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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Mentholation triggers brand-specific shifts in the bacterial microbiota of commercial cigarette products

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Abstract

Bacterial communities are integral constituents of tobacco products. They originate from tobacco plants and are acquired during manufacturing processes, where they play a role in the production of tobacco-specific nitrosamines. In addition, tobacco bacterial constituents may play an important role in the development of infectious and chronic diseases among users. Nevertheless, tobacco bacterial communities have been largely unexplored, and the influence of tobacco flavor additives such as menthol (a natural antimicrobial) on tobacco bacterial communities is unclear. To bridge this knowledge gap, time series experiments including 5 mentholated and non-mentholated commercially available cigarettes—Marlboro red (non-menthol), Marlboro menthol, Newport menthol box, Newport menthol gold, and Newport non-menthol—were conducted. Each brand was stored under three different temperature and relative humidity conditions. To characterize bacterial communities, total DNA was extracted on days 0 and 14. Resulting DNA was purified and subjected to PCR of the V3V4 region of the 16S rRNA gene, followed by sequencing on the Illumina HiSeq platform and analysis using the QIIME, phyloseq, metagenomeSeq, and DESeq software packages. Ordination analyses showed that the bacterial community composition of Marlboro cigarettes was different from that of Newport cigarettes. Additionally, bacterial profiles significantly differed between mentholated and non-mentholated Newports. Independently of storage conditions, tobacco brands were dominated by *Proteobacteria*, with the most dominant bacterial genera being *Pseudomonas*, unclassified *Enterobacteriaceae*, *Bacillus*, *Erwinia*, *Sphingomonas*, *Acinetobacter*, *Agrobacterium*, *Staphylococcus*, and *Terribacillus*. These data suggest that the bacterial communities of tobacco products differ across brands and that mentholation of tobacco can alter bacterial community composition of select brands.

Key Points

- *Bacterial composition differed between the two brands of cigarettes.*
- *Mentholation impacts cigarette microbiota.*
- *Pseudomonas and Bacillus dominated the commercial cigarettes.*

Keywords Bacterial microbiota · Commercial cigarettes · Menthol

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Introduction

Tobacco smoking is the leading cause of lung cancers (Hecht 1999) and is a major contributor to pulmonary associated diseases (Galvin and Franks 2009), strokes (Nomura et al. 1974), and cardiovascular diseases (Ewald and Cochran 2000). The key ingredients of a commercial cigarette are tobacco, chemical additives, a filter, and wrapping paper. Chemical characterizations of tobacco have identified nearly 5000 chemical and heavy metal constituents (Talhout et al. 2011; Rodgman and Perfetti 2013). Recently, the US Food and Drug Administration (FDA), through the Federal Food, Drug, and Cosmetic Act (FD&C Act), has listed 93 of these constituents as "harmful and potentially harmful constituents" (HPHCs) (compounds of tobacco products or smoke that can lead to adverse health effects among smokers as well as exposed non-smokers). As such, the FDA requires tobacco manufacturers and importers to report the levels of HPHCs found in their tobacco products and tobacco smoke.

In addition to chemical constituents, bacterial communities are integral constituents of tobacco products (Kurup et al. 1983; Rooney et al. 2005). They originate from tobacco plants and are acquired and selected for during the tobacco curing and manufacturing processes, where they play an active role in the production of tobacco-specific nitrosamines (Wiernik 1995). However, the current HPHC list does not include any tobacco-associated microorganisms to which tobacco users may be chronically exposed, despite the possibility that they may play a key role in the development of both infectious and chronic diseases. Moreover, despite their potential impact on public health, there are limited data regarding the bacterial constituents of tobacco.

Regarding the few studies that have been published, commercial cigarette brands have been shown to harbor rich and diverse bacterial populations, with bacteria ranging from common soil microorganisms to potential human pathogens (Kurup et al. 1983; Rooney et al. 2005; Sapkota et al. 2009). Using cultivation approaches, previous studies have identified species of *Actinomycetes* (Kurup et al. 1983), *Erwinia* (Larsson et al. 2008), *Bacillus* (Rooney et al. 2005), *Kurthia* (Rooney et al. 2005), and *Mycobacterium* (Eaton et al. 1995) in tobacco particles, smoked filters, and cigarette filters (Eaton et al. 1995). However, traditional culture–based methods are limited with regard to the number of microorganisms that can be characterized due to the small percentage of bacteria that can be cultured in the laboratory. To address this issue, Sapkota et al. (2009) utilized a microarray-based approach providing the first evidence that the number of bacterial species present in cigarettes may be as vast as the number of chemical constituents. This study identified 15 different classes of bacteria and a broad range of potentially pathogenic microorganisms, including species of *Acinetobacter*, *Bacillus*, *Clostridium*, *Klebsiella*, *Pseudomonas*, and *Serratia* in five commonly smoked cigarettes. However, low representation of bacterial diversity represented on the microarray prevented a comprehensive evaluation of total bacterial community composition in the tested products.

To bridge this knowledge gap, high-throughput next-generation sequencing methods were then employed by our group to evaluate total bacterial diversity of commercially available tobacco products (Chopyk et al. 2017a, b; Chattopadhyay et al. 2019; Smyth et al. 2019). These studies highlighted the breadth of bacterial diversity across different brands of cigarettes, smokeless tobacco products, and little cigars (Han et al. 2016; Chopyk et al. 2017a, b; Chattopadhyay et al. 2019; Smyth et al. 2019). Nevertheless, the specific impact of menthol (a natural antimicrobial and popular flavor additive to commercial cigarettes) on tobacco bacterial diversity remained unclear. Hence, this study aimed to not only investigate the bacterial community composition of the most popular commercial brands of cigarettes but also perform a direct comparison between mentholated and non-mentholated cigarettes of the same commercially available brand.

Materials and methods

Sample collection

We characterized five commercially available cigarette brands: Marlboro red (non-menthol), Marlboro menthol (Philips Morris Inc., Richmond, VA, USA), Newport menthol box, Newport menthol gold, and Newport non-menthol (Lorillard Tobacco Co., Greensboro, NC, USA) that were purchased online and shipped to College Park, MD, USA. We selected these brands because Marlboro and Newport are among the most advertised manufacturers and continue to be the preferred manufacturers among young cigarette smokers (CDC 2018). Marlboro is the most popular manufacturer in the USA with 40% of the market share per 2017 sales data (CDC 2018). In addition, including these brands enabled us to perform true comparisons between mentholated and non-mentholated varieties of the same brand.

The cigarettes were incubated in the laboratory for 14 days under three different experimental conditions to simulate regular user storage conditions: room (20 °C and 50% relative humidity), refrigerator (5 °C and 18% relative humidity), and pocket (25 °C and 30% relative humidity). Three lots of each of the five brands of cigarettes were tested under each condition in replicate on days 0 and 14, resulting in a total of 180 samples tested during the study.

DNA extraction

Total DNA was extracted using both enzymatic and mechanical lyses in accordance with the previously published

methods (Chopyk et al. 2017a, b). Briefly, cigarettes were aseptically dissected, and 0.2 g of the tobacco was weighed into Lysing Matrix B tubes (MP Biomedicals, Solon, OH, USA) under sterile conditions. Then, 1 ml of ice-cold 1X molecular grade PBS buffer (Gibco by Life Technologies, Grand Island, NY, USA), 5 μl lysozyme from chicken egg white (10 mg/ml, Sigma_Aldrich, St. Louis, MO, USA), and 5 μl lysostaphin from *Staphylococcus staphylolyticus* (5 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) were added to the tobacco-containing lysing matrix tubes and incubated at 37 °C for 30 min. A second enzymatic addition consisting of 10 μl proteinase K (20 mg/ml, Invitrogen by Life Technologies, Grand Island, NY, USA) and 50 μl of SDS (10% w/v, BioRad, Hercules, CA, USA) was performed. The tubes were then incubated for 55 °C for 45 min and subjected to mechanical lysis using the FastPrep Instrument FP-24 (MP Biomedicals, Santa Ana, CA, USA) at 6.0 m/s for 40 s. Lysates were then centrifuged for 3 min at 10,000 rcf, and DNA was purified using the QIAmp DSP DNA mini kit 50, v2 (Qiagen, Valencia, CA, USA), per the manufacturer's protocol. To ensure no exogenous DNA contaminated the samples during extraction, negative extraction controls were included. Nanodrop (Thermo Scientific, Waltham, MA, USA) measurements and gel electrophoresis of the samples were performed to ensure DNA quality control.

16S rRNA gene PCR amplification and sequencing

Extracted DNA was PCR amplified for the V3V4 hypervariable region of the 16S rRNA gene using the universal primers 319F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) and sequenced on the Illumina HiSeq2500 300 bp-PE (Illumina, San Diego, CA) using a method developed at the Institute for Genome Sciences (Fadrosh et al. 2014) and described previously in detail (Chopyk et al. 2017a, b; Holm et al. 2019). PCR reactions were carried out using Phusion High Fidelity DNA polymerase (Thermo Fisher, Waltham, MA, USA) and 2 ng of template DNA in a total reaction volume of 25 μl. Additionally, 0.375 μl of bovine serum albumin (BSA; 20 mg/ml) was added to PCR reactions to overcome PCR inhibition. Negative controls without DNA template were performed for both primer sets. The following PCR conditions were used: 30 s at 98 °C, followed by 30 cycles of 10 s at 98 °C, 15 s at 66 °C, and 15 s at 72 °C, with a final step of 10 min at 72 °C. Amplicon presence was confirmed using gel electrophoresis, after which the SequalPrep Normalization Kit (Invitrogen Inc., Carlsbad, CA, USA) was used for cleanup and normalization (25 ng of 16S PCR amplicons from each sample was included), prior to pooling and sequencing.

Sequencing quality filtering and data analysis

Following sequencing, 16S rRNA paired-end read pairs were assembled using PANDAseq (Masella et al. 2012), demultiplexed, trimmed of artificial barcodes and primers, and assessed for chimeras using UCHIME in de novo mode implemented in Quantitative Insights Into Microbial Ecology (QIIME; release v.1.9.1) (Caporaso et al. 2010). Quality-trimmed sequences were then clustered de novo into operational taxonomic units (OTUs), and taxonomic assignments were performed using VSEARCH (Rognes et al. 2016) with a minimum confidence threshold of 0.97. The SILVA 16S database (v.123) (Quast et al. 2012) in QIIME 1.9.1 (Caporaso et al. 2010) was used for taxonomic assignments. Downstream data analysis and visualization were done in R Studio (v.1.1.423) using the following packages: biomformat (v.1.2.0) (McMurdie and Paulson 2017), vegan (v.2.4.5) (Oksanen et al. 2017), ggplot2 (v.3.1.0) (Wickham 2009), phyloseq (v.1.19.1) (McMurdie and Holmes 2013), Bioconductor (v.2.34.0) (Huber et al. 2015), and metagenomeSeq (v.1.16.0) (Paulson et al. 2013). All sequences taxonomically assigned to the phylum *Cyanobacteria* and likely tobacco chloroplasts were removed from further downstream analysis. When appropriate, data were normalized with metagenomeSeq's cumulative sum scaling (CSS) (Paulson et al. 2013) to account for uneven sampling depth. Prior to normalization, alpha diversity was measured using both the observed richness metric and the Shannon diversity index (Shannon 1948). The Bray-Curtis dissimilarity was used for calculating beta diversity and was compared using analysis of similarities (ANOSIM) on normalized data (999 permutations).

The DESeq2 (v.1.14.1) (Love et al. 2014) package in R studio was used to determine statistically significant (p value cutoff of 0.05) differences in bacterial OTU composition between manufacturers and types of cigarettes (non-mentholated and mentholated) at alpha = 0.05 on OTUs > 0.1% abundance.

In addition, bacterial taxa were summarized and CSS (cumulative sum scaling) normalized using several R packages—vegan (v.2.4.5) (Oksanen et al. 2017), dplyr (v.0.7.8) (Wickham et al. 2018), circlize (v.0.4.5) (Gu et al. 2014), reshape2 (v.1.4.3) (Wickham 2007), and stringr (v.1.3.1) (Wickham 2019)—and those with a maximum relative abundance greater than 1% in at least one sample were used to build the shared and unique data based on the brand.

Availability of data

Data concerning the samples included in this study are deposited in the NCBI BioProject database under BioProject accession number PRJNA601146.

Results

Sequencing dataset

A total of 180 samples were successfully PCR amplified and sequenced. A total of 12,113,675 sequences were generated across all samples and clustered into 4956 operational taxonomic units (OTUs). Across all samples, the minimum number of reads was 64, and the maximum was 137,628, with an average number of sequences per sample of 67,298.19 (± 32,003 SD). The Good's estimate of coverage was calculated for all samples, and samples with Good's value < 0.90 (1 Marlboro menthol sample, 1 Marlboro red sample, and 3 Newport non-menthol samples) were removed, which ensured all samples included in the final dataset had appropriate sequence coverage (Supplementary Fig. S1). Additionally, we removed two more samples, 1 Marlboro menthol and 1 Newport non-menthol sample, which were identified as outliers based on comparisons to the corresponding replicates. After removal of *Cyanobacteria* and pruning of low abundance taxa (OTUs with less than 10 sequences), the final dataset analyzed contained 6,716,339 sequences clustered into 2586 OTUs from 170 samples.

Alpha and beta diversity analysis of all brands

Alpha diversity metrics (observed species and Shannon diversity) among samples from the two manufacturers (Marlboro and Newport) and among specific brands on day 0 and day 14 were calculated on both rarefied (after downsampling each sample to 1187 reads) (Fig. 1) and non-rarefied data (Supplementary Fig. S2). Rarefied and non-rarefied datasets were analyzed in order to assess potential biases due to differences in sequence coverage; however, no difference in alpha diversity was observed between the rarefied and non-rarefied analyses. Tobacco-associated bacteria from Newport cigarettes showed lower observed alpha diversity (observed, 504.3 ± 179.1; Shannon, 4. ± 0.47) for all conditions and time points when compared to Marlboro cigarettes (observed, 949.5 ± 194.8; Shannon, 4.33 ± 0.59) (Fig. 1a and b).

Beta diversity analyses were performed on the CSS-normalized (non-rarefied) dataset and computed using PCoA plots of Bray-Curtis dissimilarity (Fig. 2). Comparisons of manufacturers (ANOSIM R, 0.397; $p = 0.001$) and brand (ANOSIM R, 0.431; $p = 0.001$) showed 22.5% variance between bacterial communities along the first principle component axis (axis 1) and 6.3% along the second principle component axis (axis 2). Additionally, bacterial communities were significantly ($p = 0.001$) different between the three Newport brands, whereas the microbiota associated with the Marlboro cigarettes did not differ ($p > 0.05$) between the Marlboro menthol and Marlboro red brands (Fig. 2). Storage condition and days of incubation did not have a significant effect ($p > 0.05$) on overall bacterial community structure (data not shown).

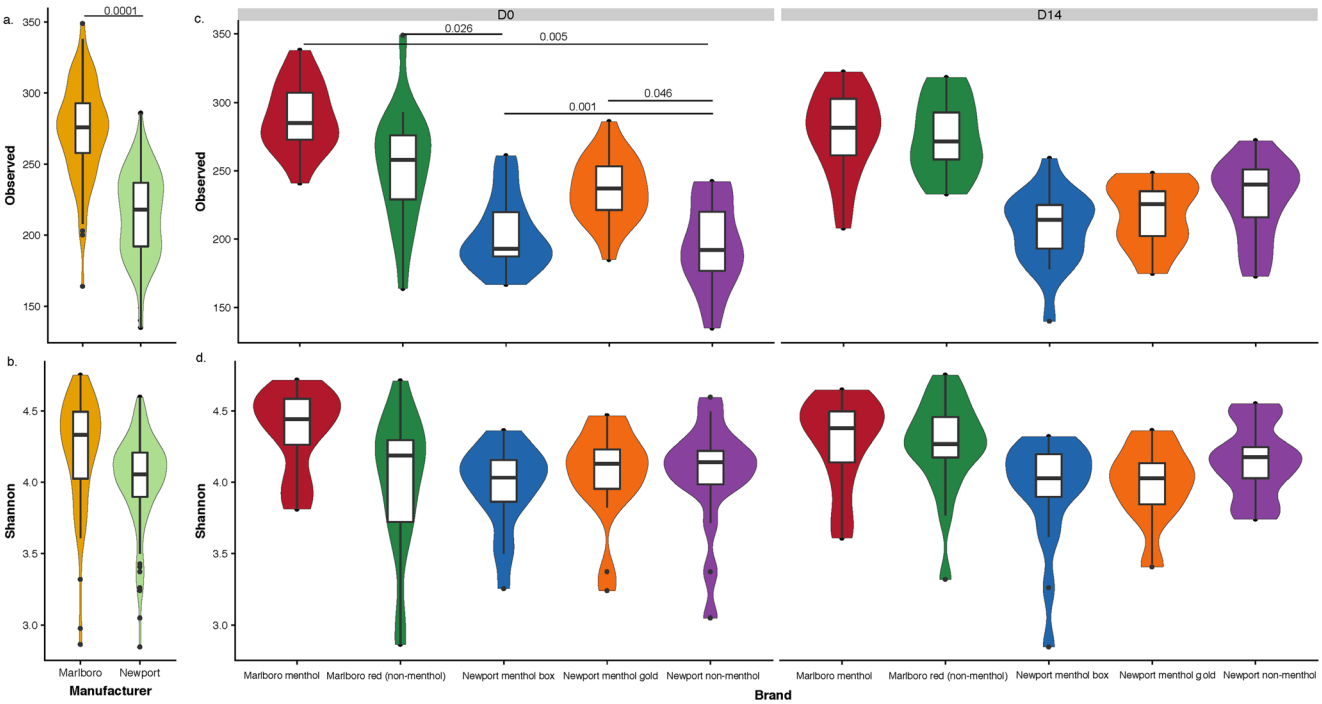

Fig. 1 Violin box plots showing alpha diversity (observed number of species and Shannon index) across samples on rarefied data to minimum sampling depth. Bars are colored by manufacturer: orange, Marlboro, and light green, Newport (left panels **a** and **b**). Tobacco brands: red, Marlboro menthol; green, Marlboro red (non-menthol); blue, Newport menthol box; orange, Newport menthol gold; and purple, Newport non-menthol (right panels **c** and **d**)

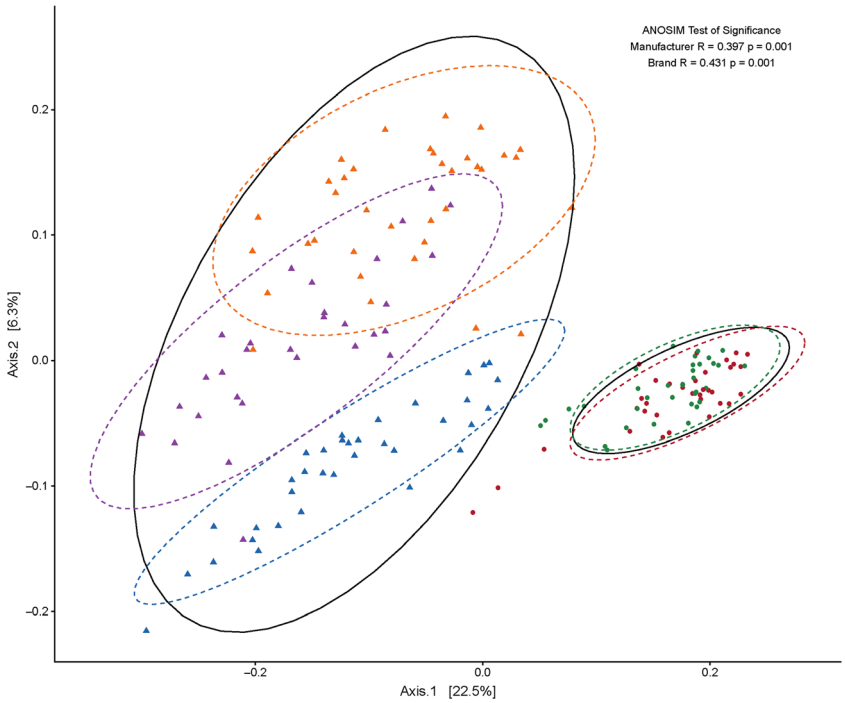

Fig. 2 Principle coordinate analysis plots of Bray-Curtis computed distances between manufacturer and brand using all time points and storage conditions. The colors represent the different tobacco brands: red, Marlboro menthol; green, Marlboro red (non-menthol); blue, Newport menthol box; orange, Newport menthol gold; and purple, Newport non-menthol. Shape represents the manufacturer: circle, Marlboro, and triangle, Newport. Solid ellipses are drawn at 95% confidence intervals for manufacturer, while dashed-colored ellipses are drawn at 95% confidence intervals for brands

Compositional differences between the two cigarette manufacturers

The top five bacterial phyla identified across all samples based on manufacturer (Marlboro ($n = 69$) and Newport ($n = 101$)) were *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Thermi*. The most predominant phyla with an average abundance of 64.73% (min, 37%; max, 99%) were *Proteobacteria* (range 59.16–70.29%), followed by *Firmicutes* ranging from 23.17 to 29.79% with an average abundance of 26.48% (min, 0.5%; max, 54%). *Actinobacteria* ranged from 4.204 to 9.04% with an average abundance of 6.62% (min, 0.1%; max,34%) and *Bacteroidetes* and *Deinococcus-Thermus* ranged from 1.67 to 2.07% and 0.06 to 0.27%, respectively.

In total, 2586 OTUs were assigned to the genus level of which only 255 could be identified to the species level. The top bacterial genera identified when manufacturer (Marlboro and Newport) and cigarette type (menthol and non-menthol) were considered were *Pseudomonas*, *Bacillus*, *Staphylococcus*, *Erwinia*, *Sphingomonas*, *Terribacillus*, *Acinetobacter*, and *Agrobacterium*, all of which dominated across cigarettes from both manufacturers (Fig. 3). At the species level, irrespective of the type of cigarettes characterized, we observed the presence of *Bacillus clausii*, *Bacillus coagulans*, *Bacillus flexus*, *Brevibacterium aureum*, *Corynebacterium stationis*, *Erwinia dispersa*, *Methylobacterium adhaesivum*, *Paenibacillus barengoltzii*, *Erwinia agglomerans*, *Pseudomonas pseudoalcaligenes*, *Pseudomonas veronii*, *Pseudomonas viridiflava*, *Saccharibacillus kuerlensis*, *Staphylococcus equorum*, *Xanthomonas axonopodis*, and *Acinetobacter rhizosphaerae*. The relative abundance of the top 100 species level taxonomic assignments, across manufacturers, brands, storage condition, lots, and days, is shown in Supplementary Table S1.

Differential abundance analyses are shown in Fig. 4 for bacterial genera that were significantly different ($p = 0.001$) between cigarette manufacturers and between cigarette types (menthol vs. non-menthol). *Sphingobacterium multivorum*, *B. clausii*, *B. flexus*, *M. adhaesivum*, *E. dispersa*, *X. axonopodis*, and *Acinetobacter schindleri* are some of the species that were present at a significantly higher abundance in Marlboro menthol cigarettes compared to non-menthol Marlboros, while *Brevibacterium aureum* was found to be present at a significantly higher abundance in non-menthol Marlboros (compared to menthols) and Newport menthol cigarettes (compared to non-menthol Newports). Additionally, *M. adhaesivum*, *P. pseudoalcaligenes*, and *A. rhizosphaerae* were present at a higher relative abundance in Newport non-menthols compared to Newport menthols, while *P. veronii* was found to be present at a higher relative abundance in Newport menthols compared to non-menthols.

Shared and unique bacterial taxa by cigarette brand and type

Bacterial taxa unique to Marlboro red were unclassified *Enterobacter*, while those unique to Marlboro menthols were unclassified *Staphylococcus* and unclassified *Enterobacteriaceae* (Fig. 5a). Shared bacterial taxa between

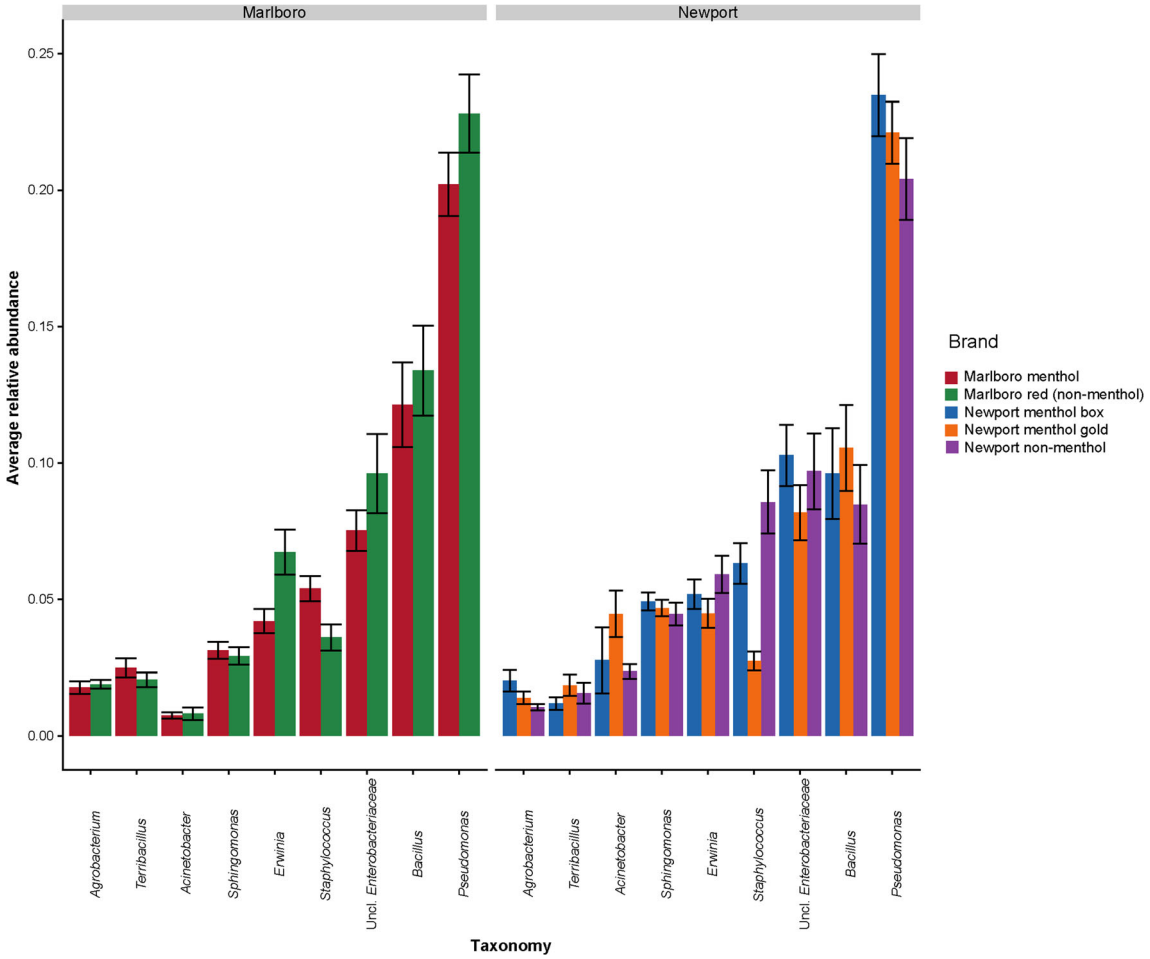

Fig. 3 Relative abundance of top 10 bacterial genera present in tobacco brands made by two manufacturers: Marlboro and Newport

Marlboro cigarette brands were *B. clausii*, *B. coagulans*, *E. dispersa*, *P. viridiflava*, unclassified *Aerococcus*, unclassified *Bacillus*, unclassified *Erwinia*, unclassified *Methylobacteriaceae*, unclassified mitochondria, unclassified *Pseudomonas*, and unclassified *Terribacillus* (Fig. 5a).

In Newport cigarettes, unique bacterial taxa were observed only among Newport menthol golds (unclassified *Aerococcus*) and Newport non-menthols (*P. viridiflava*). Shared bacterial taxa among the three Newport cigarette brands were *B. clausii*, *P.* veronii, *E. dispersa*, unclassified *Achromobacter*, unclassified *Bacillus*, unclassified *Enterobacteriaceae*, unclassified *Enterobacter*, unclassified *Erwinia*, unclassified *Methylobacteriaceae*, unclassified mitochondria, unclassified *Pseudomonas*, unclassified *Terribacillus*, unclassified *Sphingomonas*, and unclassified *Staphylococcus* (Fig. 5b).

Discussion

Our findings provide additional evidence that bacterial communities harbored in cigarette tobacco are diverse and differ significantly across manufacturers and brands. Most interestingly, we observed that the addition of menthol, a popular flavor additive and natural antimicrobial, can alter tobacco bacterial community composition but only in brands manufactured by Newport. Perhaps, the specific menthol formulation utilized by Newport has greater antimicrobial properties compared to that utilized by Marlboro.

Although flavored cigarettes (except for menthols) have been banned in multiple countries, flavors added to the majority of other tobacco products remain largely unregulated globally (Kowitt et al. 2017). In October 2009, The Family Smoking Prevention and Tobacco Control Act banned flavored cigarettes (except for menthols) to deter smoking in the USA, particularly among young adults and adolescents (Food and Drug Administration Center for Tobacco 2019). Mentholated or flavored cigarettes appeal to younger or beginning smokers largely because menthol and other flavors tend to mask the harshness and discomfort of smoking tobacco products (Kreslake et al. 2008). Previous studies also have shown that menthol users face greater addiction, increased nicotine dependence which could lead to higher risks of tobacco-attributable diseases (e.g., cancer, stroke, heart and

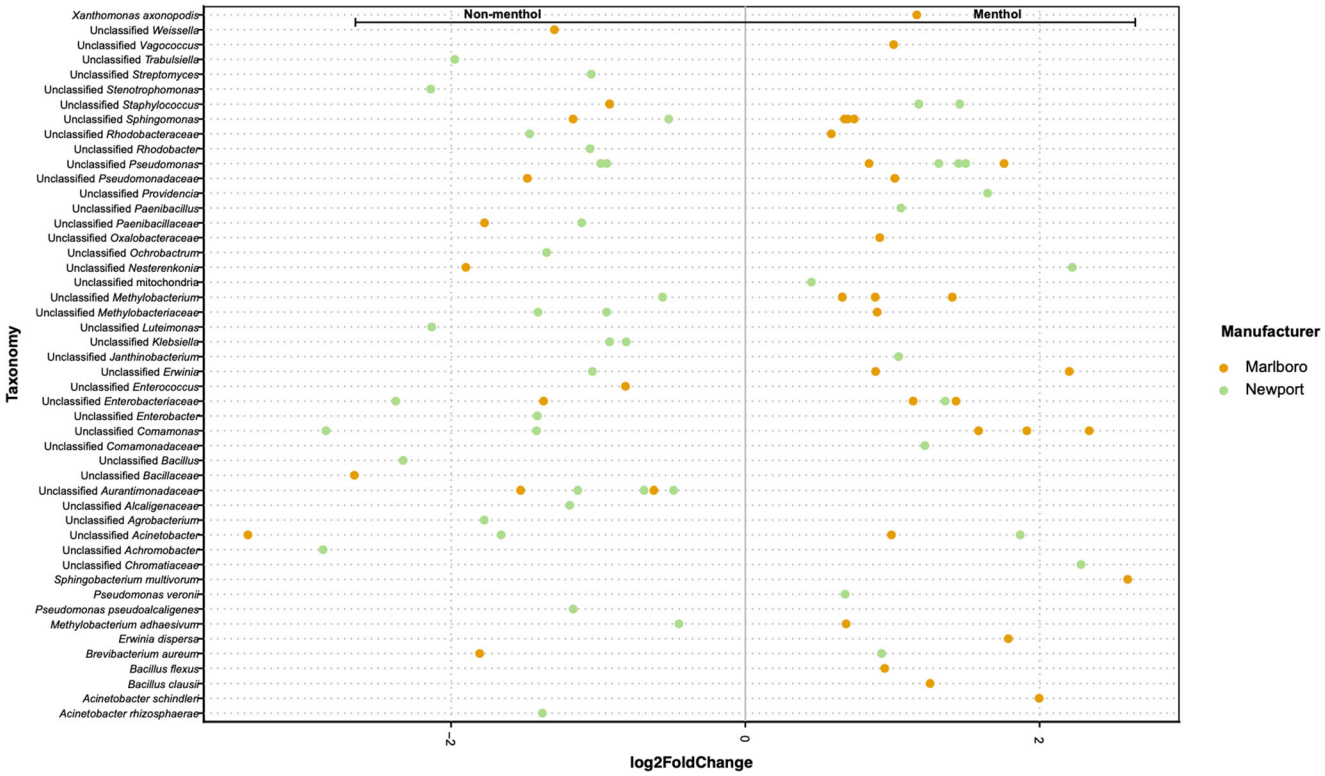

Fig. 4 Differential abundances of bacterial genera that were statistically different ($p < 0.05$) between cigarette types: non-menthol vs. menthol. A positive log2-fold change value denotes an OTU that is significantly higher in menthol cigarettes, while a negative log2-fold change indicates an OTU that is significantly higher in non-menthol cigarettes. The gray line and arrows highlight the conversion in log2-fold change from negative to positive values. The orange circle denotes Marlboro cigarettes, while the green circle denotes Newport cigarettes

respiratory diseases) (Garten and Falkner 2004), and decreased success in quitting tobacco products (Ahijevych and Garrett 2010; Foulds et al. 2010).

Menthol, while known for its antimicrobial properties, has been shown to affect the cigarette microbiota by selecting for bacteria that are tolerant to harsh environmental conditions as well as potentially pathogenic bacteria (Chopyk et al. 2017a). Here, we showed that unclassified *Pseudomonas*, *P. veronii*, *B. aureum*, and unclassified *Staphylococcus* were more abundant in Newport menthols compared to non-mentholated brands (Fig. 4). While Marlboro menthols were characterized by *B. clausii*, *B. flexus*, *E. dispersa*, *M. adhaesivum*, *A. schindleri*, *S. multivorum*, and *X. axonopodis* compared to non-mentholated brands (Fig. 4).

As observed previously, a high relative abundance of *Pseudomonas* species was observed in all cigarette brands irrespective of manufacturer, brand, and mentholation status (Chopyk et al. 2017a). Pseudomonads are common Gram-

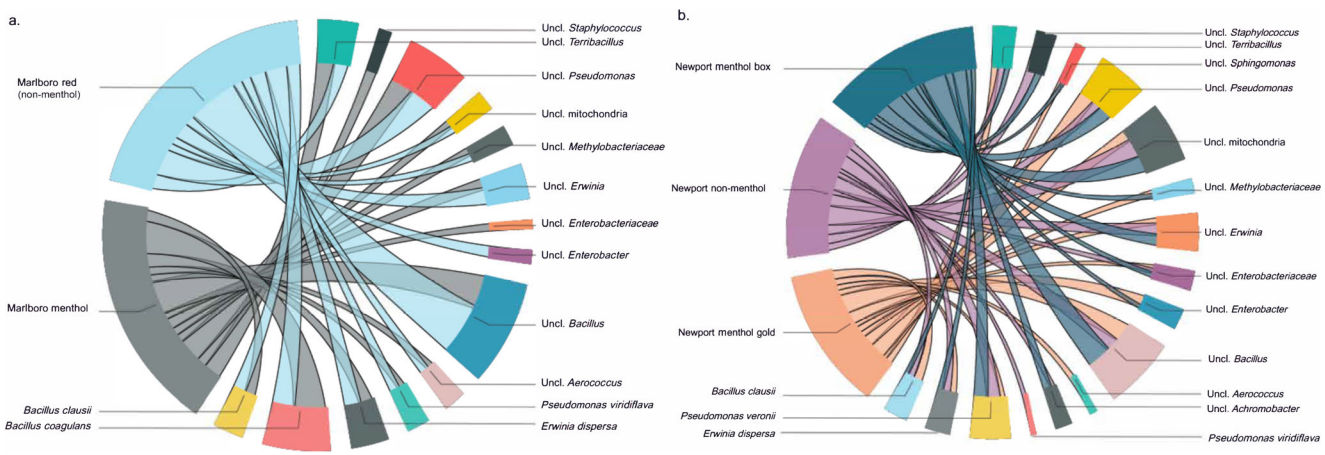

Fig. 5 Bacterial profiles of shared and unique genera between Marlboro brands (**a**) and Newport brands (**b**) visualized by chord plots

negative bacteria that are ubiquitous in nature, inhabiting soil, water, plants, and animals (Palleroni 2015). Hence, their presence in cigarettes is not surprising. However, species within the *Pseudomonas* genus can also be opportunistic pathogens, and therefore, their high relative abundance in cigarettes could be concerning with regard to the health of exposed smokers. In multiple instances, *Pseudomonas* species have been associated with chronic lung infections and cystic fibrosis (Erb-Downward et al. 2011; Fodor et al. 2012). In this study, we observed *P. pseudoalcaligenes* in Newport non-menthols which has been encountered infrequently in human infections (Gilardi 1972) but has been isolated from buccal cavities and bronchial washes (Gavini et al. 1989). Also, we observed the presence of *P. veronii* in Newport menthols (Fig. 4), which was first isolated from natural springs in France (Elomari et al. 1996) and has been used for bioremediation of contaminated soils (Nam et al. 2003; Onaca et al. 2007) but has not been shown to be pathogenic to date. Additionally, *P. veronii* is known to degrade toluene, which is shown to be present in mainstream smoke (Moldoveanu et al. 2008). Previous studies have isolated *Pseudomonas* species that can degrade nicotine (Li et al. 2010), and hence, understanding how *Pseudomonas* can biotransform nicotine and toluene might help in reducing tobacco-induced damages among cigarette users.

The other most abundant bacterial genera detected across all cigarette brands were *Bacillus* and *Staphylococcus*. Species within these genera can be opportunistic pathogens, and some, including *B. pumilus*, *B. cereus*, *and B. subtilis*, have been associated with respiratory infections and pneumonia among smokers (Rooney et al. 2005; Sapkota et al. 2009). In our study, we identified the overexpression of *B. clausii* and *B. flexus* in Marlboro menthols, but to date, they have not been shown to be pathogenic to humans. In contrast, *B. clausii* has been shown to prevent recurrent respiratory infections in children (Marseglia et al. 2007). On the other hand, *B. flexus* has the ability to tolerate arsenic (Jebeli et al. 2017), and it has been shown that inorganic arsenic is present in mainstream smoke (Lazarević et al. 2012). Several species of *Staphylococcus* (e.g., *S. aureus*, *S. haemolyticus*, and *S. epidermis*) are known to colonize the skin and upper respiratory tract of mammals and birds (Kloos 1980) and are usually harmless. The widespread, indiscriminate use of antibiotics has resulted in the selection of antibiotic resistance among *Staphylococcus* species especially *S. aureus*. Durmaz et al. (2001) identified the presence of methicillin-resistant *S. aureus* (MRSA) in the nasal cavity of smokers and cigarette factory workers compared to the non-smoker control group (Durmaz et al. 2001). In our study, we identified the presence of *S. equorum* in both cigarette brands which has been previously isolated from human clinical specimens (Novakova 2006).

Other bacterial genera observed across both cigarette brands were *Acinetobacter* spp. and *S. multivorum*, both of which have been associated with respiratory tract infections (Hanlon 2005; Lambiase et al. 2009). Multidrug-resistant *Acinetobacter* species have immerged as a major concern in hospital settings (Hanlon 2005), and *A. schindleri*, which we observed in Marlboro menthols (Fig. 4), is regarded as an opportunistic pathogen (Nemec et al. 2001). *S. kuerlensis*, which was detected in all cigarettes in this study, is an aerobic Gram-positive bacterium that was previously detected in tobacco leaves from Zimbabwe (Su et al. 2011).

In order for cigarette-associated bacteria to enter the human body via mainstream smoke, these organisms would have to survive the cigarette combustion process. Previous work has demonstrated the presence of viable bacteria (e.g., *Mycobacterium avium*) (Eaton et al. 1995) and other microbial constituents, including lipopolysaccharides, peptidoglycans, and fungal biomass (Pauly et al. 2010), in mainstream smoke, suggesting that bacteria and their constituents can survive combustion and be transferred to the upper respiratory system, potentially influencing respiratory health. Additionally, ongoing studies in our lab, utilizing both culture-based approaches and DNA labeling coupled with sequencing, are demonstrating evidence of viable bacteria in both tobacco products and the mainstream smoke of cigarettes (data not shown). Further studies are necessary to explore whether viable bacteria in mainstream smoke can be transferred to cigarette users, colonize the upper respiratory tract, and potentially contribute to respiratory illnesses.

Strengths of this study include the sample size, the analysis of multiple time points, and the head-to-head comparison of mentholated vs. non-mentholated brands from the same manufacturer. Like all 16S rRNA gene–based studies, our study also had limitations, including inherent biases from PCR amplification, our limited ability to assign species level classifications, and the inability to differentiate between live/metabolically active and relic/dead bacterial communities in the tested cigarette brands. Ongoing work in our lab is beginning to tease out the metabolically active fraction of tobacco bacterial communities by utilizing DNA labeling coupled with 16S rRNA sequencing (data not shown).

Despite the noted limitations, our study demonstrated that mentholation may alter bacterial community composition in certain cigarette brands and potentially select for bacteria including *Pseudomonas*, *Bacillus*, and *Staphylococcus*, in mentholated brands. Therefore, cigarette users' exposures to bacterial constituents originating from cigarette tobacco may be impacted differentially based on the users' specific brand of choice.

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Authors' contributions LM performed bioinformatics analysis, wrote, and edited the manuscript. SC and PK performed laboratory analyses. PIC contributed to study design and manuscript review. ARS and EFM contributed to the study design, protocol development, data analysis, and

manuscript preparation. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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