



Conventional tobacco products harbor unique and heterogenous microbiomes

Suhana Chattopadhyay^a, Padmini Ramachandran^b, Leena Malayil^a, Emmanuel F. Mongodin^{c,1}, Amy R. Sapkota^{a,*}

^a Maryland Institute of Applied Environmental Health, School of Public Health, University of Maryland, College Park, MD, USA

^b Food and Drug Administration, Office of Regulatory Science, Division of Microbiology, HFS-712, College Park, MD, USA

^c Institute for Genome Sciences and Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD, USA

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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 (using Linear discriminant analysis Effect Size (LEfSe)), a 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. *Methylobacterium* 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.

1. 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) (FDA, 2020). 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 (FDA, 2021). 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 (Kurup et al., 1983; Eaton et al., 1995;

* Corresponding author.

E-mail address: ars@umd.edu (A.R. Sapkota).

¹ Current address: National Institutes of Health (NIH), National Heart, Lung and Blood Institute, (NHLBI), Division of Lung Diseases, Bethesda, MD.

Larsson et al., 2008; Sapkota et al., 2010; Tyx et al., 2016; Chopyk et al., 2017b; Smyth et al., 2017; Hani et al., 2018; Malayil et al., 2020a). 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 (Kurup et al., 1983; Eaton et al., 1995). Subsequently, multiple bacterial genera (e.g., *Bacillus*, *Pseudomonas aeruginosa*, *Klebsiella*, and *Methylobacterium*) were identified in cigarettes via 16S rRNA-based microarrays, cloning and sequencing (Sapkota et al., 2010), while *Halomonas*, *Staphylococcus*, and *Pseudomonas* were detected in hookah tobacco (Hani et al., 2018), and *Bacillus*, *Staphylococcus*, and *Lactobacillus* were identified in smokeless tobacco products (Tyx et al., 2016; Al-hebshi et al., 2017; Smyth et al., 2017) via either qPCR or 16S rRNA V1–V4 sequencing. More recently, our group and others have extensively characterized tobacco microbiomes using 16S rRNA gene 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 (Tyx et al., 2016; Chopyk et al. 2017a, 2017b; Hani et al., 2018; Chattopadhyay et al., 2019; Smyth et al., 2019; Malayil et al. 2020a, 2022b).

The high-throughput sequencing approaches used in these studies have enabled us to catalog the vast array of bacteria (including low abundant members) associated with 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 (Gast et al., 2018; Ni et al., 2020; Fareed Mohamed Wahdan et al., 2020; Malayil et al., 2020b; Hirohara et al., 2021; Millette et al., 2021). 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 (Hamasaki, 2006; Allison et al., 2007; McGuire et al., 2010; Robbins et al., 2011; Galand et al., 2013b; Tada and Grossart, 2014; Treseder et al., 2014; Malayil et al., 2020b; Ambaye et al., 2020; Wahdan et al., 2021; Pold et al., 2021). 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.

2. Methods

2.1. 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),

Table 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
	Cheyenne menthol (CMB)	Menthol	
Little cigars (n = 108)	Cheyenne full flavor (CFF)	Non menthol	
	Swisher sweets cherry (SSC)		Cherry
	Swisher sweets original (SSO)		Non flavored
Cigarillos (n = 36)	NRC 102 (0.44 mg/g nicotine)	Non menthol	
	NRC 200 (1.33 mg/g nicotine)	Non menthol	
	NRC 300 (2.38 mg/g nicotine)	Non menthol	
	NRC 400 (5.02 mg/g nicotine)	Non menthol	
	NRC 404 (7.74 mg/g nicotine)	Non menthol	
	NRC 600 (15.54 mg/g nicotine)	Non menthol	
	NRC 103 (0.62 mg/g nicotine)	Menthol	
	NRC 201 (1.22 mg/g nicotine)	Menthol	
	NRC 301 (2.38 mg/g nicotine)	Menthol	
	NRC 401 (5.18 mg/g nicotine)	Menthol	
Research cigarettes (n = 72)	NRC 501 (12.44 mg/g nicotine)	Menthol	
	NRC 601 (15.42 mg/g nicotine)	Menthol	

Marlboro red (MRB), Newport non-menthol (NNM), Newport menthol (NMB2) and Newport menthol gold (NMG). The research cigarettes were custom-made SPECTRUM cigarettes (obtained from the National Institute of Drug Abuse, MD (NIDA)) and included six pairs of mentholated and non-mentholated varieties with varying concentrations of nicotine. 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).

2.2. 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 (Fadrosh et al., 2014; Chopyk et al., 2017b; Holm et al., 2019). 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. Negative control samples contained 1X PBS and no tobacco. 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 staphyloxyticus* (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 (ACTCCTACGGGAGG-CAGCAG) 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 (Fadrosh et al., 2014; Holm et al., 2019).

2.3. 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 μ L 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 (Urbach et al., 1999; Malayil et al., 2020b) and sequenced as mentioned above.

2.4. Sequencing reads analysis

16S rRNA reads were screened for low quality and short length and assembled using PANDAseq, demultiplexed, and chimera trimmed using UCHIME. Taxonomic assignments and relative abundance of bacterial taxa (for each sample) used for comparative analyses were determined with the Kraken 2 classification tool (Wood and Salzberg, 2014) and Bracken abundance estimator (Lu et al., 2017) from quality trimmed pair-end reads. Quality reads for BrdU and NO TRT samples were incorporated into QIIME v1.9, clustered de-novo using VSEARCH and taxonomies were assigned using the Greengenes database, using a 0.97 confidence threshold. Downstream statistical analysis and visualization of data were performed in R Statistical computing software (v. 0.99.473), Microbiome Analyst (Dhariwal et al., 2017; Chong et al., 2020), 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.

2.5. Statistical analysis

After quality trimming and removing samples that had a Good's 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 (Segata et al., 2011) to detect differentially abundant taxa and their effect size. The False Discovery Rate (FDR)-adjusted p -value was set to < 0.05 and the LDA was set to > 2.0 .

2.6. 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. Results

3.1. 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 (Fig. 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 (Fig. 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 (Fig. 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* (Fig. 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 (Fig. 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.

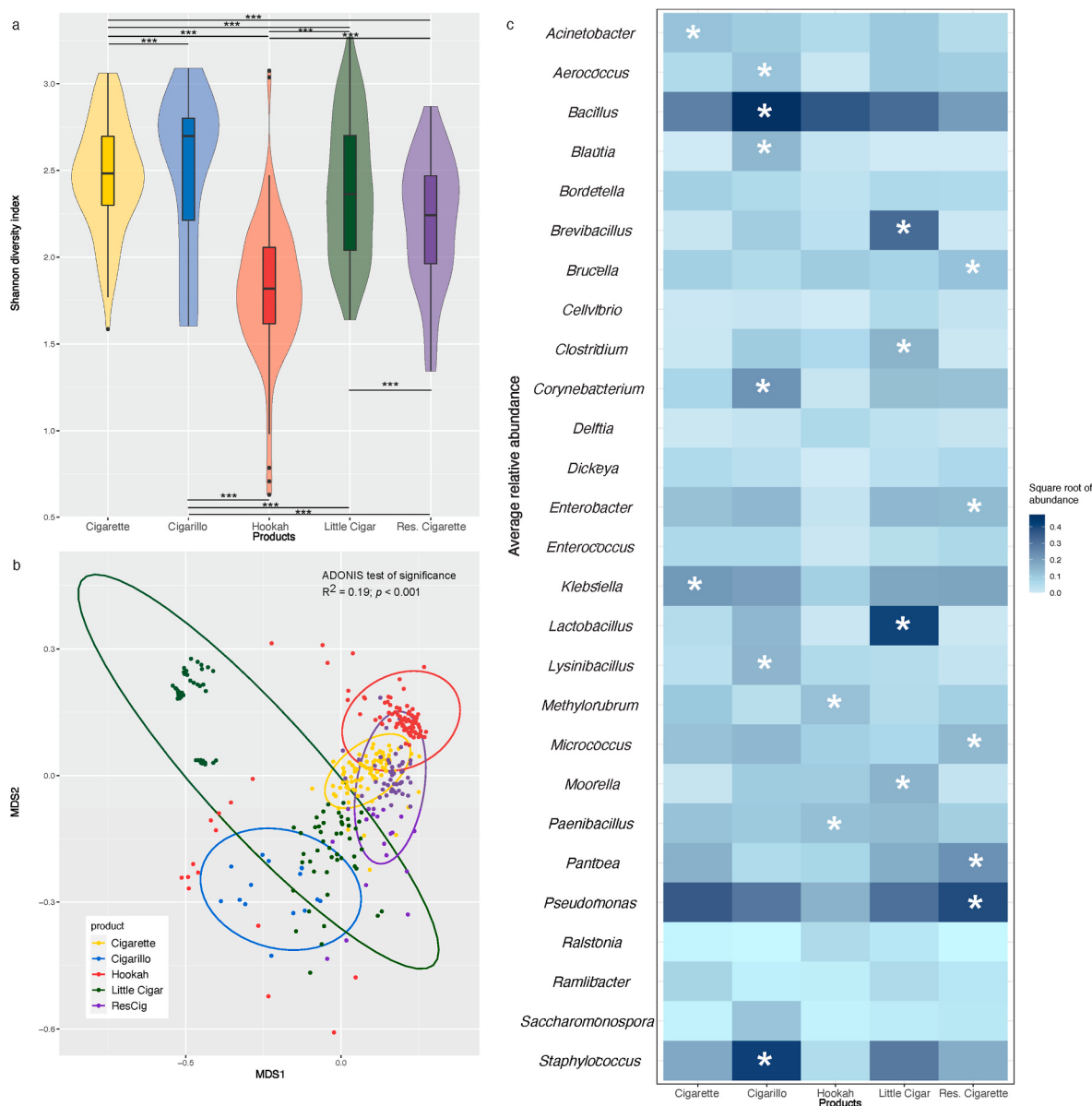


Fig. 1. Diversity and abundance of bacterial communities across all tobacco products. (a) Alpha diversity across the five tobacco products measured using the Shannon diversity index. Statistical significance between products was determined using the Kruskal-Wallis test followed by a *post hoc* Dunn's 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.

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 (Fig. 3). Unique (absent in other products above 5% relative abundance) bacterial communities in commercial cigarettes comprised *Ramlibacter tataouinensis*, *Bordetella genomsp. 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.

3.2. Core and shared microbiomes across products

Comparing across products, *B. subtilis*, *K. pneumoniae*, *P. aeruginosa*, *P. putida*, and *P. alcaligenes* were present in all five tobacco products forming the core microbiome across all products (Fig. 4). *M. thermoacetica*, *Bacillus amyloliquefaciens* and *A. urinaeequi* was shared between 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 *S. saprophyticus* and *Micrococcus luteus*. *P. stutzeri*, *P. oryzae*, and *C. ammoniagenes* was shared between little cigars and research cigarettes, while *P. stutzeri* and *C. ammoniagenes* was shared with hookah products and *P. oryzae* was shared with commercial cigarettes. Commercial cigarettes and little cigars shared *B. genomsp. 13*, *A. baumannii*, and *L. fermentum*.

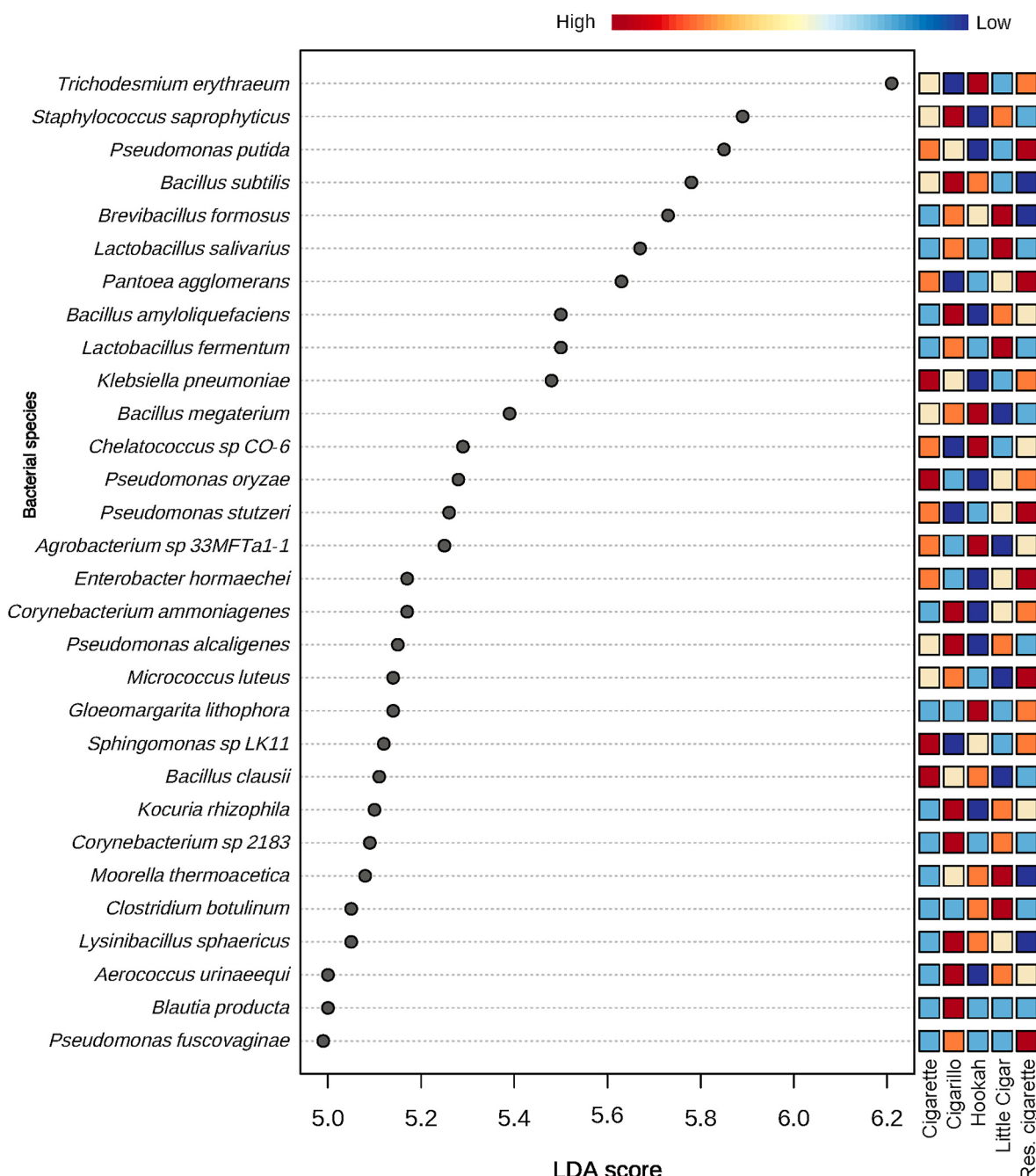


Fig. 2. LefSe analysis on the differential abundance of bacterial species across tobacco products. The color gradient on top shows the differential abundance of the taxa from low (blue) to high (red). Res. cigarette = Research cigarette. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Meanwhile, some bacterial species were shared between two to four products, and each of the tobacco products harbored two to three unique bacterial species. *Bacillus megaterium*, *Dickeya fangzhongdai*, *B. genomosp.* 13, *A. baumannii*, *L. fermentum*, *B. formosus*, and *C. botulinum* were each shared by two products. While *B. megaterium* was shared between cigarillo products and hookah products, *B. formosus* and *C. botulinum* were shared between cigarillos and little cigars. While *P. agglomerans* and *Bacillus clausii* were absent in cigarillos, *Enterobacter hormaechei* was absent in hookah products. Cigarillos had three unique bacterial species while hookah and little cigars had two unique species each. The cigarette products each had a single unique bacterial species (Fig. 4).

3.3. 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.

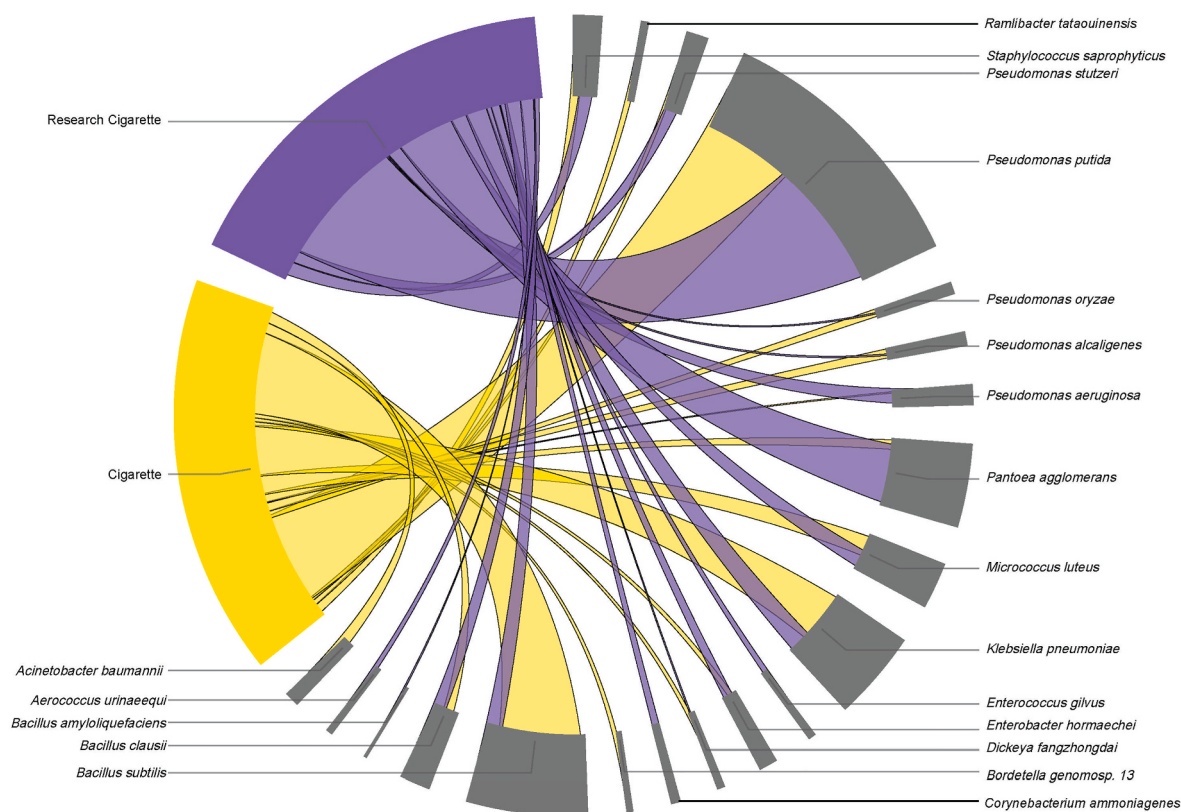


Fig. 3. Chord plot showing the average relative abundance (above 5%) of bacterial species between commercial (yellow) and research (purple) cigarettes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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*, *Methylobacterium*, *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 (Fig. 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 $\pm$ 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 $\pm$ 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. 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 (Fig. 6a). 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. 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*.

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 (Fig. 6b). The Al Fakher flavor, two apple, did not share any bacterial species with any of the other products. Fumari ambrosia and white gummy bear harbored three and one unique bacterial species respectively. Two other hookah 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.*

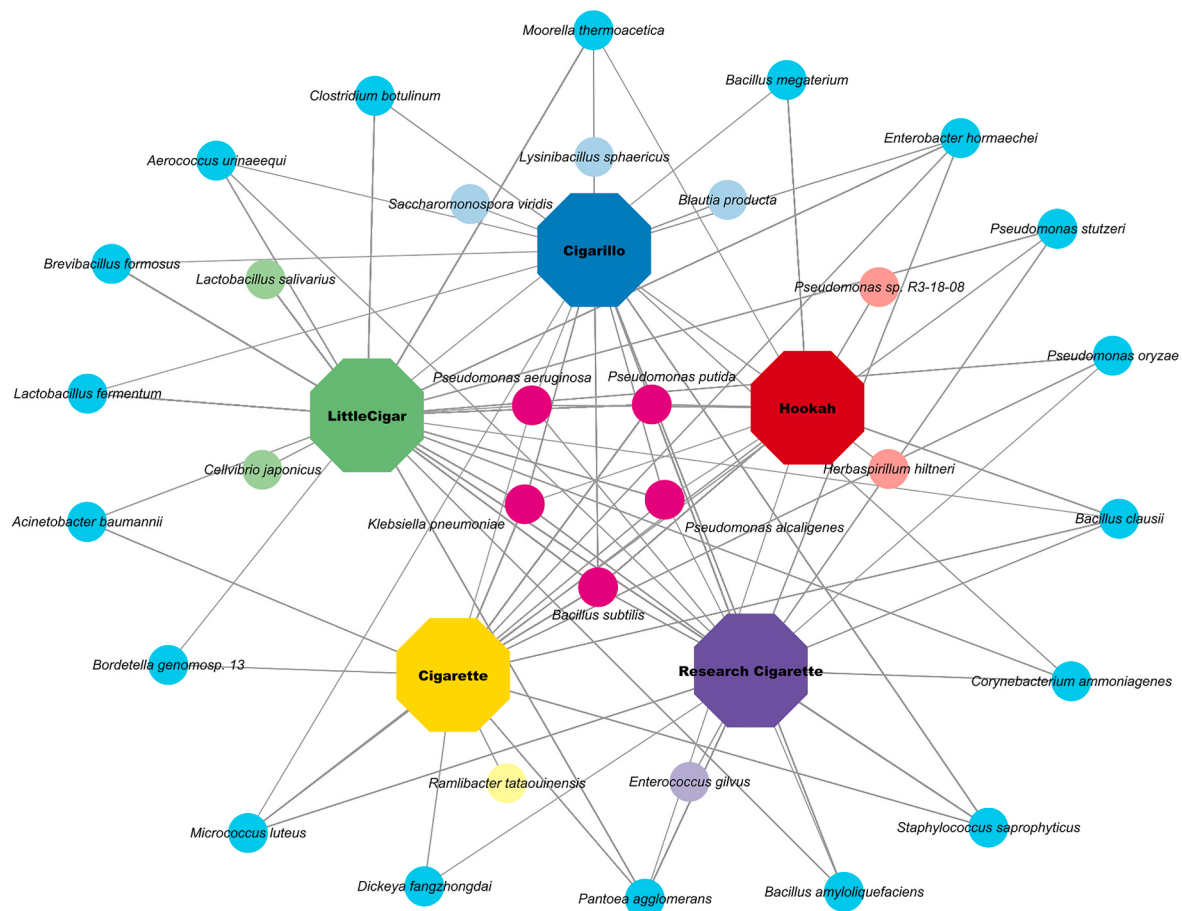


Fig. 4. Network analysis showing shared and unique bacterial species across all tobacco products. The circles represent each bacterial species, and the hexagons represent the tobacco products. The nodes joining the shapes indicate the identification of the taxa within the product.

luteus, *P. putida*, *P. aeruginosa* and *S. saprophyticus* comprised the core microbiome of both menthol and nonmenthol research cigarettes (Fig. 6c). While five unique bacterial species were found among the menthol research cigarettes, only one species was unique to the non-menthol varieties.

Among the commercial cigarettes, there were no core bacterial species (>1% relative abundance) that were identified (Fig. 6d). *B. subtilis* and *P. putida* were shared between 4 brands of commercial cigarettes except for Newport non-menthols. Three of the five cigarette brands (Newport menthol gold, Newport non-menthol and Marlboro red) had unique bacterial species, while the other two (Newport menthol 2 and Marlboro menthol) did not have any unique bacterial species.

3.5. Metabolically-active bacterial communities across products

Comparing our subset of samples tested for total (NOTRT) and metabolically-active (BRDU) bacterial communities, alpha diversity was 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 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 *Gardnerella* (Fig. 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% including *Atopobium vaginae*, *Alcaligenes faecalis*, *Bacillus cereus*, *Haemophilus parainfluenzae*, *Rothia mucilaginosa*, *Streptococcus agalactiae*, and *Propionibacterium acnes* (Table S3).

4. Discussion

In this study, we characterized total and metabolically-active 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 (Rooney et al., 2005; Sapkota et al., 2010; Chopyk et al. 2017a, 2017b; Smyth et al. 2017, 2019; Hani et al., 2018;

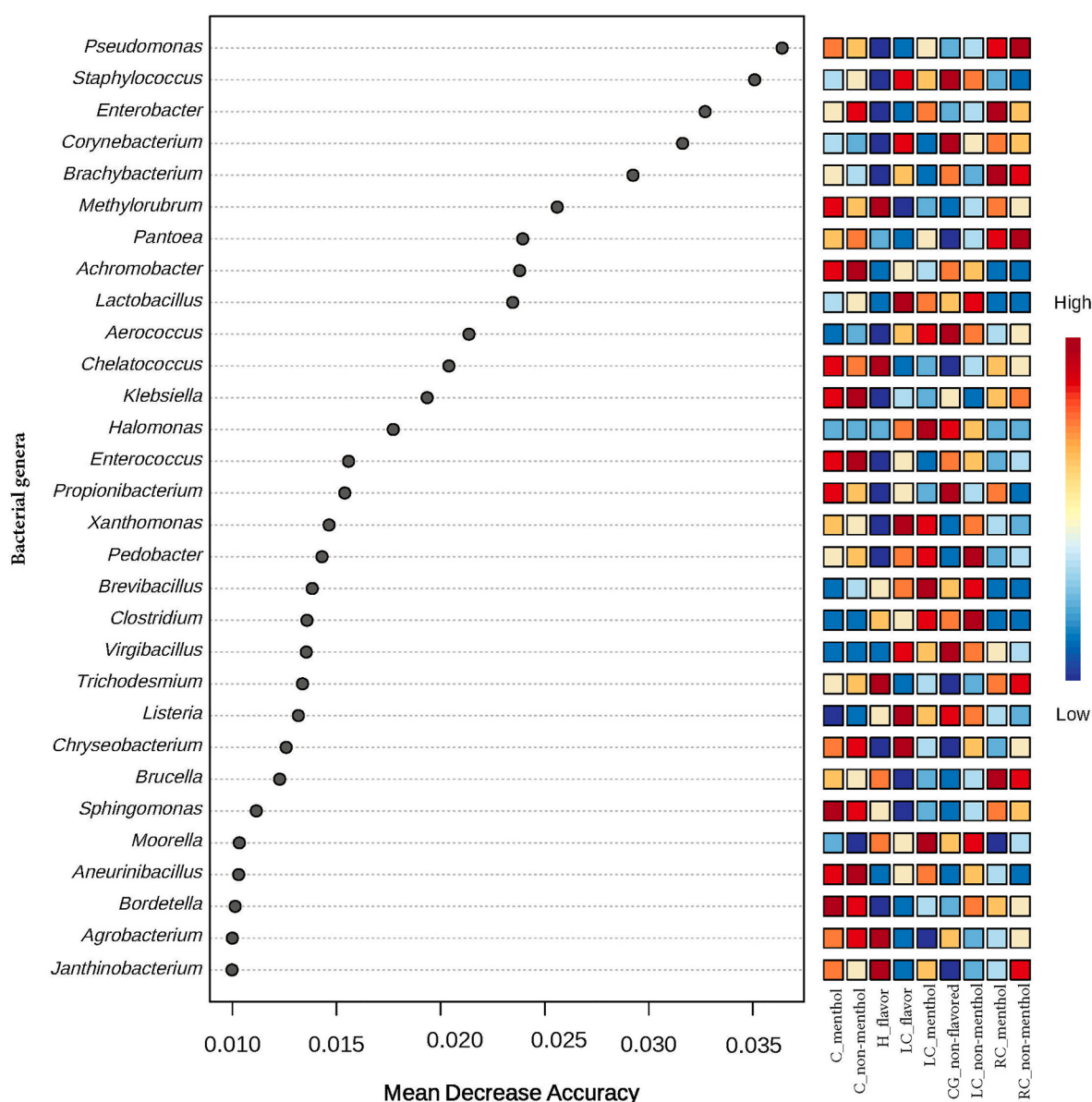


Fig. 5. Predicted association between the top 30 bacterial genera and specific tobacco products with/without additives. The color gradient shows the degree of association from low (blue) to high (red). C: cigarette, LC: little cigar, CG: cigarillo, H: hookah, and RC: research cigarette. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Chattopadhyay et al., 2019; Malayil et al., 2020a). 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* can cause chronic infections, particularly in people with chronic lung diseases like cystic fibrosis, bronchiectasis, and chronic obstructive pulmonary disease and is known to cause irreversible airway decline, poorer quality of life, and early mortality in these sensitive sub-populations (Erb-Downward et al., 2011; Fodor et al., 2012; Gellatly and Hancock, 2013; Moradali et al., 2017; Jacobs et al., 2020). 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 (Gu et al., 2019). Lastly, one of the most common nosocomial pathogens, *K. pneumoniae*, is known to express antibiotic resistance and virulence factors that aid the bacterium in spreading through the respiratory system, causing pneumonia, bloodstream infections and meningitis (Martin and Bachman, 2018; Gonzalez-Ferrer et al., 2021).

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 (Chattopadhyay et al., 2019; Smyth et al., 2019). 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 (Khater et al., 2008; Uebelacker et al., 2019). 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 (Roger et al., 1992; Nalawade et al., 2015). 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

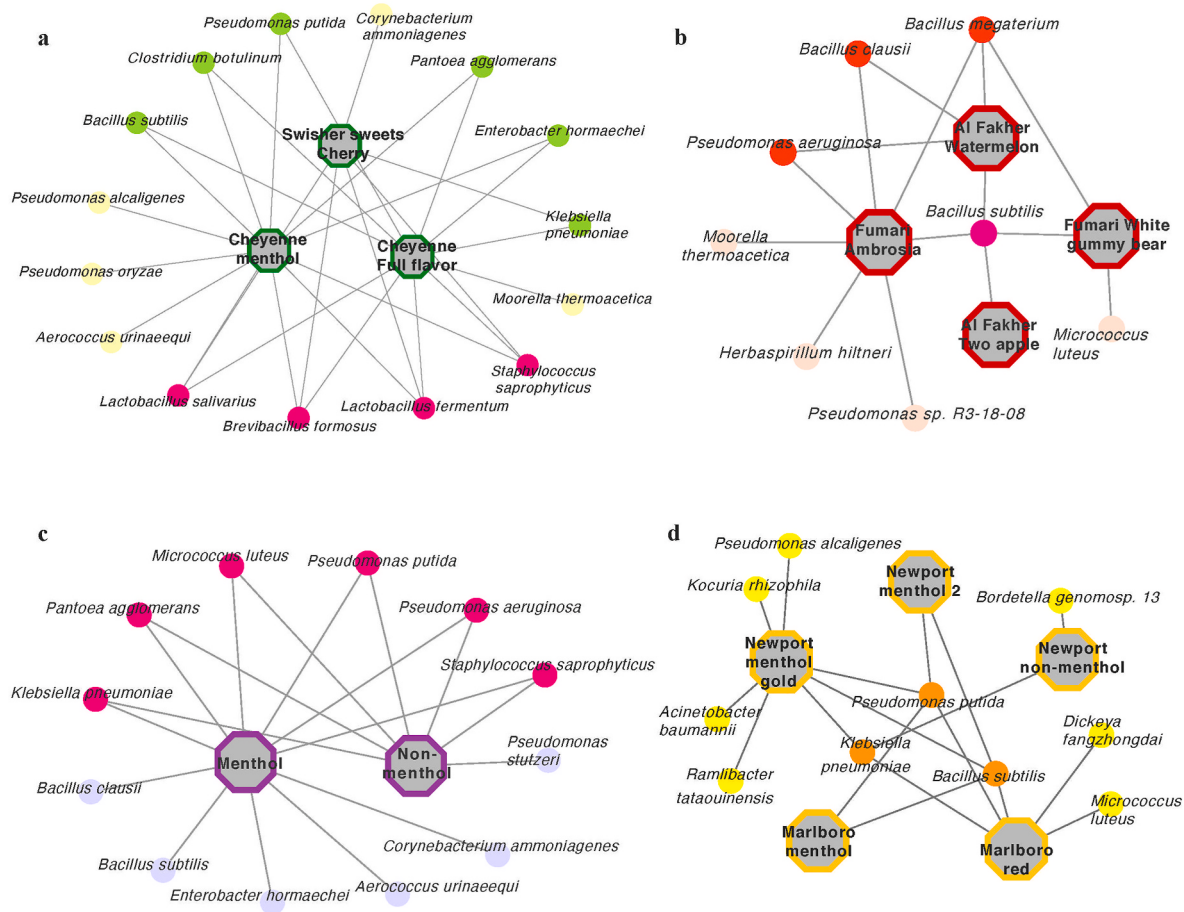


Fig. 6. Network plots showing the shared and unique bacterial species within a) hookah, b) little cigars, c) cigarettes, and d) research cigarettes. The circles represent each bacterial species, and the hexagons represent the tobacco products. The nodes joining the shapes depict the identified taxa within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

against commensal bacterial populations (Cleusix et al., 2008; De Weirdt et al., 2010). Similarly, honey—which is one of the oldest known antimicrobials—also exhibits bactericidal activity against a variety of bacteria (Morroni et al., 2018; Almasaudi, 2021).

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 (Chattopadhyay et al., 2019; Chattopadhyay et al., 2019; Smyth et al., 2019; Malayil et al., 2020a), and Chopyk et al. (2017a) found that mentholated products harbor taxa that are known to survive harsh conditions such as *Anoxybacillus* and *Schlegella* (Chopyk et al., 2017a).

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 (Tian et al., 2016; Zhao et al., 2018). 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 (Vrabec et al., 2015; Rasmussen, 2016). 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 (Khan et al., 2019) and *E. hormaechei* is a well-known causative agent of nosocomial infections, exhibiting resistance to multiple antibiotics (Monahan et al., 2019; Wang et al., 2020; Gou et al., 2020).

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 (Abdel-Moein and Hamza, 2016), *L. sphaericus* produces insecticidal proteins leading to cytopathological effects in insect larvae (de Fátima Gomes Cavados et al., 2017). A recent case report also described *L. sphaericus* as a causative agent of severe sepsis (Wenzler et al., 2015). 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

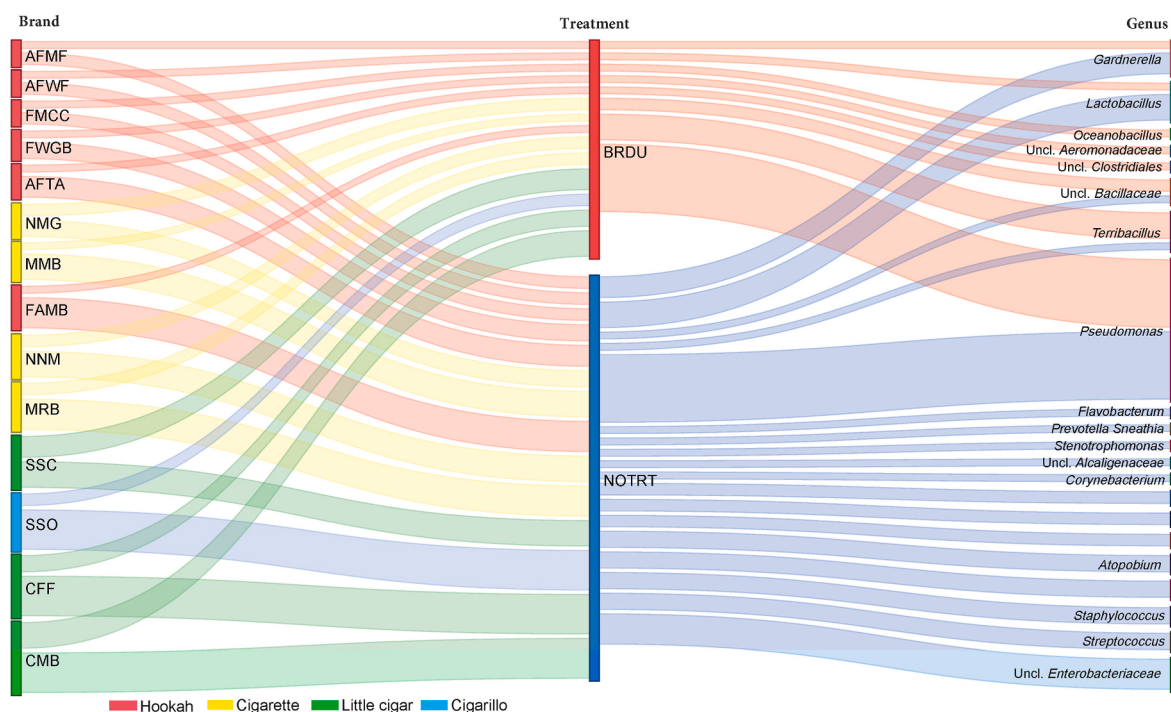


Fig. 7. Sankey plot showing the metabolically-active (BrdU) bacterial species identified within the total bacterial community (NOTRT) across all tobacco brands and flavors. AFMF: Al Fakher mint, AFWF: Al Fakher watermelon, FMCC: Fumari mint chocolate chill, FWGB: Fumari white gummy bear, AFTA: Al Fakher 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. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

lung disease), and humidifier fever (Pati et al., 2009).

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 (Malayil et al., 2020b). 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 *Cutibacterium 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 (Malayil et al., 2022a). While previous studies have described a few of the identified species in our study as pathogenic (*A. faecalis*, *B. cereus*, *Pseudomonas viridiflava*, *Rothia aeria*, *R. mucilaginosa*, *S. agalactiae*) (Bottone, 2010; Michon et al., 2010; Geng et al., 2012; Sarris et al., 2012; Maraki and Papadakis, 2015; Huang, 2020; Greve et al., 2021), some species (*Methylobacterium adhaesivum*, *Acinetobacter johnsonii*, *A. lwoffii*) (Kozłńska et al., 2014; Szwetkowski and Falkinham, 2020) 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) (Portillo et al., 2013; O'Neil et al., 2016). 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 (Wood and Salzberg, 2014; Valenzuela-González et al., 2016; Lu et al., 2017). The kmer matching technique used by Kraken has been shown to supersede those pipelines using gene markers in terms of sensitivity and specificity (Miossec et al., 2020; Lu and Salzberg, 2020), reducing runtime and memory usage (Wood et al., 2019) and reducing sensitivity to structural variations (e.g., inversions) within reads (Pearman et al., 2020). 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 (Wood et al., 2019).

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 radio-labeled precursors (like thymidine and leucine) can have methodological limitations, including contrasting uptake of these compounds by different bacterial phyla (Pérez et al., 2010; Hellman et al., 2011; Galand et al., 2013a). Finally, since this labeling technique is limited to bacterial taxa that are capable of incorporating exogenous nucleotide precursors into replicating DNA (Urbach et al., 1999), 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.

Author contributions

SC conducted laboratory processing, and statistical analyses following sequencing, and wrote and edited the manuscript. PR and LM performed bioinformatic and laboratory analyses respectively. EFM and ARS contributed to the study design, protocol development, data analysis and interpretation, and manuscript preparation. All authors read and approved the final manuscript.

Disclaimer

Dr. Mongodin contributed to this article as an employee of the University of Maryland School of Medicine. The views expressed are his own and do not necessarily represent the views of the National Institutes of Health or the United States Government.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envres.2022.115205>.

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