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Viable bacteria abundant in cigarettes are aerosolized in mainstream smoke☆

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ABSTRACT

Multiple studies have demonstrated that cigarettes harbor bacterial pathogens. Yet, to our knowledge, there are no published data to date on whether or not these microorganisms can be aerosolized and transmitted to the respiratory tract of users. To address this knowledge gap, we characterized cigarette bacterial communities and evaluated whether or not they could be aerosolized in mainstream smoke. Filtered and unfiltered cigarettes were tested. Non-smoked tobacco leaf, enriched non-smoked tobacco leaf extract and enriched mainstream smoke extract samples (n = 144) were incubated on trypticase soy agar, and resulting bacterial colonies were sequenced. Total DNA was also extracted, followed by PCR amplification of the 16S rRNA gene, sequencing and analysis using UCHIME, QIIME and R packages. The predominant bacterial genera cultured from the mainstream smoke of unfiltered cigarettes were *Bacillus*, *Terribacillus, Paenibacillus* and *Desulfotomaculum*. Culturable bacteria were not recovered from the smoke of filtered products. However, sequencing data demonstrated no significant differences in bacterial community diversity in the smoke of filtered versus unfiltered cigarettes, suggesting that other non-culturable bacteria may be aerosolized in mainstream smoke as well. Our study provides novel evidence that tobacco-associated bacterial communities are viable, can be aerosolized in mainstream smoke, and could potentially be transferred to the oral cavity and respiratory tract of smokers.

1. Introduction

Tobacco products contain more than 4000 chemicals, including nicotine, other toxicants and carcinogens (Stedman, 1968), that have been associated with adverse health effects experienced by tobacco users (Gupta et al., 1996). Recently, the U.S. Food and Drug Administration (FDA) established a list of 93 harmful and potentially harmful constituents (HPHCs) and required tobacco manufacturers to report on levels of HPHCs found in their tobacco products and smoke (US FDA, 2019). The list includes a variety of carcinogens, as well as respiratory, cardiovascular, reproductive and developmental toxicants, but is missing an entire group of tobacco constituents that could potentially affect the health of users: microorganisms.

Tobacco, being an agricultural product, is rich in microorganisms that naturally colonize the plants (Beattie and Lindow, 1999). Nevertheless, the microbiological constituents of tobacco products and their potential health impacts have received little attention despite an increasing number of studies demonstrating the presence of diverse microorganisms across a range of commercially available tobacco products (Chattopadhyay et al., 2019; Chopyk et al., 2017a, 2017b; Dygert, 1957; Eaton et al., 1995; Kurup et al., 1983; Larsson et al., 2008, 2012; Malayil et al., 2020; Rooney et al., 2005; Sapkota et al., 2010; Smyth et al., 2017, 2019; Tyx et al., 2016, 2020; Verweij, 2000). A recent review from our group reported that over 89 unique bacterial genera and 19 fungal genera have been characterized in the tobacco of cigarettes, cigars, cigarillos, hookah and smokeless products (Chattopadhyay et al., 2021).

Early studies using culture dependent methods identified

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microorganisms such as allergenic fungi (Kurup et al., 1983), thermophilic *Actinomycetes* (Kurup et al., 1983), *Pantoea* (Larsson et al., 2008), *Bacillus* (Rooney et al., 2005), *Kurthia* (Rooney et al., 2005) and *Mycobacterium avium* (Eaton et al., 1995) in cigarettes. One of the first studies to employ 16S rRNA taxonomic microarrays to evaluate total bacterial diversity in four brands of cigarettes identified 15 classes of bacteria, including potentially pathogenic species: *Acinetobacter, Bacillus, Burkholderia, Clostridium, Klebsiella, Pseudomonas aeruginosa, Serratia, Campylobacter, Enterococcus, Proteus* and *Staphylococcus* (Sapkota et al., 2010). More recent studies have applied next-generation sequencing techniques to confirm earlier results and improve the understanding of the tobacco microbiome, highlighting the breadth of bacterial diversity across commercially-available tobacco products, including cigarettes, smokeless tobacco products and little cigars (Chattopadhyay et al., 2019; Chopyk et al., 2017a, 2017b; Malayil et al., 2020; Sapkota et al., 2010; Smyth et al., 2019). Specifically, focusing on cigarette tobacco, *Bacillus*, *Pseudomonas* and *Staphylococcus* have been identified as the dominant bacterial genera in these products (Chopyk et al., 2017a, 2017b; Malayil et al., 2020; Sapkota et al., 2010).

Nevertheless, to our knowledge, no published studies have taken it a step further to evaluate whether viable bacteria identified in cigarette tobacco could be aerosolized and transferred to users via particles present in mainstream smoke. Bacteria derived endotoxins (present in the outer membrane of Gram-negative bacteria) (Barnes and Glantz, 2007; Hasday et al., 1999; Pauly and Paszkiewicz, 2011), and muramic acid (a peptidoglycan marker for gram-positive bacteria) (Larsson et al., 2008) have been detected as active components in tobacco smoke. Beyond identifying these bacterial markers in mainstream smoke, only one study has been able to recover viable *Mycobacterium avium* from smoked cigarette filters (Eaton et al., 1995), providing evidence that bacteria within the central portion of a cigarette can survive high temperatures and gases generated when the end of the cigarette is lit. However, besides this work, no studies have evaluated whether viable, particle-associated bacterial cells can be aerosolized in mainstream cigarette smoke. To address this knowledge gap, we performed a detailed characterization of the total bacterial diversity of filtered and unfiltered varieties of two different commercially available cigarette brands, and detected viable bacteria in their associated mainstream smoke using traditional culture dependent techniques and next-generation sequencing.

2. Materials and methods

2.1. Sample collection

Cigarettes were purchased from three convenience stores in the Washington D.C. metropolitan area. Four commercially available cigarette products manufactured by R.J. Reynolds Tobacco Company (Winston-Salem, NC, USA) were included: Camel Non-Filter (unfiltered), Camel Red (filtered), Pall Mall (unfiltered), and Pall Mall Red (filtered). Camel and Pall Mall brands were chosen because they are the only cigarette brands among the top five selling cigarettes in the U.S (CDC, 2020) that have a filtered and an unfiltered variety (Oren et al., 2020). A total of 32 cigarettes (two cigarettes per pack, 16 cigarettes per brand) were randomly chosen for analysis.

Each cigarette was dissected into three sections for separate experimentation (Fig. 1). The first section, 1 cm in length, was dissected from the end of the cigarette (the end that is not inserted into the mouth) under sterile conditions, placed into 25 ml of trypticase soy broth (TSB) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and incubated at 37 °C for 24 h. Eight replicates were included from each of the four cigarettes varieties (filtered and unfiltered from two brands), making a total of 32 “enriched non-smoked tobacco leaf extract” samples.

The second section, also 1 cm in length, was similarly dissected from the cigarette under sterile conditions. This section was aseptically transferred to 1 ml of ice cold 1× molecular grade PBS Buffer (Gibco by Life Technologies, Grand Island, NY, USA) in Lysing Matrix B tubes (MP Biomedicals, Solon, OH, USA), and stored at −80 °C until total DNA extraction. Similar to the first section, eight replicates were included from each of the four varieties, making a total of 32 “non-smoked tobacco leaf” samples.

The third and final section was the remaining length of the cigarette. This section (filter-end first for filtered cigarettes) was physically connected to the front of a sterile filter cartridge (GE Healthcare – Whatman, MA, USA) containing a 0.22 μm, 47 mm polyethersulfone membrane filter (Pall Corporation, Port Washington, NY, USA) (Fig. 1). Next, the rear of the filter cartridge was connected to a HEPA filter, which was then connected to a vacuum pump, both via sterile tubing. The free end of the cigarette was ignited with a conventional lighter and allowed to burn continuously for up to 5 min, with vacuum pressure

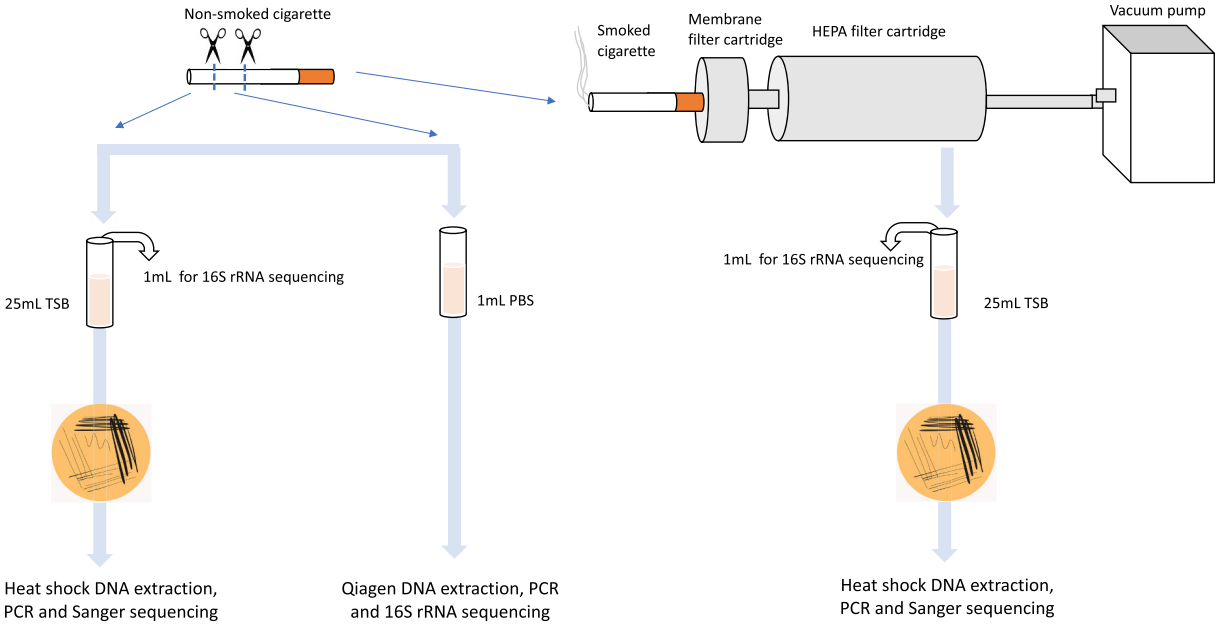

Fig. 1. Diagrammatic representation of the experimental setup.

incrementally increased from 0 to 10 Hg to sustain the burn. After 5 min, the burnt cigarette remains were disconnected from the cartridge and disposed of. The filter cartridge was disassembled to retrieve the membrane filter, which was then incubated at 37 °C for 24 h in 25 ml of TSB for further enrichment. Similar to the other two cigarette sections, a total of 32 "enriched smoke extract" samples were further analyzed.

The smoking apparatus was set up inside a chemical fume hood and before any cigarettes were smoked, a hood negative control was collected to ensure that there was no ambient aerial bacterial contamination: the vacuum was allowed to run for 30 s at 5 Hg with a filter-containing filter cartridge lacking an attached cigarette. Additionally, between each cigarette smoking, the disassembled filter cartridge was submerged in 70% ethanol for 3 min and then allowed to air dry for 20 min. After all cigarettes were smoked, a cigarette negative control was tested to ensure that the interspersed ethanol sterilizations were effective: an autoclaved sterile cigarette was attached and ignited in a similar fashion to the experimental cigarettes. For all negative controls, the filters from the filter cartridge were retrieved and incubated under the same conditions: 37 °C for 24 h in 25 ml TSB for further cultivation.

2.2. Culture-dependent technique for bacterial cultivation

From the incubated TSB enriched samples (non-smoked tobacco leaf extract and smoke extract): 1) 1 ml aliquot was recovered and stored in lysing matrix tubes at −80 °C until DNA extraction; and 2) 100 μl of each of sample was spread plated onto trypticase soy agar (TSA) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and incubated at 37 °C for another 24 h. After incubation, three colonies were picked and isolated onto fresh TSA plates, which were then incubated again for 24 h at 37 °C. The next day, one colony from each isolation plate was chosen for DNA extraction via a quick heat-shock lysis method described below.

2.3. Single colony DNA extraction

Isolated colonies were picked with a sterile loop and suspended in 200 μl of molecular-grade water and vortexed thoroughly. Next, cells were subjected to two consecutive rounds of heating at 100 °C for 5 min followed by cooling on ice for 5 min. Samples were briefly vortexed and then centrifuged at 10,000 rcf for 1 min to remove cell debris. Extracted DNA was later used for 16S rRNA PCR amplification.

2.4. Total DNA extraction

Total DNA extraction of 96 samples (enriched non-smoked tobacco leaf extract, n = 32; non-smoked tobacco leaf, n = 32; and enriched smoke extract, n = 32) was performed as previously described (Chopyk et al., 2017a, 2017b). Briefly, to achieve robust enzymatic lysis, 5 μl lysostaphin from *Staphylococcus staphylolyticus* (5 mg/ml, Sigma-Aldrich, MO), 5 μl lysozyme from chicken egg white (10 mg/ml, Sigma-Aldrich, MO), and 15 μl of mutanolysin from *Streptomyces globisporus* ATCC 21553 (1 mg/ml, Sigma-Aldrich, MO) were added to lysing matrix B tubes (Company, City, State, USA). Tubes were then incubated at 37 °C for 30 min. A second enzymatic cocktail consisting of 50 μl of SDS (10% w/v, BioRad) and 10 μl Proteinase K (20 mg/ml, Invitrogen by Life Technologies, Grand Island, NY, USA) was added to the tubes, which was followed by an incubation at 55 °C for 45 min. Samples were then mechanically lysed at 6.0 m/s for 40 s via a FastPrep Instrument FP-24 (MP Biomedicals, CA). Following mechanical lysis, samples were centrifuged for 3 min at 10,000 rcf. DNA was subsequently cleaned with a QIAmp DSP DNA mini kit 50, v2 (Qiagen, CA) using the manufacturer's protocol. Negative extraction controls were included to ensure that no contamination occurred during the extraction process. Purified DNA was later used for 16S PCR rRNA gene amplification.

2.5. 16S rRNA gene PCR amplification and sequencing

For single colony extracted DNA, the universal 16S primers were tailed with the universal M13 sequence for ease of downstream Sanger sequencing: 319F-M13 (CAGGAAACAGCTATGACACTCCTRCGGGAGGCAGCAG) and 806R-M13 (GTAAAACGACGGCCAGGGACTACHVGGGTWTCTAAT). 25 μl PCR reactions were run in a DNA Engine Tetrad 2 thermo cycler (Bio-Rad, USA) using the following cycling parameters: 3 min at 94 °C, followed by 25 cycles of 30s at 94 °C, 30s at 58 °C, and 60s at 72 °C, with a final step of 10 min at 72 °C. PCR amplicon concentrations were quantified with spectrophotometry via a NanoDrop™ (Thermo Scientific, City, State) and visualized via standard agarose gel electrophoresis. The amplicons were then sent to GENEWIZ (South Plainfield, NJ) for Sanger sequencing.

From total DNA extractions, the V3–V4 region of the 16S rRNA gene was amplified and sequenced using the 319F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) universal primers barcoded for each sample that also included a linker sequence required for Illumina HiSeq 300 bp paired-ends sequencing, and a 12-bp heterogeneity spacer index sequence. Amplification of sample DNA and negative controls was completed using previously published thermocycler parameters (Chopyk et al., 2017a, 2017b). Amplicon presence was confirmed with gel electrophoresis and cleaned up using the SequelPrep Normalization Kit (Invitrogen Inc., Carlsbad, CA, USA). Samples were pooled at a concentration of 25ng/PCR amplicon and sequenced on an Illumina HiSeq2500 (Illumina, San Diego, CA) at the Institute for Genome Sciences, Baltimore, MD.

2.6. Analysis of 16S rRNA gene sequences

For single-colony Sanger sequencing reads, taxonomic identification was completed based on comparing the sequence to the closest 16S rRNA gene sequences in the GenBank databases using the NCBI BLAST (Altschul et al., 1990) search tool.

16S rRNA Illumina HiSeq reads were screened for low quality and short read length, assembled using PANDAseq (Masella et al., 2012), demultiplexed and chimera trimmed using UCHIME (Edgar et al., 2011). Quality reads were then incorporated into QIIME v1.9 (Caporaso et al., 2010) and clustered de-novo using VSEARCH and taxonomies were assigned using the SILVA database v.132 (Quast et al., 2012), using a 0.97 confidence threshold. Downstream data analysis and visualization were completed in RStudio (v.1.1.423) using R packages: biomformat (v.1.2.0) (McDonald et al., 2012) 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) (Joseph Nathaniel Paulson, 2017). All sequences taxonomically assigned to the Phylum *Cyanobacteria* were removed from further downstream analysis. When appropriate, data were normalized with metagenomeSeq's cumulative sum scaling (CSS) (Joseph Nathaniel Paulson, 2017) 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). Bray-Curtis dissimilarity was used for calculating beta diversity and was compared using analysis of similarities (ANOSIM) on normalized data (999 permutations). In addition, bacterial taxa 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 and sample type using several R packages: vegan (v.2.4.5) (Oksanen et al., 2017), dplyr (v.0.7.8) (Wickham, 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 the plots were constructed using Cytoscape (v.3.7.2).

2.7. Availability of data

Data concerning the samples included in this study have been deposited under the NCBI BioProject accession number PRJNA686982.

3. Results

3.1. Data summary

From the TSA plates, 138 isolates (Camel unfiltered, n = 42; Camel Red (filtered), n = 24; Pall Mall unfiltered, n = 48; and Pall Mall Red (filtered), n = 24) were picked, and DNA extracted. Of these, 125 isolates were identified with Sanger sequencing. From the enriched smoke extract samples, 16 out of 24 colonies picked from Pall Mall unfiltered samples and 14 out of 18 colonies picked from Camel unfiltered samples were positively identified, while no colonies could be cultured from the filtered variety of both cigarette brands. From the enriched leaf extract samples, all 24 isolates from each of the Camel (unfiltered and filtered) and the Pall Mall Red (filtered) cigarettes were positively identified through sequencing. Only one isolate out of 24 isolates picked from Pall Mall unfiltered cigarettes could not be identified.

From the 16S rRNA Illumina sequencing, a total of 3,194,032 sequences were obtained from 96 samples and clustered into 2022 operational taxonomic units (OTUs). The minimum number of sequences recorded was 184 and the maximum was 57,368 (average of 33.271.17; SD ± 13,754.74). To ensure appropriate sequence coverage, a Good's coverage cutoff was set at 0.85. Sequences above this cutoff were checked for quality and filtered for downstream analysis. After removing cyanobacterial sequences and OTUs with less than 10 sequences, a total of 2,822,768 sequences were obtained from 95 samples, with a maximum of 55,136 and a minimum of 321 (average 29,756.92; SD ± 12,232.64) sequences across all samples. Overall, these sequences were clustered into 1128 OTUs.

3.2. Diversity metrics

Alpha diversity metrics (observed number of species and Shannon indices) were calculated on rarefied 16S rRNA sequences from 95 samples (Fig. 2a). Within each brand of cigarette, irrespective of sample types (non-smoked leaf, enriched non-smoked leaf extract and enriched smoke extract), samples from the filtered cigarettes [Camel brand (Observed: 37.70 ± 23.11; Shannon: 2.12 ± 1.18) Pall Mall brand (Observed 49.57 ± 23.89; Shannon: 2.83 ± 0.65)] were not significantly different ($p > 0.05$) in terms of alpha diversity indices when compared to those from the unfiltered cigarettes [Camel brand (Observed: 33.79 ± 18.30; Shannon: 1.99 ± 0.98) Pall Mall brand (Observed 44.50 ± 18.66; Shannon: 2.66 ± 0.54)]. However, statistically significant effects of sample type (ANOVA $p < 0.001$) were observed with regard to bacterial alpha diversity in both brands of cigarettes (Camel and Pall Mall). Specifically, enriched smoke extract samples had lower alpha diversity compared to the other two sample types (non-smoked leaf, enriched non-smoked leaf extract), irrespective of the filter variety. Additionally, a direct comparison of enriched smoke extract samples between the two brands of cigarettes revealed that the smoke from Camel cigarettes (Observed: 20.56 ± 19.28; Shannon: 0.99 ± 1.11) had significantly (ANOVA $p < 0.001$) lower alpha diversity indices when compared to that of Pall Mall cigarettes (Observed: 30.69

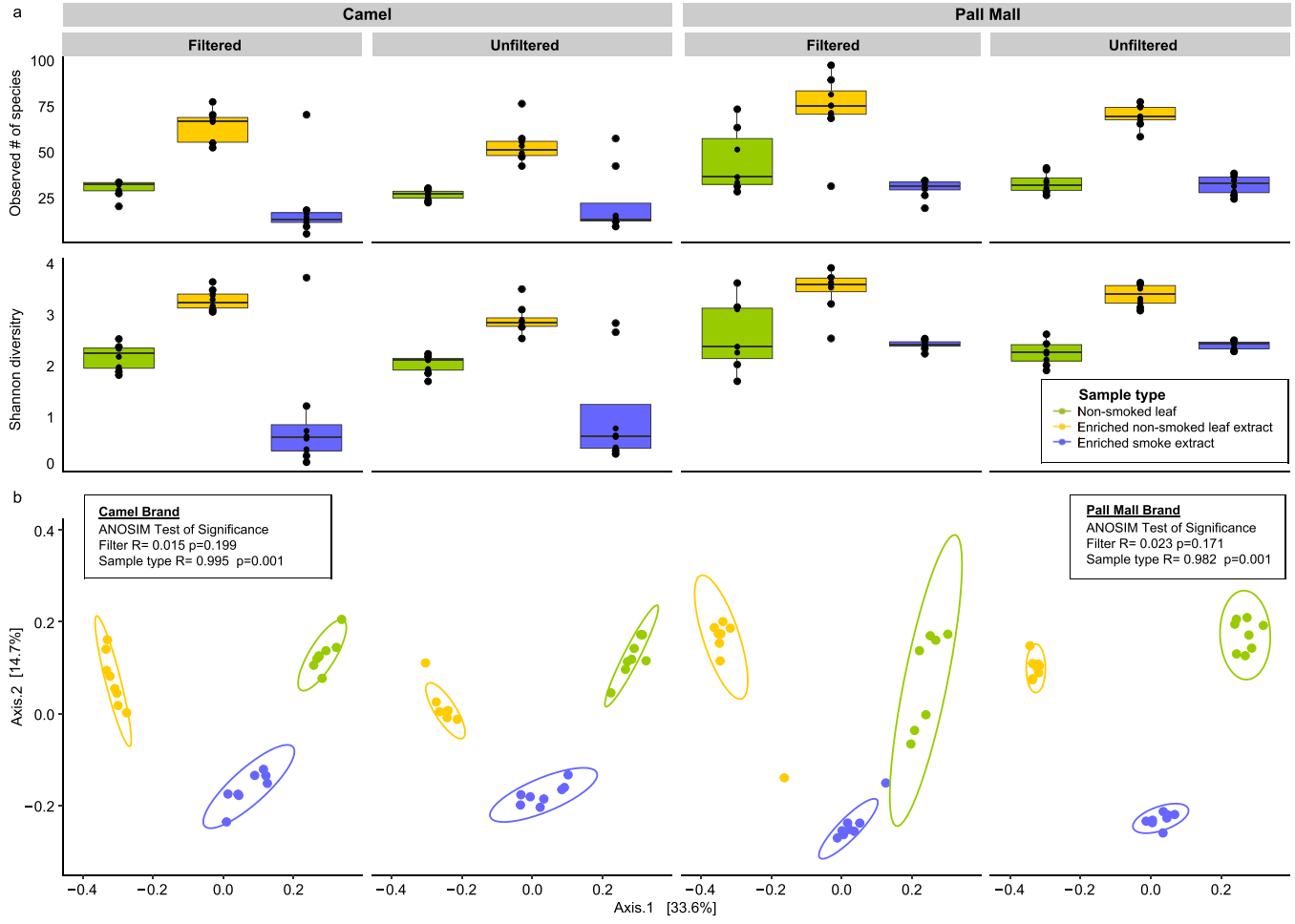

Fig. 2. Bacterial diversity across samples on rarefied data. (a) Box plots showing alpha diversity variation across Camel and Pall Mall cigarettes. (b) PCoA analysis plots of Bray-Curtis computed distances between cigarette products. Bars and points colored by sample type: *green*—non-smoked leaf; *yellow*—enriched non-smoked leaf-extract; *blue*—enriched smoke-extract. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

± 5.14; Shannon: 2.34 ± 0.09).

Beta diversity was computed on CSS normalized (non-rarefied) data using PCoA analysis of Bray-Curtis dissimilarity (Fig. 2b). Sample type (non-smoked leaf, enriched non-smoked leaf extract and enriched smoke extract) had a significant effect ($p < 0.001$) on the bacterial diversity in both brands, explaining over 98% of the variation within the bacterial community. In contrast, bacterial diversity was not impacted ($p > 0.05$) by the presence (or absence) of a filter within each brand. Additionally, comparing among the non-smoked leaf extracts samples from the same brand, confidence interval ellipses demonstrated higher heterogeneity within the filtered variety when compared to its unfiltered counterpart. This was not true for enriched smoke extract and non-smoked leaf samples.

3.3. Bacterial community composition: culture-dependent vs. culture-independent data

The top genera cultured from Camel cigarettes irrespective of the filter variety (filtered or unfiltered) and sample type was *Bacillus* spp. (Fig. 3a). Of the 58 *Bacillus* isolates, 11 were recovered from enriched smoke extract of the unfiltered variety, 24 were recovered from enriched non-smoked leaf extract of the unfiltered variety, and 23 were recovered from enriched non-smoked leaf extract of the filtered variety. The other bacterial genera cultured from the Camel brand included three isolates of *Terribacillus* spp. from enriched smoke extract from unfiltered cigarette samples and one isolate of *Lysinibacillus* spp. from the enriched non-smoked leaf extract of a filtered cigarette sample. As mentioned earlier, no colonies were recovered from the enriched smoke extract from Camel Red (filtered) products.

Culture-independent data identified *Bacillus, Paenibacillus,* and *Pseudomonas* as the top bacterial genera in Camel cigarettes irrespective of the sample type and filter variety (Fig. 3b). Among the enriched smoke extract samples, *Bacillus* was the predominant bacterial genus, with an average relative abundance of 57.14% (min 0.17%; max 94.01%) in Camel Red (filtered) samples and 36.73% (min 0.06%; max 96.25%) in Camel unfiltered samples. Additionally, at the species level, *B. cereus* was identified in Camel Red samples at an average relative abundance of 13.56% (min 0.02%; max 97.22%). *B. clausii* and *B. flexus* were identified in Camel unfiltered samples at an average relative abundance of 11.20% (min 0.01%; max 89.37%), and 10.97% (min 0.01%; max 87.64%), respectively. We also observed the presence of *Terribacillus* in the enriched smoke extract of unfiltered Camel cigarettes in both our culture-dependent and -independent data.

For Pall Mall cigarettes, irrespective of the filter variety (filtered or unfiltered) and sample type, the top genera cultured was *Bacillus* spp. (Fig. 4a). Among the total 57 *Bacillus* isolates, 12 were recovered from enriched smoke extract from unfiltered cigarettes, 21 were recovered from enriched non-smoked leaf extract of unfiltered cigarettes, and 24 were recovered from enriched non-smoked leaf extract of filtered cigarettes. Three isolates of *Paenibacillus* spp. and one isolate of *Desulfotomaculum* spp. were cultured from enriched smoke extract, and two isolates of *Pseudomonas* spp. were recovered from the enriched non-smoked leaf extract of unfiltered cigarettes.

Similar to Camel cigarettes, from our culture-independent data we observed *Pseudomonas, Bacillus,* and *Paenibacillus* to be among the top bacterial genera in Pall Mall cigarettes (Fig. 4b). But unlike Camel cigarettes, enriched smoke extract from Pall Mall samples was dominated by *Pseudomonas* at an average relative abundance of 57.14% (min 0.17%; max 94.01%) in filtered cigarettes and 36.73% (min, 0.06%; max, 96.25%) in unfiltered cigarettes. Other bacterial taxa observed in enriched smoke extract were *P. veronii*, *Janthinobacterium lividum*, unclassified *Aeromonadaceae*, unclassified *Sphingomonas*, unclassified *Curvibacter*, unclassified *Oxalobacteriaceae*, and unclassified *Citrobacter*.

Comparing between brands, culture-independent data (Figs. 3b and 4b) were similar. For example, the relative abundance of *B. cereus* was found to be highest in the non-smoked leaf samples of both brands. The relative abundance of unclassified *Paenibacillus* was found to be highest among the non-smoked leaf samples from both brands of cigarettes when compared to other sample types (smoke and leaf extract). Among the Pall Mall cigarettes, the relative abundance of *J. lividum* was found to be significantly higher in the enriched smoke extract samples and lowest in the non-smoked leaf samples. In contrast, the Camel cigarettes were not characterized by significant differences between sample types. Irrespective of cigarette brand, the relative abundance of *Lysinibacillus*

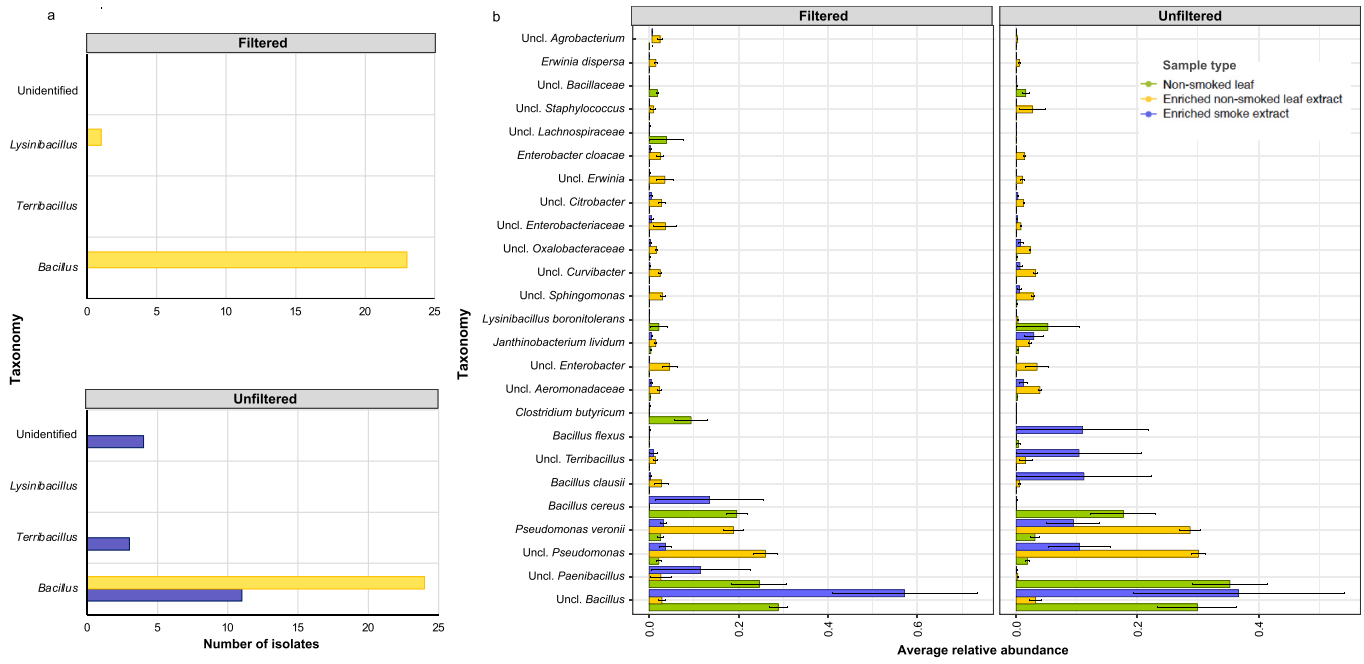

Fig. 3. (a) Bacterial isolates identified from Camel cigarettes (filtered and unfiltered) via the culture-dependent method. (b) Relative abundance of top 25 bacterial genera identified via the culture-independent method in Camel cigarettes. Bars colored by sample type: *green*—non-smoked leaf; *yellow*—enriched non-smoked leaf-extract; *blue*—enriched smoke-extract. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

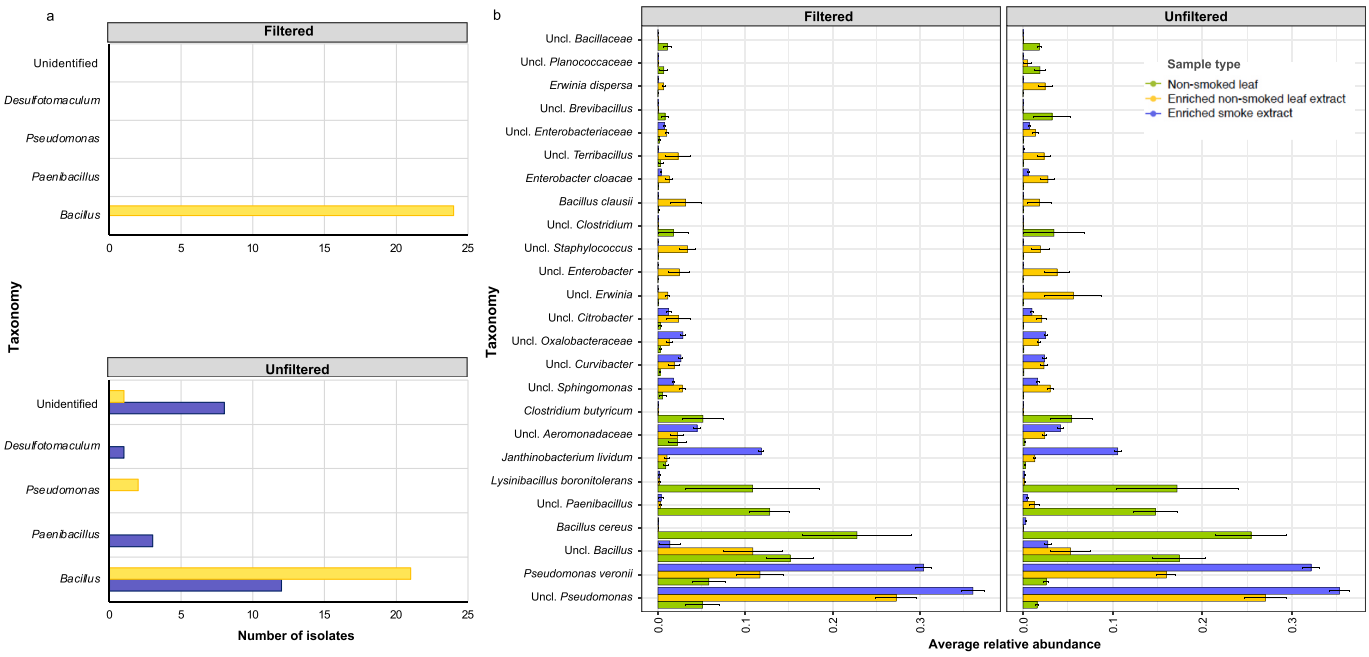

Fig. 4. (a) Bacterial isolates identified from Pall Mall cigarettes via the culture-dependent method. (b) Relative abundance of top 25 bacterial genera identified via the culture-independent method in Pall Mall cigarettes. Bars colored by sample type: *green*—non-smoked leaf; *yellow*—enriched non-smoked leaf-extract; *blue*—enriched smoke-extract. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

boronitolerans and *Clostridium butyricum* was significantly higher in the non-smoked leaf samples when compared to that of enriched non-smoked leaf extract and enriched smoke extract samples.

3.4. Unique and shared bacterial taxa by cigarette brand and sample type

In Camel cigarettes, unique bacterial taxa were observed among the three sample types: enriched non-smoked leaf-extract (*Erwinia*, *Aeromonadaceae*, *Enterobacter*, *Sphingomonas*, *Curvibacter*, and *Citrobacter*); enriched smoke extract (*B. clausii*, *Terribacillus* and *B. flexus*); and non-smoked leaf (*L. boronitolerans* and *C. butyricum*). Shared bacterial taxa among the three sample types were *Bacillus*, *Pseudomonas* and *P. veronii*. *B. cereus* and *Paenibacillus* were found to be shared among the non-smoked leaf and enriched smoke extract (Fig. 5a).

In Pall Mall samples, three bacterial taxa (*Bacillus*, *Pseudomonas* and *P. veronii*) that were shared among the three sample types (non-smoked leaf, enriched non-smoked leaf extract and enriched smoke extract) were similar to that found among the Camel samples. Additionally, we also observed that *Aeromonadaceae* and *Curvibacter* were shared among enriched smoke-extract and enriched leaf-extract samples. The unique bacterial taxa observed among the three Pall Mall sample types were as follows: enriched leaf-extract (*Staphylococcus*, *Enterobacter*, *E. cloacae*, *Sphingomonas*, *Citrobacter*, *B. clausii*, *Erwinia* and *Terribacillus*); enriched smoke-extract (*J. lividum* and *Oxalobacteraceae*); and non-smoked leaf samples (*Clostridium*, *C. butyricum*, *Brevibacillus*, *Paenibacillus*, *B. cereus* and *L. boronitolerans*) (Fig. 5b).

4. Discussion

To our knowledge, this study provides the first evidence that viable bacterial communities present in commercial cigarette tobacco can be aerosolized in the mainstream smoke of unfiltered cigarettes. Interestingly, we were unable to culture viable bacteria from the mainstream smoke of filtered cigarettes. However, our culture-independent sequencing data demonstrated no significant differences in bacterial community diversity in the mainstream smoke of filtered cigarettes

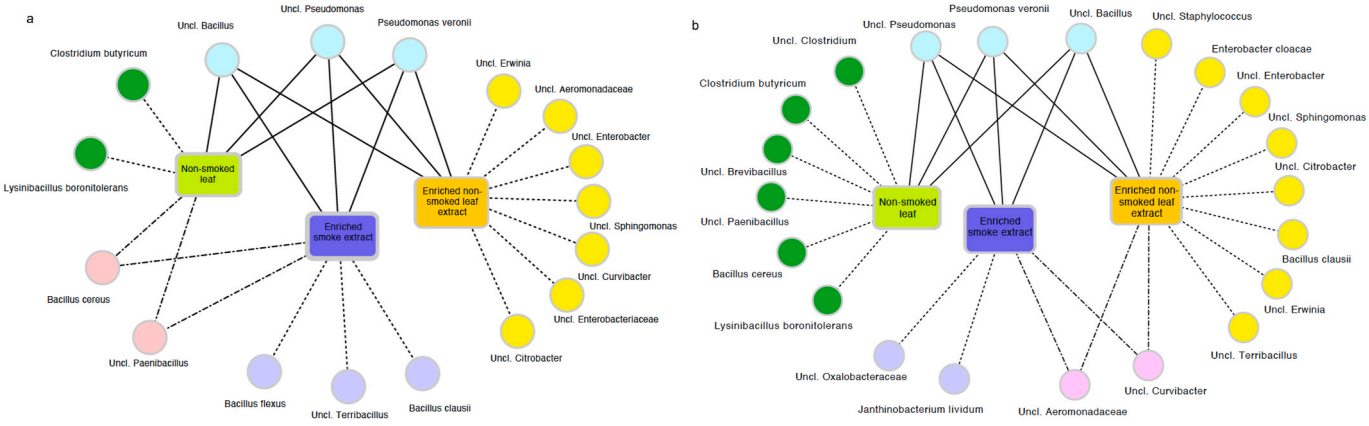

Fig. 5. Network analysis plots showing shared and unique bacterial operational taxonomic units (OTUs) between three sample types in (a) Camel cigarettes (b) Pall Mall cigarettes. Solid lines represent shared bacterial OTUs across all three sample types and dashed lines represent unique OTUs. Dashed-dotted lines represent shared OTUs between two sample types.

compared to their unfiltered counterparts. These discrepant results could suggest that while cigarette filters can trap some bacteria in mainstream smoke, culture-independent sequencing techniques might be more sensitive in detecting other bacterial species present in cigarette smoke that cannot be cultured. There is a general consensus among environmental microbiologists that less than 2% of bacterial species are culturable (Wade, 2002), and that amplicon sequencing is not only more accurate (Gupta et al., 2019) but also more sensitive with regard to bacterial detection (Manaka et al., 2017) when compared to traditional culture techniques. It is also worth noting that our culture technique employed non-selective media (TSB and TSA) under aerobic conditions, which were likely incapable of detecting many of the bacterial genera and species that were identified with our sequencing approach.

Nevertheless, we were able to identify viable *Bacillus*, *Terribacillus, Paenibacillus* and *Desulfotomaculum* in cigarette smoke extract through traditional culture techniques. Using amplicon sequencing, we also identified *Bacillus* spp. (*B. cereus, B. clausii*), *Pseudomonas* spp. (*P. veronii*), *Paenibacillus*, *Citrobacter* spp., *Curvibacter* spp., *Sphingomonas* spp., unclassified *Aeromonadaceae*, and *J. lividum* in all tested smoke extract samples.

The most abundant Gram-positive bacteria identified in our study (through both culture-dependent and -independent techniques) was *Bacillus* spp. Although we were able to culture *Bacillus* from both filtered and unfiltered enriched non-smoked leaf extract, we were only able to recover it from unfiltered enriched smoke samples. Previous studies using culture-independent techniques have identified *B. pumilus*, *B. coagulans* and *B. clausii* spp. in the tobacco of commercially available cigarettes (Camel, Marlboro and Newport) (Chopyk et al., 2017a, 2017b; Malayil et al., 2020). Additionally, Sapkota et al. (2010) identified *B. pumilus* in other commercially-available cigarettes (Kool Filter Kings and Lucky Strike) using a combination of microarrays, cloning and sequencing (Sapkota et al., 2010). As a part of the U.S. Army's investigation of a series of acute eosinophilic pneumonitis cases, eight species of *Bacillus* (including *B. cereus)* were identified in regionally representative cigarettes samples (Rooney et al., 2005). *Bacillus* spp. has also been recovered from fresh tobacco leaves (Larsson et al., 2008), cured tobacco leaves (Kaelin and Gadani 2000), little cigars and cigarillos (Chattopadhyay et al., 2019), and smokeless tobacco (Smyth et al., 2017; Tyx et al., 2016).

However, all of the previous studies mentioned above identified *Bacillus* spp. on either tobacco plant leaves, or in the tobacco product, but lacked data demonstrating the viability of any bacteria in mainstream smoke. As mentioned above, an earlier study recovered viable *Mycobacterium avium* from the filters of smoked cigarettes, suggesting that bacteria from tobacco could potentially pass into and possibly through the filter during the smoking process (Eaton et al., 1995). The present study builds upon this work and definitively demonstrates that viable *Bacillus* spp., including *B. cereus* and *B. clausii,* are not only present in enriched leaf extract but also can be recovered from the mainstream smoke of unfiltered cigarettes.

Another Gram-positive bacteria that is widely prevalent in the environment, *Paenibacillus* spp.*,* was also identified in all sample types through both culture-dependent and -independent techniques. This naturally occurring, nitrogen-fixing bacteria provides protection to plants through the production of antimicrobials and insecticides (Grady et al., 2016). Previous studies have identified *Paenibacillus* spp. in tobacco products (Chopyk et al., 2017a, 2017b; Li et al., 2014), and a few species within this genera have been associated with acute respiratory distress syndrome (Yoon et al., 2015) and mediastinitis after cardiac surgery (Anikpeh et al., 2010). Both *Bacillus* and *Paenibacillus* are spore forming genera, which are being increasingly isolated from multiple kinds of tobacco products (Chattopadhyay et al., 2021), and their potential virulence properties could lead them to behave as pathogens/opportunistic pathogens (Brown et al., 2012; Celandroni et al., 2016).

Pseudomonas spp. were the predominant Gram-negative bacteria that were isolated from all of the sample types of the tested cigarettes. *Pseudomonas* spp. has been shown to be highly prevalent in multiple tobacco products (Chopyk et al., 2017a, 2017b; Dygert, 1957; Malayil et al., 2020; Sapkota et al., 2010), including species that are known opportunistic pathogens. Specifically, *P. aeruginosa, P. stutzeri, P. fulva, P. oryzihabitans, P. pseudoalcaligenes, P. viridiflava, P. veronii, P. thermotolerans, P. putida*, and *P. cichorii* have been identified in commercial cigarettes (Chopyk et al., 2017a, 2017b; Malayil et al., 2020; Sapkota et al., 2010), little cigars (Chattopadhyay et al., 2019), and smokeless tobacco products (Smyth et al., 2017; Tyx et al., 2016, 2020).

Besides being potentially pathogenic, a number of *Pseudomonas* species and strains are able to degrade nicotine (Chen et al., 2008; Hu et al., 2019; Wang et al., 2012; Xia et al., 2019). In the present study, we were able to culture *Pseudomonas* from Pall Mall leaf extract and identified *P. veronii* in all sample types (mainstream smoke and leaf extract) of both cigarette brands (Camel and Pall Mall) using our 16S rRNA sequencing data. *P. veronii* is a toluene degrader and toluene has been shown to be present mainly in the vapor phase of mainstream cigarette smoke (Moldoveanu et al., 2008), and can contribute to both acute and chronic health effects in humans. Perhaps these microorganisms may play a role in mitigating the negative impacts of multiple chemical constituents in tobacco, including toluene and nicotine. For instance, Chen et al. (2008) described *Pseudomonas* spp. that were able to degrade nicotine in tobacco leaves without causing any loss of desirable taste, flavor and smoking properties (Chen et al., 2008). Hence, improving understanding of nicotine and toluene biotransformation by tobacco-associated microorganisms and applying this knowledge to modify the tobacco curing and manufacturing process might help to reduce tobacco-induced damage among users without compromising tobacco aesthetics.

Other Gram-negative bacteria that were isolated from all of the sample types of the tested cigarettes tested were *Sphingomonas* spp., *Citrobacter* spp., *Aeromonas* spp., and *Curvibacter* spp. Similar to *Pseudomonas*, the bacterial genera *Sphingomonas* and *Aeromonas* have been detected in commercial cigarettes (Chopyk et al., 2017a, 2017b; Malayil et al., 2020) and contain species that have been regarded as opportunistic pathogens (Johnson et al., 2018; Nikiforov et al., 2014). In contrast, *Citrobacter* spp., and *Curvibacter* spp. have not been previously detected in tobacco products. However, the periodontal infection of a 78-year-old heavy smoker was attributed to *Citrobacter koseri* (Ando et al., 2019), and *Curvibacter* spp. have been isolated from patients with chronic obstructive pulmonary disease (COPD) (Cullen and McClean, 2015).

Our study had several limitations. First of all, we included only two commercially-available cigarette brands. Previous studies have demonstrated significant differences in bacterial diversity between brands and lots (Chopyk et al., 2017a, 2017b; Malayil et al., 2020). While we chose the third and fourth largest selling cigarette brands in the U.S. (because these brands included filtered and unfiltered counterparts that could be compared), a future study should include other popular cigarette brands, as well as other types of smoked tobacco products (e.g., cigars, cigarillos, and hookah). Moreover, we did not use selective media for the growth of specific bacteria. Identifying other viable bacteria (including pathogens and opportunistic pathogens) that could be transferred to tobacco users via mainstream smoke, is an extremely important area for future work. In addition, our sequencing data (like all sequencing data) are limited in that we could not distinguish between metabolically-active (live) bacteria vs relic DNA (from dead organisms) within the total bacterial community members that were identified. Future studies, incorporating coupled DNA-labeling and sequencing methods that can tease out the metabolically-active bacteria within the total bacterial communities detected can help address this challenge. Finally, we focused on characterizing only the bacterial communities of cigarette tobacco and cigarette smoke, while previous studies have demonstrated the presence of fungal markers (e.g., ergosterol) and fungi (or fungal biomass) in multiple tobacco products and/or

mainstream smoke (Larsson et al., 2008). Hence, to gain a more complete understanding of tobacco and smoke microbiomes, it is necessary to include the characterization of the fungal members residing within these products.

In conclusion, to our knowledge, this study is the first to elaborately demonstrate the aerosolization of viable bacteria from cigarette tobacco to mainstream smoke via culture-dependent and -independent methods. Although we were only able to culture select bacteria from the mainstream smoke of unfiltered cigarettes, and not from their filtered counterparts, our sequencing data suggest that other bacteria that could not be cultured using the non-selective techniques used in this study may potentially be transferred to mainstream smoke as well. Future studies are needed to further evaluate the microbiome of mainstream smoke and determine whether both viable bacterial and fungal communities could be inhaled by users, potentially impacting the microbiome of the upper respiratory tract and contributing to respiratory and other smoking-related diseases.

Authors information

LM and SC performed laboratory analyses, conducted data analyses, wrote and edited the manuscript. AB, MP, RC and LH performed laboratory analyses. ARS and EFM contributed to the study design, protocol development, data analysis and interpretation, and manuscript preparation. All authors read and approved the final manuscript.

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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.

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