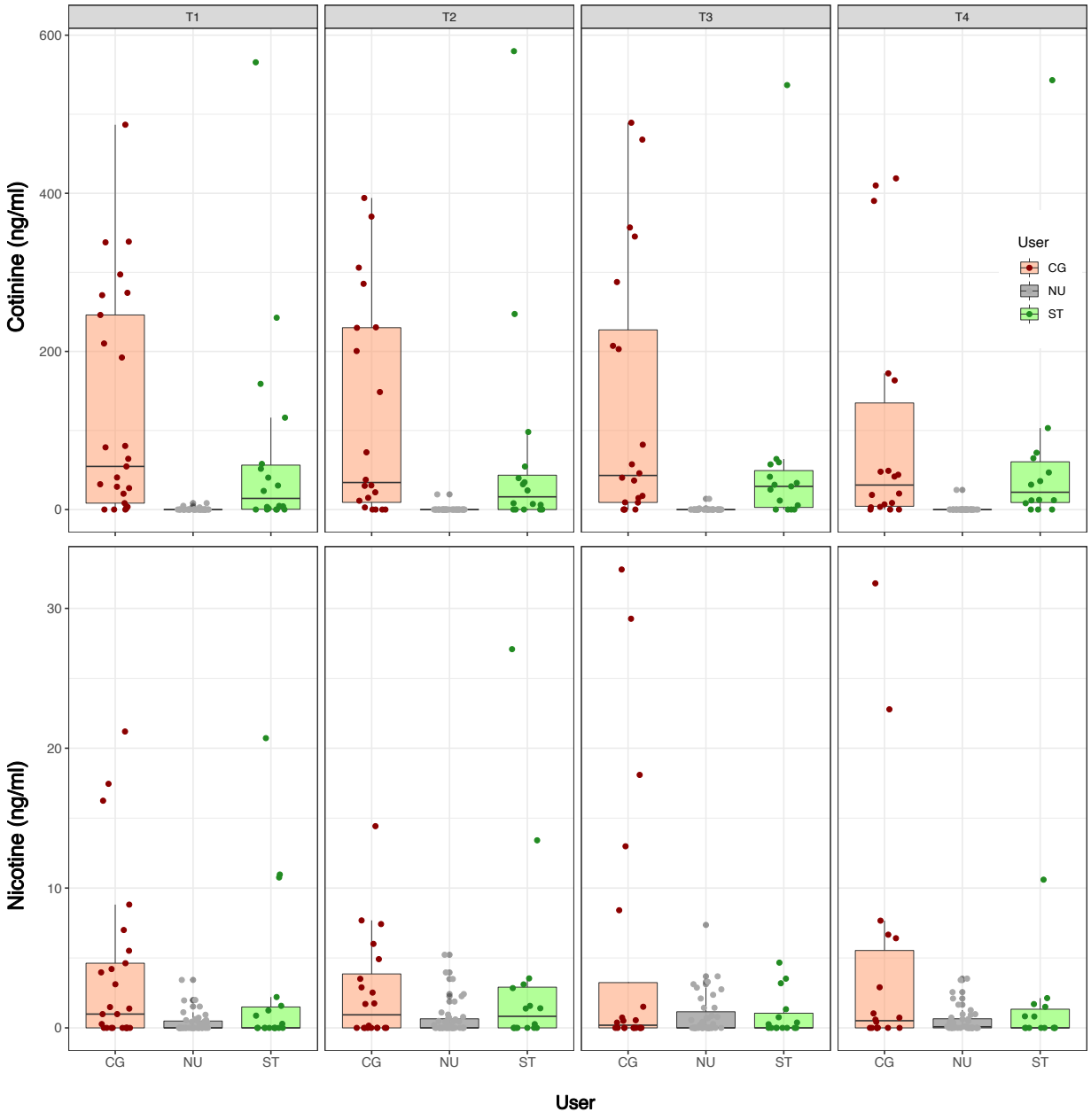
23. Figure 5.5: Relative abundance of bacterial OTUs in buccal swab samples that were statistically significantly different ($p < 0.05$) between (a) non-users (NU) and cigarette users (CG) and (b) non-users (NU) and smokeless tobacco users (ST). The OTUs are colored by their bacterial phyla. Circles represent Gram-negative and triangles represent Gram-positive bacteria. A positive log2-fold change value denotes an OTU that is significantly higher in user (CG or ST)

samples, while a negative log2-fold change indicates an OTU that is significantly higher in NU samples. The grey line and arrows highlight the conversion in log2-fold change from negative to positive values.

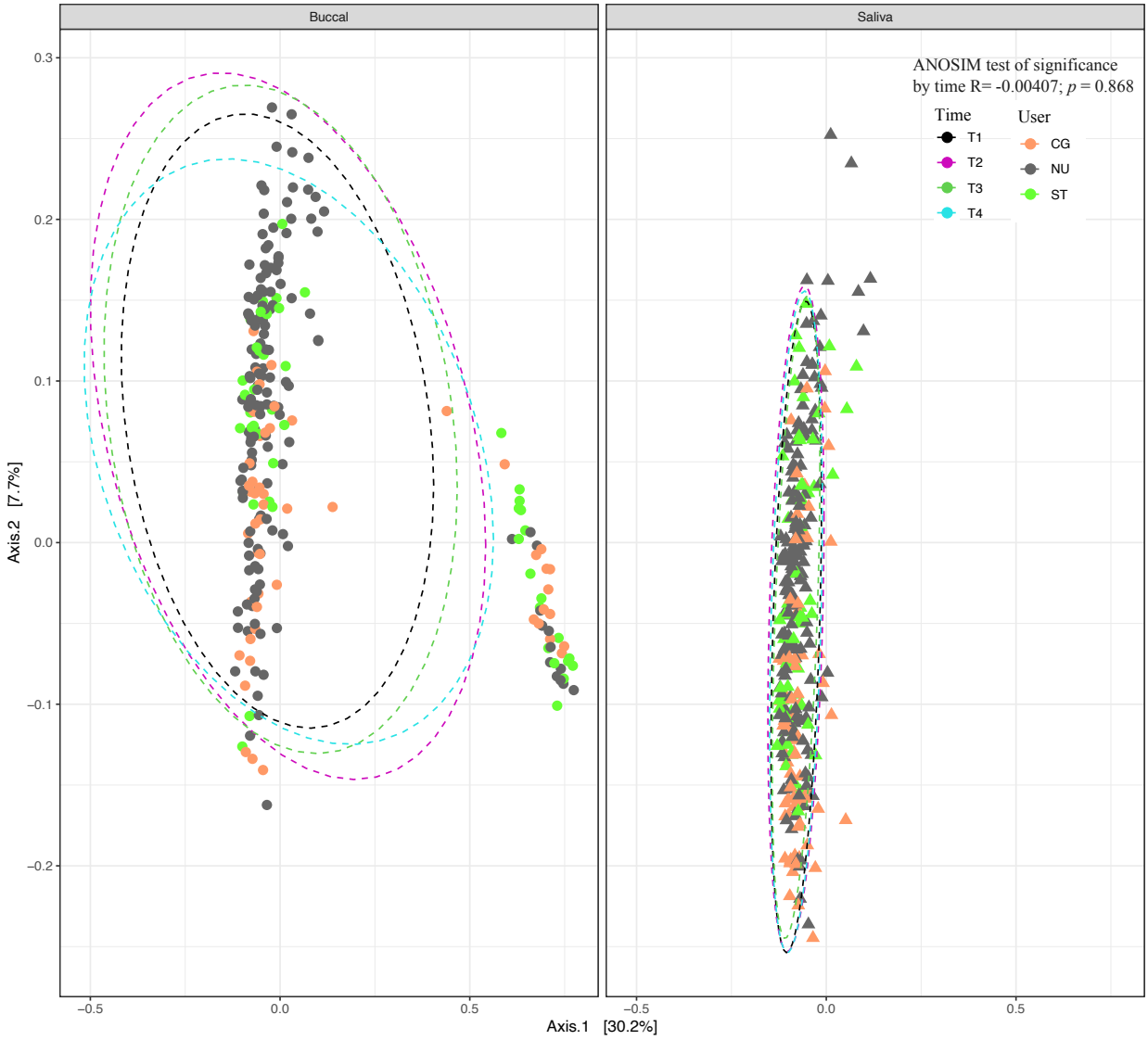


24. Figure 5.6: Relative abundance of bacterial OTUs in saliva samples that was statistically significantly different ($p < 0.05$) between (a) non-users (NU) and cigarette users (CG) and (b) non-users (NU) and smokeless tobacco users (ST). The OTUs are colored by their bacterial phyla. Circles represent Gram-negative and triangles represent Gram-positive bacteria. A positive log2-fold change value denotes an OTU that is significantly higher in user (CG or ST) samples, while a negative log2-fold change indicates an OTU that is significantly higher in NU samples. The grey line and arrows highlight the conversion in log2-fold change from negative to positive values.

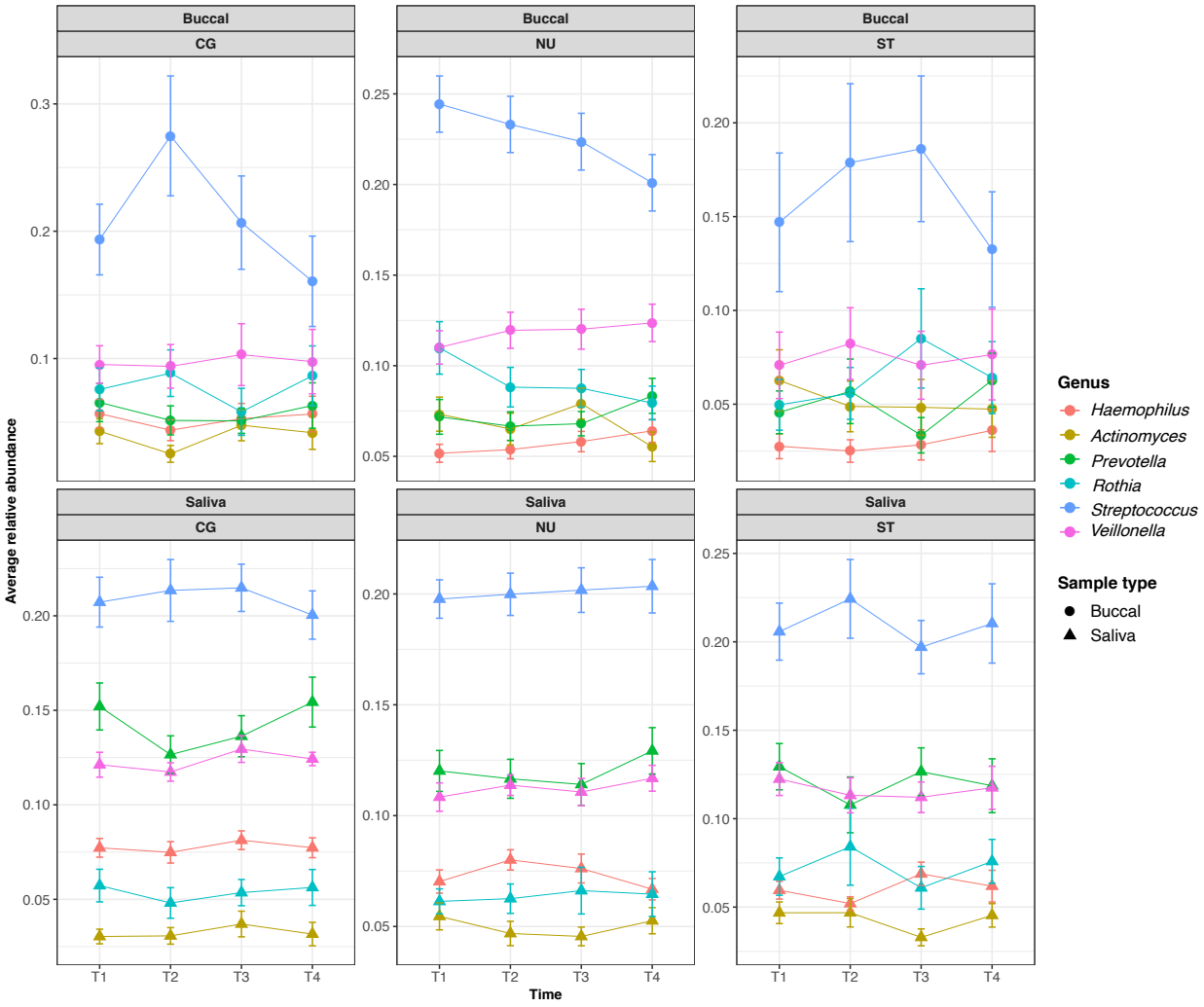
5.7 Supplementary figures



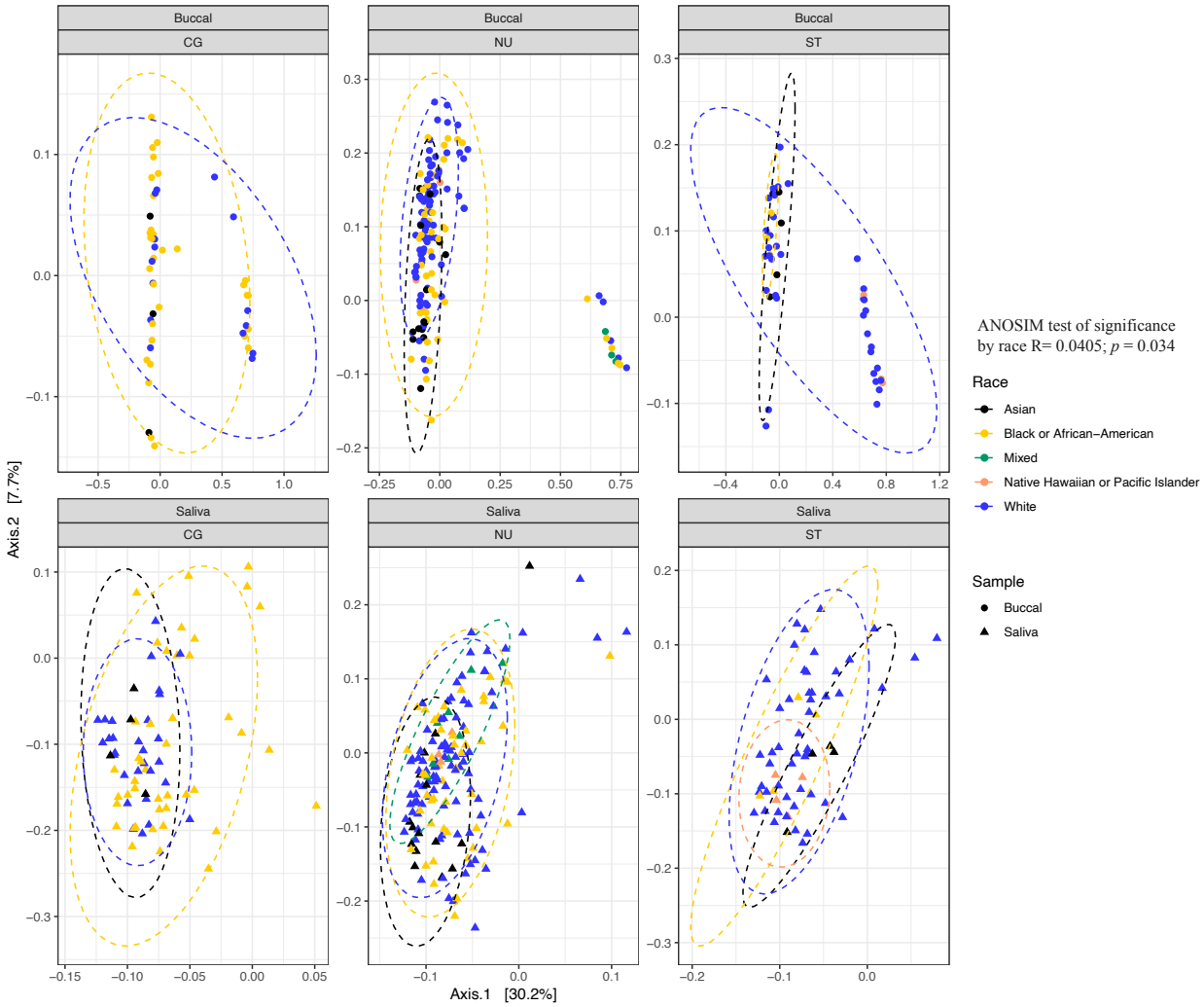
25. Figure S 5.1: Box plots of nicotine and cotinine levels detected in saliva samples from all participants, across all four-time points.



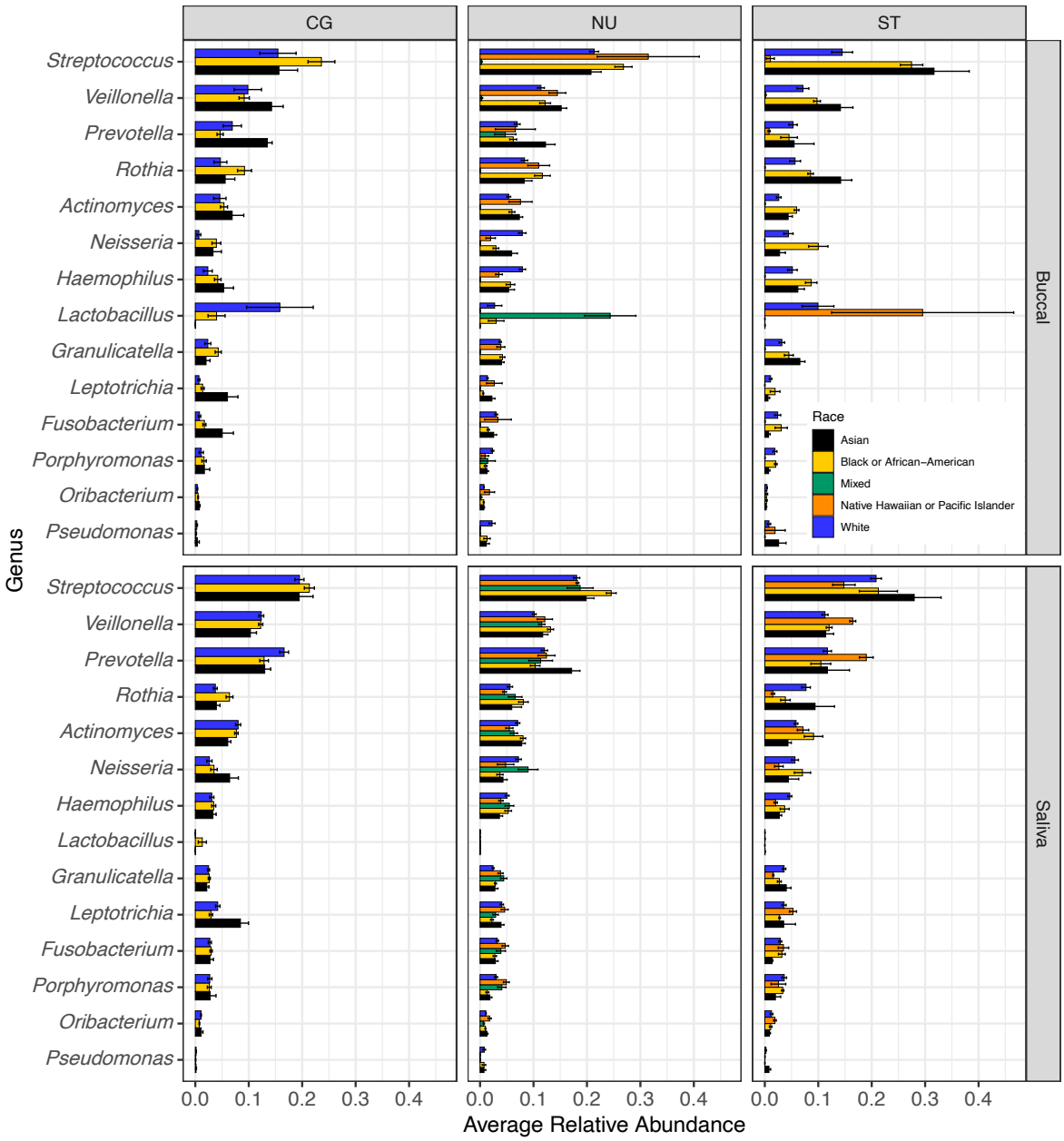
26. Figure S 5.2: Beta diversity was visualized through PCoA plots of Bray- Curtis computed distances among time points. Ellipses are drawn at 95% confidence intervals.



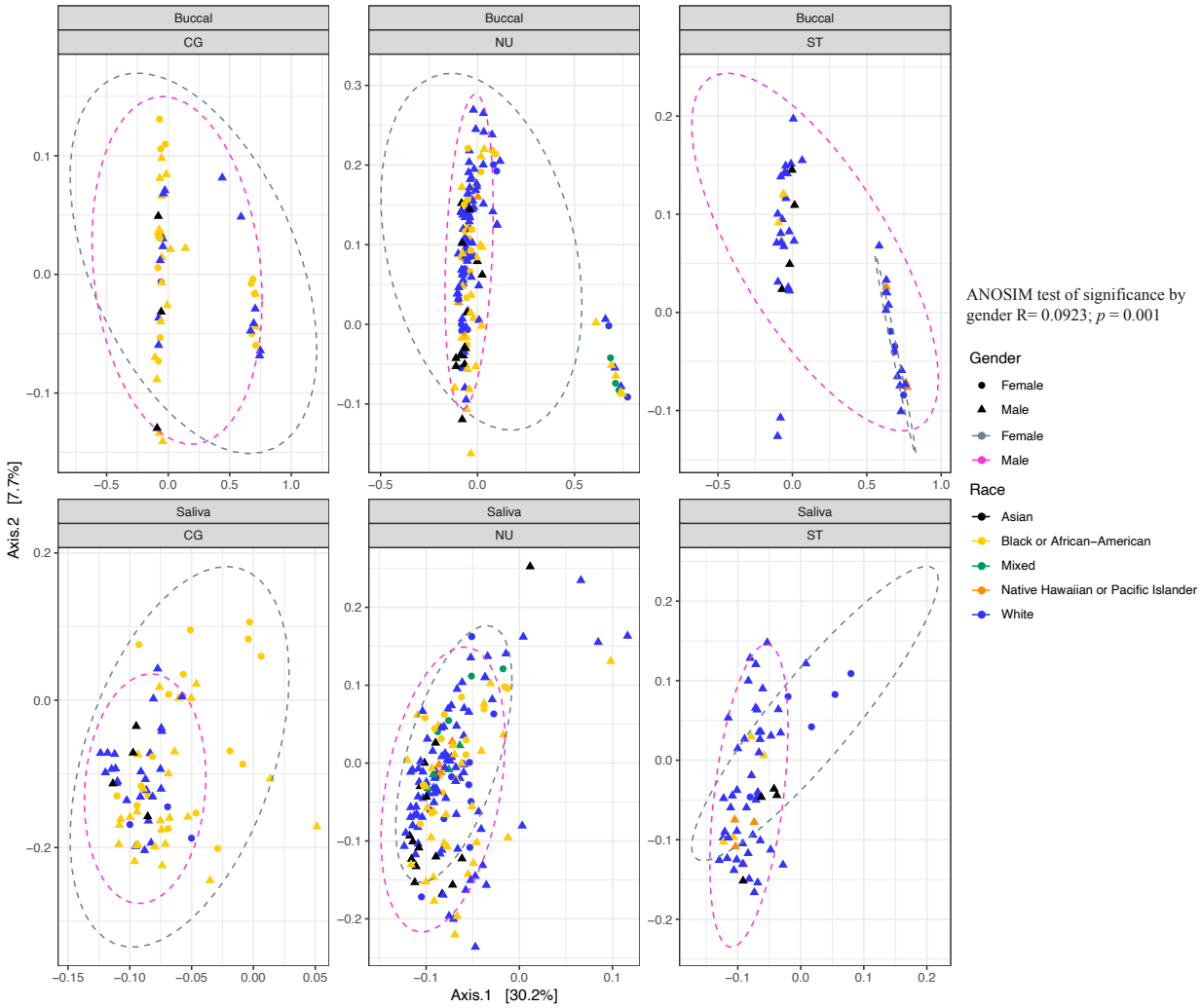
27. Figure S 5.3: Average relative abundance (± SE) of the top six bacterial genera present in all samples across four time points.



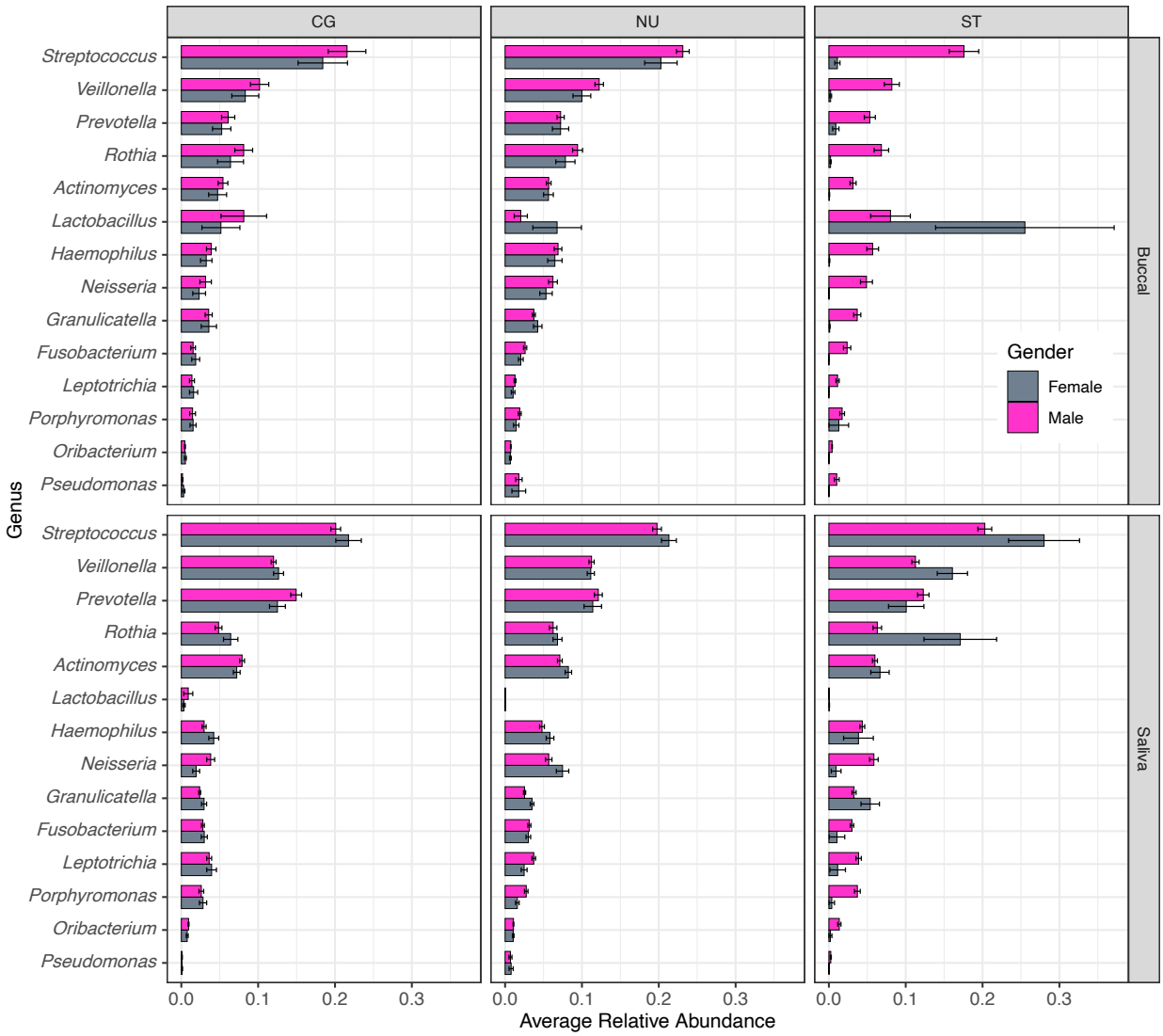
28. Figure S 5.4: Beta diversity was visualized through PCoA plots of Bray- Curtis computed distances among races. Ellipses are drawn at 95% confidence intervals.



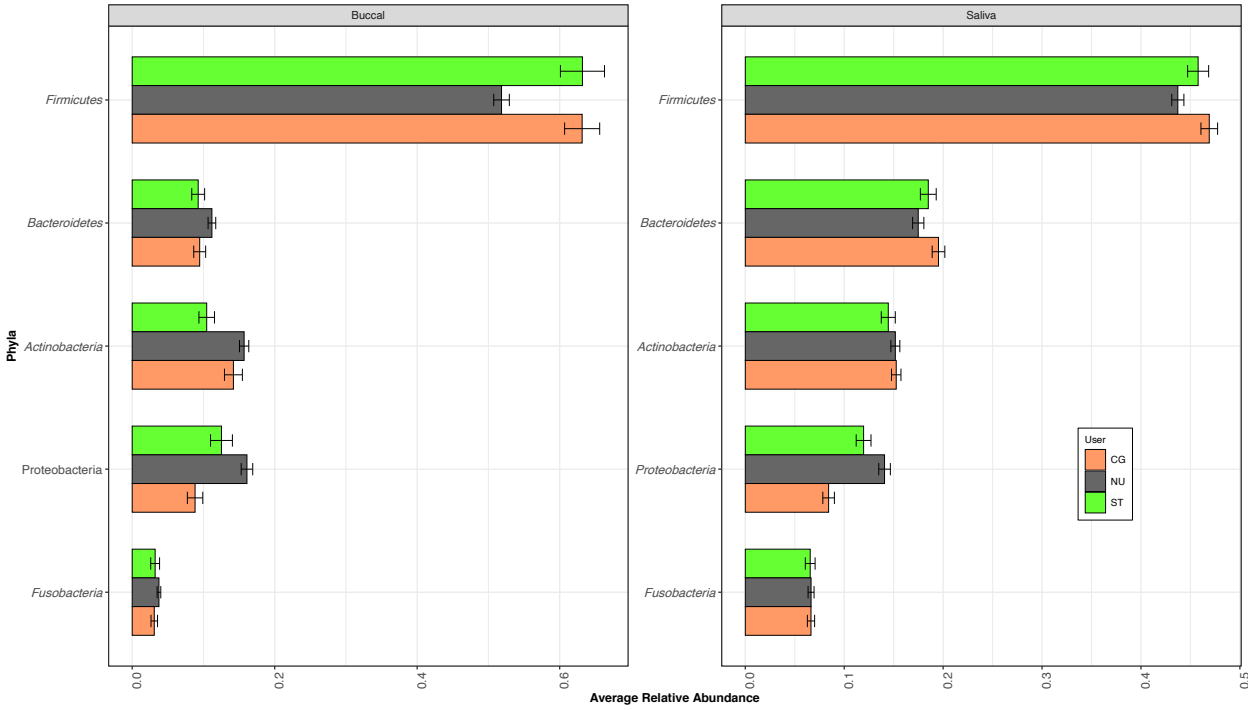
29. Figure S 5.5: Beta diversity was visualized through PCoA plots of Bray- Curtis computed distances among genders. Ellipses are drawn at 95% confidence intervals.



30. Figure S 5.6: Average relative abundance (± SE) of the top 15 bacterial genera present in all samples across the five races.



31. Figure S 5.7: Average relative abundance (± SE) of the top 15 bacterial genera present in all samples across two genders.



32. Figure S 5.8: Average relative abundance (± SE) of the top bacterial phyla present in all samples.Relative abundance was plotted for user groups (cigarette user (CG), orange; smokeless tobacco user (ST), green; non-user (NU), grey) from all sample types (buccal swab and saliva) across all time points.

33. Figure S 5.9: Box plots of relative abundance of the top 14 bacterial genera by sample type (buccal swab (red), saliva (blue) across each user group: cigarette users (CG), smokeless tobacco users (ST), and non-users (NU).

5.8 Tables

9. Table 5.1: Cigarette users (CG), smokeless tobacco users (ST) and non-tobacco users (NU) included in this study.

	CG (n=24)	**ST (n=18)**	**NU (n=43)**
	n(%)	*n(%)*	*n(%)*
Age, mean (SD)	35.70 (12.56)	28.55 (6.31)	31.02 (8.51)
Sex			
Male	17 (70.83)	17 (94.44)	35 (81.40)
Female	7 (29.17)	1 (5.56)	8 (18.60)
Marital Status			
Legally married	0	0	4 (9.30)
Living with Partner/cohabiting	1 (4.17)	0	5 (11.63)
Single, Never Married	19 (79.17)	18 (100)	33 (76.74)
Divorced	3 (12.50)	0	1 (2.33)
Separated	1 (4.17)	0	0
Ethnicity			
Hispanic/Latino	4 (16.67)	0	1 (2.33)
Non Hispanic/Latino	18 (75.0)	17 (94.44)	42 (97.67)
Refused	2 (8.33)	1 (5.56)	0
Race			
Asian	2 (8.33)	1 (5.56)	3 (6.98)
Black or African-American	14 (58.33)	1 (5.56)	13 (30.23)
Mixed	0	0	2 (4.65)
Native Hawaiian or Pacific Islander	0	1 (5.56)	1 (2.33)
White	8 (33.33)	15 (83.33)	24 (55.81)
Employment			
Full time	14 (58.33)	6 (33.33)	28 (65.12)
Military services	0	1 (5.56)	0
Part time-irregular hrs	0	0	1 (2.33)
Part time-regular hrs	3 (12.50)	5 (27.78)	2 (4.65)
Refused	1 (4.17)	0	0
Retired/Disabled	0	0	1 (2.33)
Student	3 (12.50)	5 (27.78)	11 (25.58)
Unemployed	3 (12.50)	1 (5.56)	0
Years of formal education			
<12 years	13 (54)	0	0
>12 years	11 (46)	18 (100)	43 (100)

10. Table 5.2: Tobacco use of cigarette users (CG), smokeless tobacco users (ST) and non-tobacco users (NU) included in this study.

	CG (n=24)	ST (n=18)	NU (n=43)
	n (%)	*n (%)*	*n (%)*
What brand of cigarette did you smoke most often?			
American Spirits	1 (4.17)	0 (0)	0 (0)
Marlboro	8 (33.33)	2 (11.11)	0 (0)
Newport	11 (45.83)	1 (5.56)	0 (0)
Camel	3 (12.5)	1 (5.56)	0 (0)
Djarum	1 (4.17)	0 (0)	0 (0)
Were they filtered or unfiltered?			
Filtered	22 (91.67)	4 (22.22)	0 (0)
Unfiltered	2 (8.33)	0 (0)	0 (0)
Were they menthol, non-menthol/plain, special or mild, or some other flavor?			
Menthol	15 (62.5)	1 (5.56)	0 (0)
Non-menthol/Plain	7 (29.17)	2 (11.11)	0 (0)
Special/Mild	2 (8.33)	1 (5.56)	0 (0)
Were they full flavor, light, or ultra-light?			
Full flavor	19 (79.177)	3 (16.67)	0 (0)
Ultra light	2 (8.33)	0 (0)	0 (0)
Light	3 (12.5)	1 (5.56)	0 (0)
Were they regular, Kings, 100's, or 120's?			
Regular	20 (83.33)	2 (11.11)	0 (0)
100's	2 (8.33)	2 (11.11)	0 (0)
Kings	2 (8.33)	0 (0)	0 (0)
How many cigarettes do you smoke in a normal day?			
<5	0 (0)	3 (16.67)	0 (0)
6 - 10	15 (62.5)	1 (5.56)	0 (0)
11 - 15	5 (20.83)	0 (0)	0 (0)
16 - 20	3 (12.5)	0 (0)	0 (0)
What brand of smokeless (or spitless) tobacco do you usually use?			
Copenhagen	1 (4.17)	6 (33.33)	0 (0)
Grizzley	0 (0)	5 (27.78)	0 (0)
Skoal	0 (0)	3 (16.67)	0 (0)
Other	0 (0)	4 (22.22)	0 (0)
How many days did you use smokeless tobacco in the past month?			
<10	24 (100)	1 (5.56)	43 (100)

10 - 19	0 (0)	4 (22.22)	0 (0)
20 - 30	0 (0)	13 (72.22)	0 (0)
Have you ever used or tried smoking a cigar, cigarillo, or little cigar?			
Yes	17 (70.83)	16 (88.89)	12 (27.91)
No	7 (29.17)	2 (11.11)	25 (58.14)
Was the product you tried a cigar, cigarillo, or little cigar?			
Cigar	7 (29.17)	12 (66.67)	9 (20.93)
Little cigar	5 (20.83)	1 (5.56)	0 (0)
Cigarillo	5 (20.83)	3 (16.67)	1 (2.33)
Don't know	7 (29.17)	1 (5.56)	27 (62.79)
Have you ever used or tried an electronic cigarette?			
Yes	18 (75)	10 (55.56)	3 (6.98)
No	6 (25)	8 (44.44)	34 (79.07)

Chapter 6: Conclusions, future research, and public health significance

Killing 8.7 million people each year globally, one of the world's largest preventable causes of premature death is the tobacco epidemic [326]. Tobacco use has grave health consequences ranging from inflammation, rheumatoid arthritis, diabetes to stroke and cancer. While 146 countries around the world have adopted World Health Organization MPOWER control measures to curb the use of tobacco products, more than a billion people globally still use tobacco [327]. Specifically in the U.S., with a projected reduction of 30% in tobacco use by the year 2025, currently there are over 127 million Americans who report smoking tobacco [328].

A variety of tobacco products are available in the market with the most traditional being cigarettes, cigars, little cigars, cigarillos, hookah, and electronic cigarettes. While the U.S. Food and Drug Administration (FDA)'s Harmful and Potentially Harmful Constituents (HPHC) list excludes the microbial contaminants present in tobacco products, research over the past 50 years has provided insights into the microbial contaminants that persist and potentially can be transmitted to users via aerosols and smoke [7–12]. Once transmitted to the user, these microorganisms could impact the oral microbiome, causing dysbiosis potentially leading towards disease development. Given that there are studies characterizing the bacterial microbiome in specific tobacco products, none provide a comparative analysis of the bacterial communities present in these products or evaluate the impact of smoking different tobacco products on the temporal stability of the oral microbiome over time. Therefore, to address this knowledge gap, my dissertation research utilized culture-independent next-generation sequencing technologies to provide an in-depth characterization of the bacterial microbiome that is present in conventional tobacco products and evaluate the temporal dysbiosis of the oral microbiome after tobacco use.

Specifically, Chapter 3 provided a detailed comparative characterization of the bacterial community that resides within top-selling traditional cigarettes, cigarillos, little cigars, and hookah in the U.S. and compared it to that of research cigarettes that are frequently used in clinical studies. Both bacterial diversity and abundance were significantly different with some pathogenic/potentially pathogenic strains identified across the products. We found that while bacterial diversity in hookah was the lowest, heterogeneity in bacterial community composition was highest in the little cigars. While each of the tobacco products harbored a unique microbiome with specific bacterial species, a core microbiome consisting of *Pseudomonas aeruginosa, P. putida, P. alcaligenes* and *Bacillus subtilis* were shared among all samples. We also observed the compositional shifts in bacterial community with the presence of additives like menthol and flavoring in the tobacco products. Furthermore, we implemented the use of DNA binding dye 5-bromo-2'-deoxyuridine (BrdU) to identify the metabolically-active fraction of the total bacterial community. Identification of some metabolically-active pathogens such as *Bacillus cereus* and *Haemophilus parainfluenzae* in commercial tobacco products is of concern because of the potential for these microorganisms to be transferred to users' oral cavities via mainstream smoke.

While chapter 3 was a comparative exploratory analysis, the research described in this chapter can be used to make informed decisions regarding the prospective ban on additives such as menthol and other flavors in commercial tobacco products. Mentholated tobacco products are believed to mask the harshness of smoking and hence are popular among users. But the use of mentholated products is not uniform across all demographics. The FDA estimates about 85% of non-Hispanic Black smokers use mentholated varieties of tobacco, while only 30% of non-Hispanic white smokers used menthol [142]. A recent study using nationally representative

longitudinal data also showed lower odds of quitting smoking among Black menthol smokers when compared to White or Hispanic smokers [329]. While this chapter includes evaluating the microbiome from four different types of commercially available tobacco products, there is also a need to include data on microbial contaminants that could be present in the understudied but extremely popular electronic liquids. Preliminary data from our group show the presence of viable bacterial taxa in these liquids (data not shown). While the sale of the majority of the flavoring has been banned in electronic liquids in the past year, the FDA has last month proposed a ban on menthol in traditional cigarettes and all characterizing flavors (including menthol) in cigars [142,330]. Our data will add to the current literature that is available on the microbial contaminants present in tobacco products and potentially provide evidence-based data to help make informed regulatory decisions.

While it is important to study the bacterial microbiome that is present in the different tobacco products, it is also imperative to evaluate the potential transfer of tobacco bacterial communities to the user's oral cavity and subsequent impacts on the oral microbiome of users. A recent publication from our group shows for the first time that viable bacteria (*Bacillus*, *Paenibacillus* and *Terribacillus*) can be aerosolized in mainstream cigarette smoke [16]. The fourth chapter of my dissertation evaluates the immediate transfer of bacteria from a tobacco product to a user's oral cavity after a single use of a little cigar. Our data demonstrates that there was no significant difference in bacterial diversity and community composition between pre- and post-smoking samples, potentially indicating no immediate transfer of tobacco-related bacteria to users' oral cavities.

While chapter 4 provides evidence of no immediate transfer of bacteria from little cigars to the user's mouth, studies have shown the adverse effects of toxicants from cigarette smoke

that interfere with the oral microbiome and impact the homeostasis of the host's oral microbiota [3,19,78,107,114]. For example, smoking tobacco has been shown to reduce the relative abundance of *Neisseria* while increasing that of *Streptococcus* and *Veillonella* in the oral microbiome [280]. Smoking leads to oxygen depletion which might explain the above-mentioned bacterial dysbiosis in the oral cavity [18,246,280,331]. While diversity and abundance of oral bacterial community changes, it triggers an inflammatory host response that could potentially increase the risk of disease development [19,23,107,114].

While there is consensus that tobacco smoking can cause dysbiosis of the oral microbiome, there are controversies regarding the temporal stability of this dysbiosis. Therefore, the fifth chapter of my dissertation focused on the effects of smoking on the oral microbiome over time. Characterization of the changes in the oral bacterial community demonstrated stability over four months among both tobacco users (cigarette smokers and smokeless tobacco users) and non-users. While these data provide insights into the temporal stability of the oral microbiome, they cannot be generalized for all populations.

Beyond tobacco smoking, data from both chapters 4 and 5 showed no significant effect of participants' intrinsic factors like age on oral bacterial diversity and composition. Although we found that inter-participant variation in the oral microbiome was greater than intra-participant variation (Chapter 4). Previous studies indicate the superseding effect of factors like race over smoking tobacco when evaluating changes in the oral microbiome [19]. Given the multiple intrinsic biological and extrinsic environmental factors (like smoking) that can influence the oral microbiome, more research needs to be done to evaluate the impacts of these factors on oral microbiome dysbiosis.

Comparing smokers and non-users, multiple studies have demonstrated an altered relative abundance of oral bacterial species among smokers [18,271,272]. Smoking tobacco has been associated with unstable microbial colonization, potentially increasing the risk of bacterial infections with alterations in innate and adaptive immune responses [107,332]. Many of the oral bacterial members like *Streptococcus gordonii, S. mitis, S. oralis* and *S. salivarius* possess the enzyme alcohol dehydrogenase, enabling these members to metabolize ethanol (alcohol) to carcinogenic acetaldehyde and hydroxyl radicals [325,333]. With significant loss of microbial diversity and subsequent alterations of the normal microbiome into pathogenic bacterial communities, smoking tobacco has been known to contribute to oral carcinogenesis [334].

Smoking can initiate several possible changes in the host, like immunosuppression, oxygen deprivation, and biofilm formation which can lead to the loss of beneficial bacteria, and perpetuation of pathogenic bacteria colonization in the oral cavity [107,114–116]. While dysbiosis of the oral microbiome is a hallmark condition for inflammatory diseases like periodontitis [335], the identification of specific oral microbiomes can play a major role as a diagnostic and prognostic biomarker of oral cancer [325]. Although opportunistic pathogens like *Bacillus licheniformis* and *B. pumilus* are known to be associated with lung inflammation, and *Staphylococcus epidermis* and *S. hominis* are known nitrate reducers, potentially leading to the formation of carcinogenic tobacco-specific N-nitrosamines, very few bacteria are identified as carcinogenic. Most of the carcinogens in tobacco smoke are formed from the pyrolysis of tobacco, but some bacteria act as initiators and some as promoters in the tumorigenesis process. Specifically, *Mycobacterium tuberculosis* is associated with lung cancer [336], *Fusobacterium n*ucleatum is proposed to be a risk factor for colon cancer [337–339], and *Bacillus fragilis* can act as an initiator of colon cancer [337,340]. Comparing smoking status, *Acidovorax* and *Klebsiella* were not only more abundant among

smokers' lung tissue (compared to non-smokers), but were also found to be enriched in squamous cell carcinoma tumor sections harboring TP53 (most common mutated gene in lung cancer) mutations, potentially acting as a promoter enhancing tumorigenesis [341]. *Acidovorax* has been identified in cigarette products and could metabolize organic pollutants in cigarette smoke [65,342]. Both *S. epidermis* and *S. hominis* have been identified in smokeless tobacco products [343] and these have the potential to be involved in causing bacterial endocarditis, due to potential transportation to the heart through the bloodstream. Enrichment of the oral bacteria *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* has been associated with a higher risk of pancreatic cancer [112]. While frequently associated with oral squamous cell carcinoma, one study reported an association between *Streptococcus anginosus* and DNA damage in the oral mucosa with increased synthesis of NO and cyclooxygenase [344], *S. salivarius, S. intermedius, S. mitis* and *Neisseria* sp. are known to possess alcohol dehydrogenase responsible for the production of hydroxyl ethyl radicals [333,345]. Although dysbiosis in oral microbiota has been indicated to be linked to HIV infection, gastrointestinal cancer, colorectal cancer, periodontitis, and diabetes [346–349], there remains limited data to conclusively build a causative relationship between a specific bacterium found in tobacco products and human disease.

In conclusion, Chapters 3, 4 and 5 of my dissertation provide a comprehensive characterization of the total and metabolically-active bacterial communities present in conventional tobacco products, and evaluate the potential immediate transfer and impact of these bacteria to the user's oral cavity over time. While there is potentially no transient transfer of the heterogenous tobacco-related bacteria to users' oral cavities, the oral microbiome diversity and composition are significantly different between tobacco (cigarette and smokeless tobacco) users

and non-users. Significant alterations in the prevalence and abundance of potentially pathogenic bacterial species in the oral samples of tobacco users are indicative of poor oral health. A perturbed balance in the oral microbiome associated with tobacco use and temporal stability of these bacterial communities in the oral cavity could potentially lead to oral disease development over time.

Appendix A

The predisposition of smokers to COVID-19 infection: A mini-review of global perspectives

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Abstract

With the current knowledge that both SARS-CoV-2 and smoking tobacco adversely impact the respiratory system, there is an ongoing debate in the scientific community about whether or not smokers have a stronger predisposition towards COVID-19 infections. Through this mini-review, we aimed to study the relationship between tobacco smoking and COVID-19 by conducting a comprehensive literature search using Google Scholar and PubMed databases. Peer-reviewed meta-analyses, cohort studies, scoping and systematic reviews, clinical trials, comments, and letters to editors on the effects of smoking tobacco on COVID-19 susceptibility published globally over the past two years (January 2020 – April 2022) were included. Our search identified 31 articles that demonstrated a positive or strong relationship between smoking and COVID-19, while 13 articles had contrasting results. Additionally, we evaluated mechanistic studies suggesting that, among smokers, angiotensin-converting enzyme-2 genes are upregulated, facilitating easier binding of SARS-CoV-2, thereby increasing the risk of COVID-19 infection. In conclusion, the majority of studies in this area to date provide evidence of a strong relationship between smoking and COVID-19 infection; however, the strength of this association may vary across the smoking behaviors of differing populations.

Introduction

In December 2019, the city of Wuhan, China witnessed the emergence of a new virus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It took the virus only 48 days to infect over 1,000 people, a significantly faster transmission rate compared to other similar viruses such as the 2002-2003 SARS-CoV and the 2012-2014 Middle East respiratory syndrome coronavirus (MERS-CoV). Within a short period of time, SARS-CoV-2 spread across China, and made its way across the globe, reaching nearly all countries by March 2020 [350]. As of May 5, 2022, with over 513,384,685 cases reported and close to 6,246,828 deaths globally, the COVID-19 (the disease caused by SARS-CoV-2) pandemic is still ongoing [351].

While the long-term impacts on the human body from this virus are still being studied extensively, the main organ system affected by this disease is the respiratory system [352]. COVID-19 infection often includes symptoms of the common cold, bronchitis, and pneumonia. Associated fluid saturation of the lungs leads to difficulty in breathing and more severe cases may result in acute respiratory distress syndrome, and ultimately lung failure and death. Additionally, sepsis, (when the infection spreads through the bloodstream) can also occur during a COVID-19 infection [353].

In contrast, the adverse health effects of smoking tobacco products have been studied in great detail over many decades and it is well-documented that tobacco use adversely affects the lungs [354,355]. In addition to restricting airflow, smoking inflames and irritates the lungs, causing mucus to build up and making the lungs more susceptible to a variety of bacterial and viral infections [356,357]. Tobacco smoking has also been shown to reduce the number of cilia in the lungs, causing chronic airflow limitation and reducing lung function [358,359]. These adverse health effects are not restricted to combustible traditional tobacco products alone. Electronic cigarette

(e-cigarette) users also experience lung inflammation and damage, but to a lesser degree than traditional smokers [360]. Moreover, smokers not only are 34% more likely to be infected with the flu when compared to non-smokers [361,362] but also carry a higher risk of hospital admittance [363].

Since both SARS-CoV-2 and tobacco smoking heavily affect the lungs, there is the potential that smoking could influence the transmission of COVID-19, or that a long-term smoker could be at a higher risk of acquiring the disease. To date, it has been demonstrated that the rate of COVID-19 infection, disease progression, and mortality rates in people with underlying health conditions and comorbidities are higher compared to that of healthy individuals [364,365]. While the most common comorbidities studied with COVID-19 disease progression are hypertension, diabetes, obesity, chronic obstructive pulmonary disease (COPD), cardiovascular diseases (CVD), and malignancies, multiple studies have demonstrated a statistically significant positive relationship between smoking and COVID-19 prevalence, including a higher risk of severe illness due to COVID-19 among active smokers (Table 1). In contrast, other studies point toward a potential protective benefit of smoking with regard to COVID-19 infection (Table 1). While this debate is ongoing, the objective of this mini-review was to better understand the predisposition of smokers to COVID-19 infection globally by summarizing the published studies to date in this area.

Studies reporting a direct relationship between smoking and severe COVID-19 symptoms, disease progression and mortality

The first study that demonstrated a potential link between smoking and COVID-19 was a systematic review and meta-analysis of studies completed in China [366]. The study was published online on March 20, 2020, only a few days (March 11, 2020) after the World Health

Organization (WHO) declared COVID-19 a global pandemic. This meta-analysis included five independently researched studies of which four were retrospective [357,367–369] and one was prospective [370]. Overall, the meta-analysis revealed that smokers were 1.4 times more likely to have severe symptoms of COVID-19 and were 2.4 times more likely to be admitted to an intensive care unit (ICU), need medical ventilation, or die compared to non-smokers. While the above study found that smokers were more likely to have severe symptoms of COVID-19, a second meta-analysis by Patanavanich and Glantz (2020) demonstrated a likelihood of COVID-19 disease progression with smoking. This study included data available from 19 peer-reviewed papers (China (16), Korea (1), and the United States (U.S.) (2)) that were accessible from PubMed on April 6, 2020 [371]. This meta-analysis included a total of 11,590 individuals among which 731 COVID-19 patients (6.3%) had a history of smoking. Out of these smokers, 29.8% demonstrated disease progression as compared to 17.6% of non-smokers that experienced disease progression. The authors concluded that patients who are smokers were significantly more likely to experience disease progression and severe COVID-19 infection compared to nonsmokers (OR = 1.91; 95% CI: 1.42-2.59).

Similarly, Karanasos et al. (2020) conducted a meta-analysis that included 22 studies of which 17 reported on severity, four on morbidity, and one on both [372]. The analysis included 6,310 patients from both the U.S. and China and found that smoking increased the risk of a more severe response to COVID-19 (OR=1.34; 95% CI: 1.07-1.65). This meta-analysis also demonstrated an increased risk in the severity of COVID-19 patients among smokers but failed to show an increase in the mortality rate. Another retrospective cohort study conducted in Kuwait examined 1,096 patients, screened for risk factors that affected admission to ICUs, and also found a significant association between hospital admission and smoking (OR = 5.86; 95%

CI: 1.40-24.47; $p = 0.015$) [373]. In addition, the authors calculated mortality risk and found a significant association (OR: 10.09; 95% CI: 1.22-83.40; $p = 0.032$) among smokers. Furthermore, two more studies evaluated the relationship between smoking and the risk of hospitalization and death from COVID-19 among 10,713 and 3,403 patients diagnosed with COVID-19 respectively [374] [375]. While the Soares et al (2020) study concluded that smoking was one of the comorbidities strongly associated with hospitalizations ($p = 0.053$), the Emami et al. (2020) meta-analysis of 10 articles noted smoking as a prevalent underlying cause among patients hospitalized for COVID-19.

Subsequently, a meta-analysis of 46 peer-reviewed papers with a total of 22,939 COVID-19 patients, demonstrated an increased risk of COVID-19 disease progression among ever smokers (OR= 1.59; 95% CI: 1.33 – 1.89) [376]. In addition, the authors found that smoking was associated with an increased risk of mortality from COVID-19 (OR= 1.19; 95% CI:1.02 – 1.39) and disease progression among younger adults. In contrast, a cross-sectional observational study of 1,769 sailors found that older adults (>50 years old) were at a significantly higher risk of COVID-19 symptoms compared to <50 years old adults (OR= 2.84; 95% CI: 1.30 – 7.5) [377]. Another meta-analysis of 13 studies including 3,027 patients demonstrated that older males (>65 years old) and smokers were at a higher risk of COVID-19 disease progression (> 65 years old males OR = 6.06, 95% CI: 3.98 - 9.22); current smoking OR = 2.51, 95% CI: 1.39 - 3.32) [378]. Finally, a multi-center observational study from Malaysia included 5,899 COVID-19 patients and showed that even though the ever-smokers were at a higher risk of having COVID-19 complications (respiratory distress syndrome, renal and liver injury), there was no significant difference between disease outcomes when compared to non-smokers [379].

The predisposition of current vs. former smokers to COVID-19 symptoms, disease progression, hospitalization and mortality

As the pandemic progressed, a few studies evaluated the effect of COVID-19 among current smokers, former smokers, and non-smokers. For example, a review article by Alqahatani et al. (2020) analyzed 15 studies from China that examined 2,473 confirmed cases of COVID-19 among current smokers [380]. The authors observed that current smokers were 1.45 times more likely to have severe symptoms from COVID-19 compared to former smokers or people who have never smoked (RR = 1.45, 95% CI: 1.03–2.04). Current smokers also had a higher mortality rate (38.5%). Similarly, in a systematic review and meta-analysis of 40 articles, Umnuaypornlert et al. (2021) found that both current and former smokers experienced statistically significantly greater COVID-19 severity (OR=1.58; 95% CI: 1.16–2.15; and OR=2.48; 95% CI: 1.64– 3.77; respectively) and an increased risk of death (OR=1.35; 95% CI: 1.12–1.62; and OR=2.58; 95% CI: 2.15–3.09; respectively) compared to non-smokers [381].

To evaluate the association between the history of smoking and the severity of COVID-19 symptoms, a meta-analysis of 16 articles was then performed [382]. While a significant association was found between a history of smoking and severe COVID-19 cases (OR = 2.17; 95% CI: 1.37–3.46; $p < 0.001$), non-smokers (10.7%) had less severe COVID-19 symptoms compared to active smokers (21.2%). The cross-sectional study on sailors mentioned above found similar results with active smokers (OR = 0.59; 95% CI: 0.45–0.78; $p < 0.001$) characterized by a significantly higher COVID-19 prevalence when compared to former smokers (OR = 0.98; 95% CI: 0.70–1.38; $p = 0.93$) [377]. A systematic review article including 47 studies (China (32), U.S. (10), Italy (2), U.K. (1), and International (2)) focused on the effects of smoking on COVID-19 [383]. Studies included in this review paper reported hospitalization and

mortality rates for COVID-19 patients from early December 2019 to early June 2020. The overall meta-analysis in this review article (n = 32,849 study participants) demonstrated an increased risk of COVID-19 among smokers (RR= 1.35; 95% CI: 1.19-1.53). This study also demonstrated that a history of smoking was associated with an increased risk of mortality among patients (RR = 1.26; 95% CI: 1.20 – 1.32). Current smokers were found to be at a statistically significantly higher risk of severe COVID-19 infection (RR= 1.80; 95% CI: 1.14- 2.85). Similar to the above-mentioned study by Vardavas and Nikitara (2020), the authors also observed an association between severe or critical cases of COVID-19 and smoking, in-hospital mortality, and disease progression ($p < 0.05$) [366]. In contrast, a retrospective cohort study from the U.S. involving 10,216 patients showed significantly more hospitalization rates from COVID-19 among former smokers (OR 2.31; 95% CI 1.94-2.74) when compared to current smokers (OR= 0.68; 95% CI: 0.60–0.99) [384].

Similar to the above-mentioned research articles, several studies from the U.K. found a significant association between current smoking and COVID-19 symptoms. First, a cross-sectional survey of 53,002 people in the U.K. found that current smoking increases the risk of COVID-19 infection [385]. In the survey, 25.7% were ex-smokers and 15.2% were current smokers. Former smokers (OR=1.07; 95% CI: 1.01-1.15) and current smokers (OR= 1.11; 95% CI: 1.03-1.20) had a higher prevalence of COVID-19 when compared to non-smokers. another study from the U.K. evaluated the risk of COVID-19 among tobacco smokers via the Zoe COVID-19 Symptom Study app used by over 2.4 million people [386]. The results showed that current smokers (11%) were not only more prone to report COVID-19 related symptoms (OR=1.14, $p < 0.05$) but also had a higher symptom burden (greater number of symptoms) (OR = 1.42, $p < 0.05$) and were more frequently admitted into the hospital (OR= 2.11, $p < 0.05$) when

compared to non-smokers. Another cohort study in England evaluating the effects of smoking via a questionnaire among 387,109 participants found that current smoking increased the relative risk of COVID-19 1.45 times (95% CI:1.16-1.83) when compared to never smoking, while past smoking increased the relative risk of COVID-19 1.34 times (95% CI: 1.15-1.56) compared to never smoking [387]. A fourth study from the U.K. examined 1,649 COVID-19 patients, 968 hospitalizations, and 444 COVID-19-related deaths [388]. Mendelian randomization analyses revealed current smokers to be at a higher odds of COVID-19-related hospitalization (OR= 1.80, 95% CI: 1.26 -2.29) when compared to non-smokers. Additionally, a higher number of cigarettes smoked per day was also positively correlated with a higher risk of all infection outcomes (OR= 2.51, 95% CI: 1.20 - 5.24). Finally, a large cohort study from England including 7,869,534 participants evaluated the risk of current and former smokers to hospitalization and mortality from COVID-19 [389]. The authors found that while all smokers had a higher risk for all-cause mortality when compared to non-smokers, current smokers were at a reduced risk of severe COVID-19 symptoms compared to former smokers who were at a higher risk of hospitalization (hazard ratio = 1.07 (1.03 – 1.11)) and death HR 1.17 (1.10 – 1.24).

Furthermore, a literature review by Farsalinos et al. (2020) of 18 relevant studies (China (15); U.S. (2); South Korea (1)) was performed to examine the relationship between adverse outcomes of COVID-19 and smoking [390]. The overall review included 6,515 patients with a smoking prevalence of 6.8%. Results from this study found that current smokers were 1.53 times more likely to experience adverse effects from a COVID-19 infection than non-smokers ($p = 0.022$). However, current smokers were 0.42 times less likely to have an adverse outcome than former smokers ($p = 0.003$). Similarly, 13 additional studies demonstrated that current smoking was more prevalent among COVID-19 patients exhibiting severe COVID-19 outcomes

compared to patients experiencing non-severe outcomes [391]. Former smokers (OR 3.46; 95% CI: 2.46–4.85) and current smokers (OR 1.98; 95% CI: 1.16–3.39) also had significantly higher odds of severe COVID-19 outcomes.

Additional risk factors and co-morbidities beyond smoking impact COVID-19 symptoms, disease progression, hospitalization and mortality

While the data from the above-mentioned articles indicate that current smoking causes a higher rate of infection from COVID-19 when compared to former smoking, there have been additional studies demonstrating that other factors may also play a significant role in exacerbating the symptoms of COVID-19. For example, a few meta-analyses have evaluated the effects of COPD and smoking on patients with COVID-19. One such meta-analysis (comprising 21 peer-reviewed articles) included 4,603 patients and found that smokers have a 1.65 times higher risk of severe COVID-19 when compared to non-smokers (OR= 1.65; 95% CI: 1.17 - 2.34) [392]. While the meta-analysis found smoking increased the risk of composite poor outcome, COPD was also associated with an increased risk of severe COVID-19 (OR= 4.62; 95% CI: 2.49 – 8.56). Another systematic review and meta-analysis by Zhao et al. (2020) included 12 Chinese studies and also analyzed the impact of COPD and smoking history on COVID-19 infection [393]. This study evaluated a total of 2,002 patients, but only 7 of the 12 studies included reported the smoking history of the individuals. A compilation of the data found that while smoking increases the risk of severe COVID-19 infection by about a factor of 2 (OR = 1.98; 95% CI: 1.29-3.05), COPD was closely linked to the development of severe COVID-19 (OR= 4.38; 95% CI: 2.34 – 8.20). However, when one of the studies that had the largest sample size (that seemed to heavily impact their results) was removed, the impact of smoking on COVID-19 severity became

statistically insignificant. The authors concluded that although their overall data indicated that smoking and COPD increases the severity of COVID-19 infection, there were limitations in the studies and further data is required to generate more conclusive results.

Other studies have assessed additional risk factors such as diabetes and hypertension, along with smoking status. For example, a meta-analysis of 109 articles involving 517,020 COVID-19 patients provided evidence of a statistically significant relationship between smoking and COVID-19 severity using a random-effects model, controlling for age, gender, and other risk factors (OR= 1.55; 95% CI: 1.41 – 1.71) [394]. Additionally, the authors observed a statistically significant association between smoking and the risk of admission to an ICU (OR=1.73, 95% CI: 1.36-2.19), increased mortality (OR=1.58, 95% CI: 1.38-1.81), and critical disease composite endpoints (e.g., acute respiratory distress syndrome, invasive ventilation or death) (OR=1.61, 95% CI: 1.35-1.93). The study also showed that other covariates that significantly affect the association between smoking status and severe COVID-19 were age (p= 0.004), hypertension (p= 0.007), diabetes (p= 0.029), and COPD (p = 0.001).

Impact of smoking non-cigarette products on COVID-19 symptom severity

In addition to cigarette smoking, a few studies have evaluated the impacts of non-cigarette products (like hookah and e-cigarettes) and nicotine replacement therapy (NRT) on COVID-19; however, no conclusive effects have been observed [395]. In the review by Kashyap et al. (2020), smoking tobacco was associated with an increase in the severity of, but not the prevalence of, COVID-19 infection [395]. The authors also suggested that smoking leads to increased lung inflammation which may be a cause of increased morbidity and mortality associated with COVID-19. Similarly, a representative population survey by Tattan-Birch et al.

(2021) found that current and past smoking increased the prevalence of COVID-19 infection [396]. The survey included 3,179 participants and examined factors including smoking status, e-cigarette use, and NRT. The odds of smokers (20.9% of the study sample) self-reporting COVID-19 were significantly higher (OR =1.34, 95% CI:1.04-1.73) when compared with never smokers (14.5%). However, the survey found no significant relationship between COVID-19 and e-cigarette use or NRT. A limitation of the study was information bias since participants were self-reporting COVID-19 symptoms. Another cohort study from the U.S. included 69,264 patients who self-reported to be either e-cigarette, traditional cigarette, or dual users [397]. While the authors found no statistically significantly higher susceptibility to COVID-19 associated with smoking e-cigarettes (OR= 0.93; 95% CI: 0.69-1.25), smoking cigarettes or both products decreased the COVID-19 diagnosis (OR= 0.43; 95% CI: 0.35 – 0.53 and OR= 0.67; 95% CI: 0.49 – 0.92), respectively. A second community-based cohort study from the U.S. studied 8,214 participants with self-reported tobacco use [398]. While smoking cigarettes or e-cigarettes was inversely related to COVID-19 infection rates (OR= 0.49; 95% CI: 0.32 – 0.74 and OR= 0.654; 95% CI: 0.32 – 1.35), respectively, using smokeless tobacco was positively associated with COVID-19 infection rates (OR= 2.17; 95% CI: 1.26 – 3.72).

In contrast to the above-mentioned work, a study by Gaiha et al. (2020) postulated that the youth e-cigarette epidemic is directly related to the COVID-19 pandemic and sought to understand a possible link between youth smokers and COVID-19 in the U.S. [399]. Surveying 4,351 people between the ages of 13 to 24, the authors revealed that youth who used e-cigarettes exclusively were five times more likely to be diagnosed with COVID-19 than their non-vaping peers (OR 5.00, 95% CI: 1.82-13.96). Additionally, youth who ever smoked both e-cigarettes and traditional cigarettes (dual users) were seven times more likely (OR 7.00, 95% CI: 1.98–

24.55) to develop COVID-19 symptoms. The study also examined the impact of more frequent cigarette use and found that youth who were dual users in the past 30 days were 6.8 times more likely to have a COVID-19 infection (OR 6.8, 95% CI: 2.40–19.55) and were also 4.7 times more likely to develop symptoms (OR 4.7, 95% CI: 3.07–7.16). A limitation of the study was that COVID-19 hospitalization or the severity of symptoms was not included.

Relationship between angiotensin-converting enzyme-2 (ACE-2), smoking and COVID-19 infection

The pathophysiology of SARS-CoV-2 demonstrates that the angiotensin-converting enzyme-2 (ACE-2) is the cell receptor for this virus [372]. Potentially, different factors that cause an increased expression of the ACE-2 enzyme on the alveolar epithelial cells, similar to the effect of smoking, may result in a higher susceptibility to and greater severity of COVID-19 infection. Chronic exposure to NO_2 from smoking can cause an increase in ACE-2 expression in the alveolar epithelial cells, suggesting that smoking likely increases the risk of COVID-19, as well as the severity and mortality from the infection. Another study summarized the mechanism of receptor-ligand function and its relationship to the behavior of SARS-CoV-2 infection among smokers [400]. These authors reported that tobacco smokers are 1.4 times more likely to develop severe symptoms of COVID-19 infection and have a 2.4 times higher mortality rate. The authors explain that the upregulation of the ACE-2 gene not only increases the risk of COVID-19 infection but also can lead to cytokine storms causing lung injury in COVID-19 patients. Additionally, the authors observed a 100-fold increase in ACE-2 activity following increased exposure to NO_2 from tobacco smoking, which explains the higher prevalence of COVID-19 among smokers.

A cross-sectional study that examined the impact of smoking on COPD also evaluated the expression of the ACE-2 gene [401]. The study found that current smokers were characterized by a significantly increased expression of the ACE-2 gene, but former smokers revealed no increase in ACE-2 gene expression. The authors concluded that active cigarette smoking upregulated ACE-2 expression, which may increase the risk of severe COVID-19 infection. Another review encompassing data from Iran, China, Italy, and South Korea showed that the SARS-CoV-2 virus has a 10-20 times higher affinity for the ACE-2 receptors than previous SARS-CoV viruses [402]. Additionally, the study also reported that men have higher smoking rates and COVID-19 infection rates than women, indicating that gender is a potential confounder of the relationship between smoking and COVID-19 infection. While this review reiterated that smoking causes the ACE-2 receptor to be upregulated, leading to higher infection rates for smokers, the authors warn that this upregulation in ACE-2 receptors not only increases the risk of infection but also the transmission of the virus. Lastly, this paper reported that users of e-cigarettes and "heat-not-burn" devices are just as likely to have these increased risks of an upregulated ACE-2 receptor. Similar to previous studies, in an evaluation of ACE-2 expression in SARS-CoV-2 infections, Li et al. (2020) and Russo et al. (2020) also found a higher expression of ACE-2 among smokers, likely leading to a greater risk of SARS-CoV-2 [403,404]. The above-mentioned studies reinforce the idea that smoking upregulates ACE-2 and facilitates easier binding to the SARS-CoV-2 virus, suggesting that long-term smokers are at a greater risk of COVID-19 infection.

Studies supporting the inverse relationship between smoking and COVID-19 prevalence

In contrast to all of the above-mentioned studies, multiple studies across different countries have demonstrated an inverse relationship between smoking tobacco and developing COVID-19 symptoms. A case-control study from Mexico included 32,583 patients (12,304 COVID-19 positive cases and 20,279 COVID-19 negative controls) of which 2.3% of cases were smokers and 4.3% of controls had a history of smoking [405]. This study not only concluded that active smokers had a decreased likelihood of developing COVID-19 (females, adjusted OR=0.49 (95% CI: 0.31 – 0.78); males, adjusted OR=0.64 (95% CI 0.51-0.81)) but also suggested that nicotine in tobacco might have a therapeutic effect. Similarly, a study with 331,298 patients in Mexico studied the clinical characteristics affecting the mortality of patients with COVID-19 [406]. According to their multivariate logistic regression model, smoking was not statistically significantly associated with mortality risk or development of COVID-19 symptoms. However, the authors suggest their multivariate analysis might be influenced by the sex of the patient.

Similar to the results from the study by Hernández-Garduño (2020), Tsigaris and Silva (2020) also reported a negative relationship ($p > 0.05$) between COVID-19 mortality and smoking and suggested that nicotine may provide some protection from the infection [353]. Comparing 38 European nations, the authors found that countries with higher smoking rates had fewer COVID-19 cases per million people than countries with lower rates of smoking after controlling for confounding variables including economic activity and COVID-19 prevention measures in different countries. Greece, which has a smoking rate of 43.4%, the highest of any country analyzed, had only 280 COVID-19 positive cases per million people, the lowest of any country analyzed. Furthermore, while the average smoking prevalence among the five countries with the highest smoking rates was 39.5%, they only had an average of 1,084 COVID-19 cases

per million and 29 COVID-19 deaths per million people. This was substantially lower when compared to the five countries with the lowest smoking rate, which had an average smoking prevalence of 18.6% but had 2,754 COVID-19 cases per million people and 133 COVID-19 mortalities per million people. However, the study did not find a statistically significant (p= 0.626) relationship between COVID-19 mortality rates and smoking.

Two cross-sectional studies evaluating the correlation between active smokers and COVID-19 positivity rates showed similar results [407,408]. The first study included 340 inpatients and 130 outpatients with a smoking rate of 4.1% and 6.1%, respectively [407]. The authors concluded that in comparison to the smoking rate among the general population (25.4%), the smoking prevalence in symptomatic COVID-19 patients was lower. The second study collected data from a primary care network in the U.K. to analyze the effect of several clinical risk factors, including smoking, on COVID-19 [408]. The study found that while active smokers had a positive COVID-19 test rate of approximately 6% less than that of non-smokers (adjusted OR = 0.49; 95% CI: 0.34-0.71), ex-smokers had a positive test rate of 0.6% less than that of non-smokers (adjusted OR = 0.87; 95% CI: 0.69-1.10). Despite these results, the authors concluded that more research is necessary before any conclusions about the impact (or lack thereof) of smoking on COVID-19 can be made.

A meta-analysis of five studies from China was published corroborating the previous negative relationship between smoking and COVID-19 infections [409]. This analysis found no significant relationship between smoking and COVID-19 (OR = 1.69; 95% CI: 0.41–6.92; and p = 0.254). Interestingly, the authors used the same five studies as Vardavas et al. (2020) but came to a different conclusion. This perhaps demonstrates the impact that limited data have on study findings and indicates that more research is needed to come to a definitive conclusion. Another

meta-analysis of 7,162 patients was performed using 20 studies from China and 2 from the U.S. [410]. The authors found no significant association between smoking and COVID-19 disease severity (OR 1.40, 95% CI 0.98–1.98) and mortality (OR 1.86, 95% CI 0.88–3.94). Lastly, after reviewing 12 peer-reviewed articles from different regions of the world, Tajlil et al. (2020) observed a statistically significantly ($p < 0.001$) lower proportion of COVID-19 patients with a smoking history compared to what was expected, given the population averages of the geographic areas studied [411].

Controversies regarding the true nature of the relationship between smoking and COVID-19 prevalence and symptoms

A spatial epidemiological approach utilizing World Health Organization (WHO) data from 175 countries showed that the prevalence of smokers significantly explained global variation in COVID-19 outbreaks [412]. Nevertheless, controversies have arisen within the scientific community regarding the true relationship between smoking tobacco and developing COVID-19 symptoms. For example, Carmona-Bayonas et al. (2020) performed a Bayesian statistical analysis of the data from the review article by Lippi and Henry (2020) [409,413]. Lippi and Henry (2020) claimed that their data showed that smoking had no relationship with COVID-19 symptoms. However, Carmona-Bayonas et al. (2020) argued that this was an example of "absence of evidence is not evidence of absence." Furthermore, in the Bayesian analysis performed, the authors found that active smoking increases the severity of COVID-19 infection (OR =1.79; 95% CI: 0.86-4.13). Similarly, Guo et al. (2020) also dissected the analysis performed by Lippi and Henry (2020) and claimed that their conclusion was incorrect [409,414]. Guo first asserts that the figures that Lippi and Henry used in their statistical analysis were not

consistent with the results of the studies that they analyzed. Performing an updated meta-analysis, Guo et al. (2020) found that the pooled OR was 2.20 (95% CI: 1.31–3.67; $p = 0.003$) and not 1.69 (95% CI: 0.41-6.92; $p = 0.254$) as suggested by Lippi and Henry (2020). This provided evidence that Lippi and Henry's (2020) conclusion that smoking does not increase the prevalence of COVID-19 may not be accurate. Subsequently, multiple myths concerning the topic of smokers being potentially protected against SARS-CoV-2 have evolved, and subsequent articles discussing the flaws in these interpretations have also been published [415,416]. The authors of these rebukes shed light on the methodical limitations of some of the articles that demonstrate an inverse relationship between smoking and COVID-19 complications.

Conclusions

Overall, the majority of the papers cited in this mini-review (31 articles) found a positive relationship between smoking tobacco (or active smokers) and COVID-19 infection, while 13 articles reported contrasting results. The papers reviewed included data concerning the impact of smoking on the risk of being infected by SARS-CoV-2 and the severity of the disease once infected. Differentiating the type of tobacco products used, some data indicated that e-cigarettes may increase susceptibility to COVID-19 but to a lesser extent than traditional smoking.

Overall, given the multitude of factors that might be responsible for exacerbating COVID-19 symptoms, there remains much to be researched. However, as evidenced by this literature review, the great majority of papers to date evaluating the associations between smoking and COVID-19 indicate that smoking may worsen COVID-19 infection and increase transmission. Moreover, some studies that have demonstrated a protective relationship between

smoking and COVID-19 have later been shown to have methodological issues, suggesting that the findings from these studies should be viewed with caution.

11. Table A1: Studies evaluating the relationship between smoking and COVID-19 symptoms, hospitalization and deaths.

	Supporting studies	Study type (#articles)	Sample size	Nature of relationship with COVID-19
Positive relationship	**Smokers vs. non-smokers**			
	Vardavas and Nikitara 2020	Systematic review (5)	1549	Symptom prevalence RR = 1.4; 95% CI: 0.98–2.00
	Patanavanich and Glantz 2020	Meta analysis (19)	11,590	Disease progression OR = 1.91; 95% CI: 1.42-2.59
	Reddy et al 2021	Systematic review (47)	32,849	RR= 1.35; 95% CI: 1.19-1.53
	Karanasos et al. 2020	Meta analysis (22)	6,310	OR=1.34; 95% CI: 1.07-1.65
	Almazeedi et al. 2020	Retrospective cohort study	1,096	Hospitalization OR = 5.86; 95% CI: 1.40-24.47
	Emami et al. 2020	Meta analysis (10)	76,993	
	Soares et al. 2020	Cohort	10,713	Hospitalization OR= 5.12; 95% CI: 3.82-6.81
	Patanavanich and Glantz 2021	Meta analysis (46)	22,939	Disease progression OR 1.59, 95% CI 1.33–1.89
				Mortality rate OR 1.19, 95% CI 1.02–1.39
	Paleiron et al. 2021	Cross-sectional	1,769	OR= 2.84; 95% CI: 1.30 – 7.5
	Zheng et al., 2020	Meta analysis (13)	3,027	OR = 2.51; 95% CI: 1.39–3.32
	Current and fomer smokers vs. non-smokers			
	Alqahtani et al. 2020	Meta analysis (15)	2,473	RR = 1.45; 95% CI: 1.03–2.04 (current)
	Umnuaypornlert et al. 2021	Meta analysis (40)		Disease severity OR=2.48; 95% CI: 1.64–3.77 (former)
				Disease severity OR=1.58; 95% CI: 1.16–2.15 (current)
				Mortality rate OR=2.48; 95% CI: 1.64– 3.77 (former)
				Mortality rate OR=1.35; 95% CI: 1.12–1.62 (current)
	Gülsen et al. 2020	Meta analysis (16)	11322	OR=1.51; 95% CI: 1.12–2.05 (current)
	Jackson et al. 2020	Cross-sectional	53,002	OR=1.07; 95% CI: 1.01-1.15 (former)
				OR= 1.11; 95% CI: 1.03-1.20 (current)
	Hopkinson et al. 2021	Survey	2.4 million	Symptoms prevalence OR = 1.42, p < 0.05
				Hospitalization rate OR= 2.11, p < 0.05
	Hamer et al. 2020	Cohort	387,109	RR=1.34; 95% CI: 1.15-1.56 (former)
				RR= 1.45; 95% CI: 1.16-1.83 (current)
	Clift et al. 2021	Observational	421,469	Hospitalization rates OR = 1.80; 95% CI: 1.26 -2.29 (current)
	Farsalinos et al. 2020	Review (18)	6,515	OR= 1.53; p = 0.022 (current)
	Sanchez-Ramirez and Mackey 2020	13		OR= 3.46; 95% CI: 2.46–4.85 (former)
				OR= 1.98; 95% CI: 1.16–3.39 (current)
	Puebla Neira et al. 2021	Retrospective cohort study	10216	Hospitalization rates OR 2.31; 95% CI 1.94-2.74 (former)
				Hospitalization rates OR= 0.68; 95% CI: 0.60–0.99 (current)
	Paleiron et al. 2021	Retrospective cohort study	1688	Symptoms prevalence OR OR = 0.98; 95% CI: 0.70–1.38 (former)
				symptoms prevalence OR = 0.59; 95% CI: 0.45–0.78 (current)
	Gao et al. 2022	Cohort	7869534	Hospitalization rates HR= 1.07; 95% CI:1.03 – 1.11 (former)
				Hospitalization rate HR = 0.36 ; 95% CI: 0.17 to 0.76 (current)
				Mortality rate HR= 1.17; 95% CI:1.10 to 1.24 (former)
				Mortality rate HR= 0.77; 95% CI:0.54 to 1.10 (current)
	Co-morbidities and smoking			
	Pranata et al. 2020	Meta analysis (21)	4,603	Symptom prevalence OR = 1.65; 95% CI: 1.17 - 2.34
	Zhao et al. 2020	Systematic review (12)	2,002	Symptom prevalence OR 1.98; 95% CI: 1.29 - 3.05
	Zhang et al. 2021	Meta analysis (109)	517,020	Mortality rate OR=1.58; 95% CI: 1.38–1.81
				Hospitalization rate OR=1.73; 95% CI: 1.36-2.19
	Jayarajan et al. 2020	Prospective	59	Mortality rate OR=1.58; 95% CI: 1.38-1.81
	Non-cigarette smoking			
	Gaiha et al. 2020	Survey	4,351	OR 5.00; 95% CI: 1.82-13.96 (e-cig only)
				OR 7.00; 95% CI: 1.98–24.55 (e-cig + cig)
	Tattan-Birch et al. 2021	Survey	3179	OR =1.34, 95% CI:1.04-1.73
	Jose et al. 2021	Cohort	69,264	OR= 0.67; 95% CI: 0.49 – 0.92; p = 0.013 (e-cig + cig)
	Duszynski et al. 2021	Cohort	8214	OR= 2.17; 95% CI: 1.26 – 3.72 (smokeless tobacco)
Negative relationship	**Smokers vs. non-smokers**			
	Hernández-Garduño 2020	Case-control	32,583	Females OR=0.49; 95% CI: 0.31 – 0.78
				Males OR=0.64; 95% CI 0.51-0.81
	Parra-Bracamonte et al. 2020		331,298	Symptom prevalence OR= 1.175; 95% CI: 1.130- 1.221
	Tsigaris and Teixeira da Silva 2020			Mortality rate p> 0.05
	de Lusignan et al. 2020	Cross-sectional		OR = 0.49; 95% CI: 0.34-0.71
	Lippi and Henry 2020	Meta analsysis (5)		OR = 1.69; 95% CI: 0.41–6.92
	Farsalinos et al. 2021	Meta analsysis (20)	7,162	Symptom prevalence OR 1.40; 95% CI 0.98–1.98
				Mortality rate OR 1.86; 95% CI 0.88–3.94
	Duszynski et al. 2021	Cohort	8214	OR= 0.654; 95% CI: 0.32 – 1.35 (e-cig)
				OR= 0.49; 95% CI: 0.32 – 0.74 (cig)
	Jose et al. 2021	Cohort	69,264	OR= 0.93; 95% CI: 0.69-1.25; p= 0.628 (e-cig only)

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