



Microbiome-scale analysis of aerosol facemask contamination during nebulization therapy in hospital

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SUMMARY

Background: Microbial contamination of aerosol facemasks could be a source of nosocomial infections during nebulization therapy in hospital, prompting efforts to identify these contaminants. Identification of micro-organisms in medical devices has traditionally relied on culture-dependent methods, which are incapable of detecting the majority of these microbial contaminants. This challenge could be overcome with culture-independent sequencing-based techniques that are suited for the profiling of complex microbiomes.

Aim: To characterize the microbial contaminants in aerosol facemasks used for nebulization therapy, and identify factors influencing the composition of these microbial contaminants with the acquisition and analysis of comprehensive microbiome-scale profiles using culture-independent high-throughput sequencing.

Methods: Used aerosol facemasks collected from hospitalized patients were analysed with culture-independent 16S rRNA gene-based amplicon sequencing to acquire microbiome-scale comprehensive profiles of the microbial contaminants. Microbiome-based analysis was performed to identify potential sources of microbial contamination in facemasks.

Findings: Culture-independent high-throughput sequencing was demonstrated for the capacity to acquire microbiome-scale profiles of microbial contaminants on aerosol facemasks. Microbial source identification enabled by the microbiome-scale profiles linked microbial contamination on aerosol facemasks to the human skin and oral microbiota. Antibiotic treatment with levofloxacin was found to reduce contamination of the facemasks by oral microbiota.

Conclusion: Sequencing-based microbiome-scale analysis is capable of providing comprehensive characterization of microbial contamination in aerosol facemasks. Insight gained from microbiome-scale analysis facilitates the development of effective strategies for the prevention and mitigation of the risk of nosocomial infections arising from exposure to microbial contamination of aerosol facemasks, such as targeted elimination of potential sources of contamination.

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Introduction

Nosocomial infections, a significant cause of global morbidity and mortality, are associated with a significant increase in healthcare burden worldwide [1]. Microbial contamination of medical devices, such as jet nebulizers, has been identified as one source of nosocomial infections [2]. Nebulizers deliver aerosolized medication deep into the respiratory tract. Pathogenic micro-organisms, if present on contaminated nebulizers, could pose particular risks of respiratory nosocomial infection in vulnerable patients with chronic respiratory conditions, including cystic fibrosis and chronic obstructive pulmonary disease [3].

When nebulizers are employed as aerosol generators, medications can be delivered to the patient with either a mouthpiece or a facemask. Facemasks are generally employed for patients who are unable to hold a mouthpiece properly. Increasing recognition that nebulizers could be a source of nosocomial infections has spurred efforts to investigate the occurrence of microbial contamination in nebulizers [4–6]. Notably, previous studies identified colonization of nebulizers by potential pathogens such as *Stenotrophomonas* spp. [7], *Pseudomonas* spp. [8] and *Burkholderia* spp. [9]. However, to the authors' knowledge, no previous studies have focused on microbial contamination of aerosol facemasks, which have greater exposure to facial skin microbiota and may get contaminated by microbial populations that are distinct from mouthpieces used for nebulizer therapy. Therefore, the microbial populations on aerosol facemasks need further investigation.

Identification of microbial contaminants in medical devices has traditionally relied on culture-based techniques [6]. It is known that a considerable majority of micro-organisms are not tractable for laboratory cultivation [10]. Even for micro-organisms that can be cultivated, certain populations could remain in the viable but unculturable state [11]. As a result, it is likely that a significant proportion of the microbial contaminants in medical devices may not be detectable by culture-based techniques. More importantly, given the potential diversity of micro-organisms present in medical devices [7,9,12], it would be infeasible for culture-based techniques to capture the majority of microbial contaminants. This challenge could be overcome with culture-independent sequencing-based techniques that are well suited for the profiling of complex microbiomes [13].

Thus, with the goal of assessing microbial populations specifically related to the use of facemasks for nebulizer therapy, the objectives of the current study were to acquire comprehensive profiles of microbial contaminants present in aerosol facemasks, and identify factors influencing the composition of these microbial profiles. Findings from this study provide information needed to develop strategies to mitigate risks arising from exposures to microbial contamination during nebulizer therapy.

Methods

Sample collection

Nebulizers with adult aerosol facemasks (Model 210, Westmed, Tucson, AZ, USA) were collected from 28 inpatients, referred to as 'subjects' hereafter, admitted to the University

of Tennessee Medical Center (UTMC) Hospital, Knoxville, TN, USA with a respiratory condition that required nebulized treatment with an aerosol facemask. Subjects (information in Table S1, see online supplementary material) gave consent to collect their used medical devices as described in University of Tennessee Graduate School of Medicine Institutional Review Board Protocol No. 4279.

Used aerosol facemasks were collected during a subject's hospital stay according to the following scheme: an unused nebulizer and facemask were provided to the patient upon admission. After being used by the subject for 24 h, the used nebulizer and facemask were collected for analysis. Similarly, a second or third set of unused nebulizer and facemask was subsequently provided to the same subject and collected after use for analysis. Aerosol facemasks were collected by certified respiratory therapists and placed in sterile plastic bags for subsequent analysis. The study was conducted in accordance with the guidelines of the Institutional Review Board of UTMC.

Sample processing

The used aerosol facemasks collected from hospitalized subjects were analysed with heterotrophic plate count (HPC) as a measure of the level of microbial contamination. Further analysis was performed with culture-independent 16S rRNA gene-based amplicon library sequencing to acquire microbiome-scale comprehensive profiles of the microbial contaminants in the aerosol facemasks. To collect microbial biomass from the aerosol facemasks, sterile polyester-tipped swabs moistened with sterile phosphate buffered saline (PBS) were used to swab the facemasks, as described previously [14]. For 16S rRNA gene-based amplicon library sequencing, the swabs were preserved at -80 °C for subsequent DNA extraction and processing.

For HPC analysis, the facemask was dissected aseptically to allow the parts to fit in 50-mL centrifuge tubes for vortexing in PBS, as described previously [15]. The resulting suspensions represented microbial biomass removed from the nebulizer parts, which were plated on to agar plates for HPC analysis according to standard protocols, as described previously [16]. HPC analysis was conducted separately for the facemask and medicine cup of the nebulizer assembly to compare the level of microbial contamination between these two parts of the nebulizer assembly.

High-throughput sequencing

High-throughput sequencing of 16S rRNA gene amplicon libraries was conducted following previously described protocols to profile microbial communities comprehensively at microbiome scale [17,18]. The FastDNA Spin Kit for Soil (MP Biomedicals, Irvine, CA, USA) was used to extract DNA from swab samples according to the manufacturer's instructions. DNA extracts were then subjected to polymerase chain reaction (PCR) amplification using 515F as the forward primer and 806R as the reverse primer, which also contained the unique 12-base specific barcode [17,18], followed by clean-up of PCR products with the ChargeSwitch Nucleic Acid Purification Technology (Invitrogen, Carlsbad, CA, USA) to remove contaminants including primer dimers. Purified PCR products were pooled to construct the amplicon library, which was quantified with the KAPA Illumina Library Quantification Kit (Kapa

Biosystems, Wilmington, MA, USA). Paired-end sequencing of the amplicon libraries was conducted with the Illumina MiSeq platform (Illumina, San Diego, CA, USA). Unused aerosol facemasks were used as controls for microbial biomass collection, DNA extraction and PCR amplification following the same procedures as the used nebulizers. However, processing of control facemasks did not yield PCR products, and subsequent DNA sequencing to acquire microbiome profiles for unused facemasks was not performed.

Data analysis

Raw sequence reads were initially processed with QIIME2 Version 2020.6 for quality filtering [19]. QIIME2 was also used for taxonomic analysis with SILVA Release 138 [20,21]. Sequences were clustered into operational taxonomic units (OTUs) with QIIME2 using the de-novo clustering protocol set at 97% similarity. Principal coordinate analysis was used to visualize Bray–Curtis dissimilarities between samples. The R package ggplot2 was used to visualize all data [22]. Raw sequence reads were deposited at the sequence read archive database of the National Center for Biotechnology Information with Accession Nos SAMN24009843–SAMN24009889 under Project PRJNA788514.

Microbiome-based source identification

Potential sources of microbial contamination in the facemasks were identified using the microbiome-based SourceTracker software, as described previously [23]. Three human-related sources – faecal, oral and skin microbiota – were targeted as potential sources of microbial contamination for facemasks. Using Bayesian statistics, SourceTracker quantitatively assigns proportions of the microbial contaminants in a facemask, represented by the facemask microbiome profile, to a set of potential sources represented by the source microbiome profiles (i.e. human faecal, oral and skin microbiota). Source microbiome profiles used in this study were retrieved from the Earth Microbiome Project, including 467 human gut and faecal samples (Accession Nos ERR1866468–ERR1867190, ERR1867465–ERR1867524 and ERR1868423–ERR1868675), 992 human skin samples (Accession Nos ERR1867196–ERR1867464 and ERR1867837–ERR1868161) and 509 human oral samples (Accession Nos ERR1868164–ERR1868674 and ERR1868679–ERR1868749). The use of human source microbiomes covering a broad population is recommended to reduce potential biases arising from the use of source microbiomes derived from individual subjects in microbiome-based source identification [23].

Results

Microbial contamination of aerosol facemasks

The extent of microbial contamination of the facemasks was measured by HPC as colony-forming units (CFUs). HPC results showed that the facemasks were contaminated by 405 ± 484 CFUs on average (Figure S1, see online supplementary material). Additionally, HPC analysis was conducted for the medicine cups, another key component of the nebulizer assembly, showing an average of 5 ± 9 CFUs (Figure S1, see online supplementary material). It is evident that microbial contamination was significantly more pronounced in facemasks

than medicine cups, likely due to the close contact between facemasks and facial skin (where there is an abundance of micro-organisms).

Microbiome-scale profiles of microbial contaminants

While results from HPC analysis (Figure S1, see online supplementary material) provided a measure of the level of microbial contamination on the facemasks, the identities of the microbial contaminants remained unknown. Given the high diversity of micro-organisms, microbiome-scale profiles were acquired for the microbial contaminants with 16S rRNA gene-based amplicon library sequencing.

Phylum-level profile

At the phylum level, the most abundant microbial contaminant was Proteobacteria, accounting for 45.5% of the sequences recovered from the aerosol facemasks (Figure S2, see online supplementary material). Firmicutes represented the second most abundant population, accounting for 29.1% of the microbial contamination, followed by Bacteroidetes, Actinobacteria and Fusobacteria, accounting for 9.5%, 9.48% and 1.6% of the microbial populations colonizing the facemasks, respectively. The remaining microbial contaminants comprised minor bacterial phyla with relative abundances <1% (Figure S2, see online supplementary material). This microbiome profile reveals that the most abundant bacterial populations belonged to Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria, collectively comprising approximately 95% of the microbial profile. The phylum-level profiles revealed that microbial contamination could be attributed to bacterial phyla which are present ubiquitously in both humans and the environment [24–27]. Therefore, analysis at finer taxonomic resolutions is warranted to understand the clinical implications of the microbial contaminants on aerosol facemasks.

Genus-level profile

Analysis of the sequences of microbial contaminants at the genus level identified 12 bacterial genera with high abundance, each representing >2% of the sequences on average (Figure 1). Of the 12 abundant genera, *Pseudomonas*, a genus in the phylum Proteobacteria, was the most abundant, contributing 13.4%, on average, to the microbial sequences present on facemasks (Figure 1). Proteobacteria was also represented by additional bacterial genera with high relative abundance, including *Stenotrophomonas*. In fact, *Stenotrophomonas* was one of only four bacterial genera with relative abundance >5%. It should be noted that both *Pseudomonas* and *Stenotrophomonas* are diverse genera comprising both pathogenic and non-pathogenic phylotypes that inhabit various human and environmental niches [28–31].

Following *Pseudomonas*, *Staphylococcus* and *Streptococcus* were identified as the second and third most abundant genera in the microbial profile, with average relative abundances of 10.1% and 9.5%, respectively. Both *Staphylococcus* and *Streptococcus* belong to the phylum Firmicutes, which comprises *Veillonella*, another genus found to be present on the aerosol facemasks with high abundance (Figure 1). The abundance of *Staphylococcus*, *Streptococcus* and *Veillonella* in the microbial profile, all known to be important constituents of the human

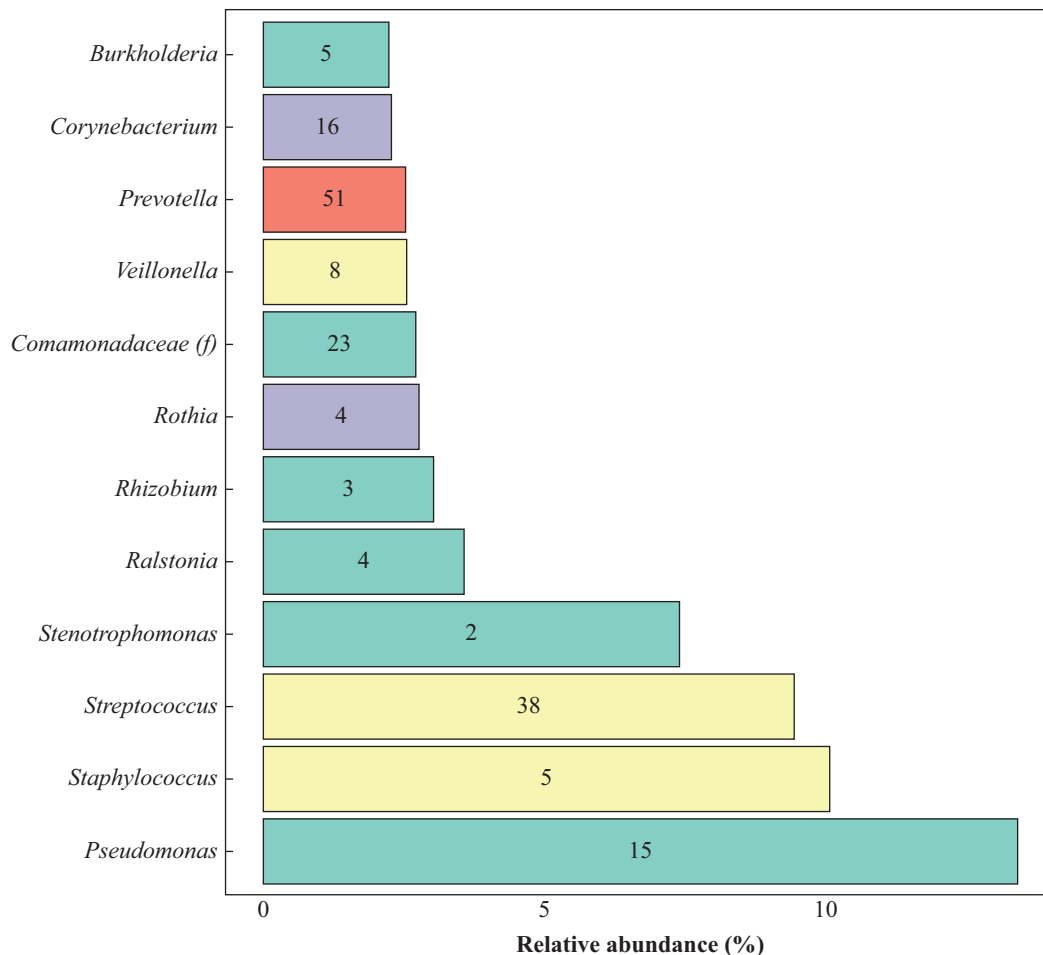


Figure 1. The most abundant microbial contaminants at genus level. Shown are bacterial genera with the average relative abundance in all samples >2%. The colour coding corresponds to the phylum to which each genus belongs (green bars, Proteobacteria; yellow bars, Firmicutes; red bar, Bacteroidetes; purple bars, Actinobacteria). The numerical values in the boxes indicate the numbers of operational taxonomic units (OTUs) classified in each bacterial genus according to 16S rRNA gene amplicon library sequencing.

microbiome [32–36], is indicative of the significance of human-associated microbiota as potential sources of microbial contaminants on used facemasks.

In addition to Proteobacteria and Firmicutes, two other phyla, Actinobacteria and Bacteroidetes, were also represented by bacterial genera with high abundance (Figure 1). *Rothia* and *Corynebacterium*, both grouped in the phylum Actinobacteria, accounted for 2.8% and 2.3% of the microbial profile, respectively. *Prevotella*, the only genus in the phylum Bacteroidetes identified with high abundance, contributed 2.5% to microbial contaminants detected on the facemasks. While populations of *Rothia*, *Corynebacterium* and *Prevotella* are common members of the human microbiome, some phylotypes of these three genera have been found to be potential pathogens [37–39].

Source identification of microbial contaminants

The acquisition of microbiome-scale profiles of microbial contaminants enabled microbiome-based source identification to determine the sources of microbial contamination [23]. Given increased infection risks of microbial contaminants originated from human sources, the human faecal, oral and skin

microbiota were targeted for source identification in this study.

Microbiome-based source identification using SourceTracker revealed that the human skin microbiota was the primary source of microbial contamination on the aerosol facemasks, contributing 21.3% on average to the microbial contaminants (Figure 2). Accounting for 9.6% of the microbial contaminants on the aerosol facemasks, the oral microbiota was a significantly less important secondary source of contamination. In contrast, the human gut microbiota was identified as a minor source, only accounting for 0.2% of the microbial contaminant profile (Figure 2). The transfer of skin microbiota to the facemask due to the long contact time between the facemask and skin likely outweighed the deposition of micro-organisms present at high abundance in droplets from the oral cavity. While the human skin and oral microbiota were identified as important sources of microbial contamination on the aerosol facemasks, it should be noted that >68% of the microbial contamination could not be attributed to any of the three human sources. It is reasonable to postulate that unidentified sources in the environment might be significant contributors to microbial contamination of the aerosol facemasks.

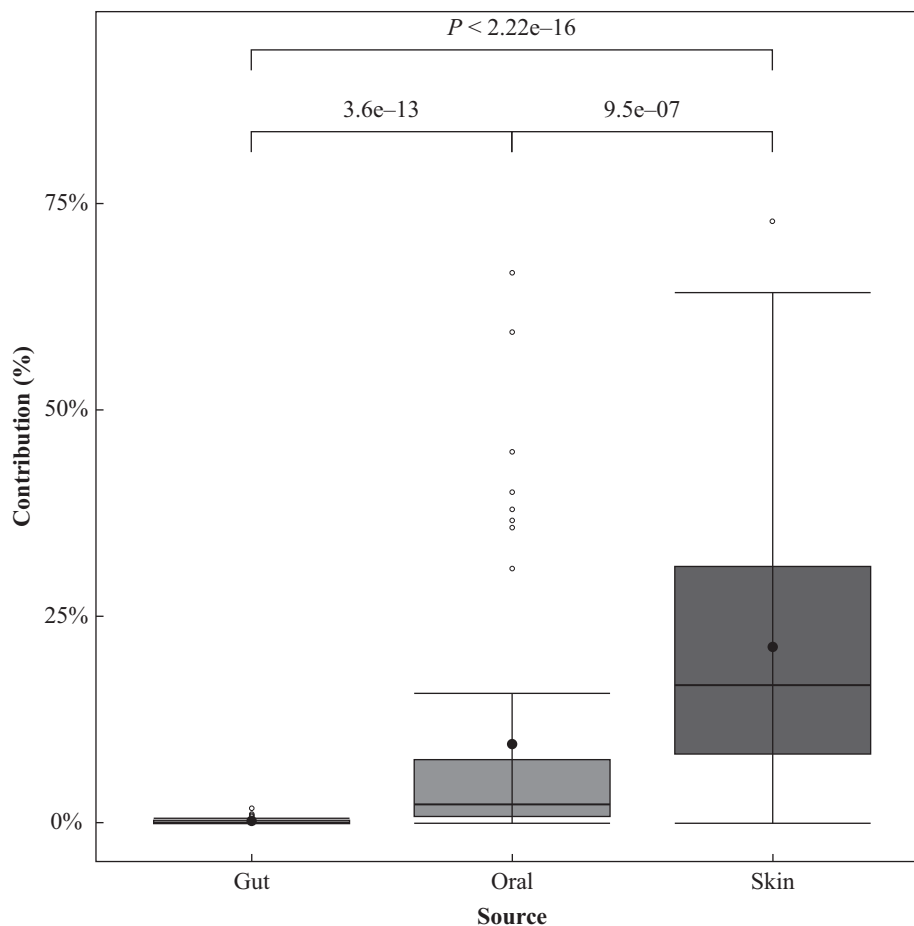


Figure 2. Comparisons of contributions to microbial contamination in aerosol facemasks from three sources: (1) human gut microbiota; (2) human oral microbiota; and (3) human skin microbiota. Data shown represent means of all samples included in this study with the error bars indicating standard deviations. Statistical significance is shown with *P*-values (Wilcoxon ranked sum test, *P* < 0.05).

Microbial contaminants characteristic of human sources

With human skin and oral microbiota revealed as significant sources of microbial contamination on the aerosol facemasks (Figure 2), it is important to identify phylotypes in the microbial contaminant profile linked to human sources. It could be hypothesized that the abundance of microbial phylotypes of human origin would correlate with the extent of human contributions to nebulizer contamination.

Spearman correlation analysis of bacterial phylotypes with high abundance on aerosol facemasks showed that four of these phylotypes, including *Streptococcus* OTU3888, *Veillonella* OTU3801, *Prevotella* OTU1419 and *Rothia* OTU0420, exhibited significant positive correlations between phylotype abundance and percentage contribution of the human oral microbiota to facemask contamination (Figure 3). The correlation coefficients ranged between 0.59 and 0.85, illustrating the strength of the positive correlation. These findings support these phylotypes of *Streptococcus*, *Veillonella*, *Prevotella* and *Rothia* as likely representatives of microbial contaminants which originated from the human oral microbiota, which is consistent with previous surveys of the human oral microbiome [40].

Further analysis revealed that *Staphylococcus* phylotype OTU0963 was strongly correlated with the contribution of human skin microbiota to facemask contamination, with a compelling Spearman correlation coefficient of 0.84 (Figure 3), supporting the likely origin of this *Staphylococcus* phylotype from the human skin microbiota, consistent with prior observations that *Staphylococcus* is a core member of the human skin microbiome [41].

It should be noted that significant correlations were not observed between any abundant bacterial phylotype and the contribution of human gut microbiota to contamination (Figure 3), which is likely due to the negligible contribution of human gut microbiota to nebulizer contamination (Figure 2).

Impact of medication on profiles of microbial contaminants

Microbiome-scale analysis of aerosol facemasks identified substantial variations in the composition of microbial contaminant profiles among subjects. Similarly, the contribution of human microbiota to microbial contamination also varied considerably among subjects, particularly that of human oral microbiota (Figure 2). Given that medication used for treatment differed substantially among the subjects (Table S2, see

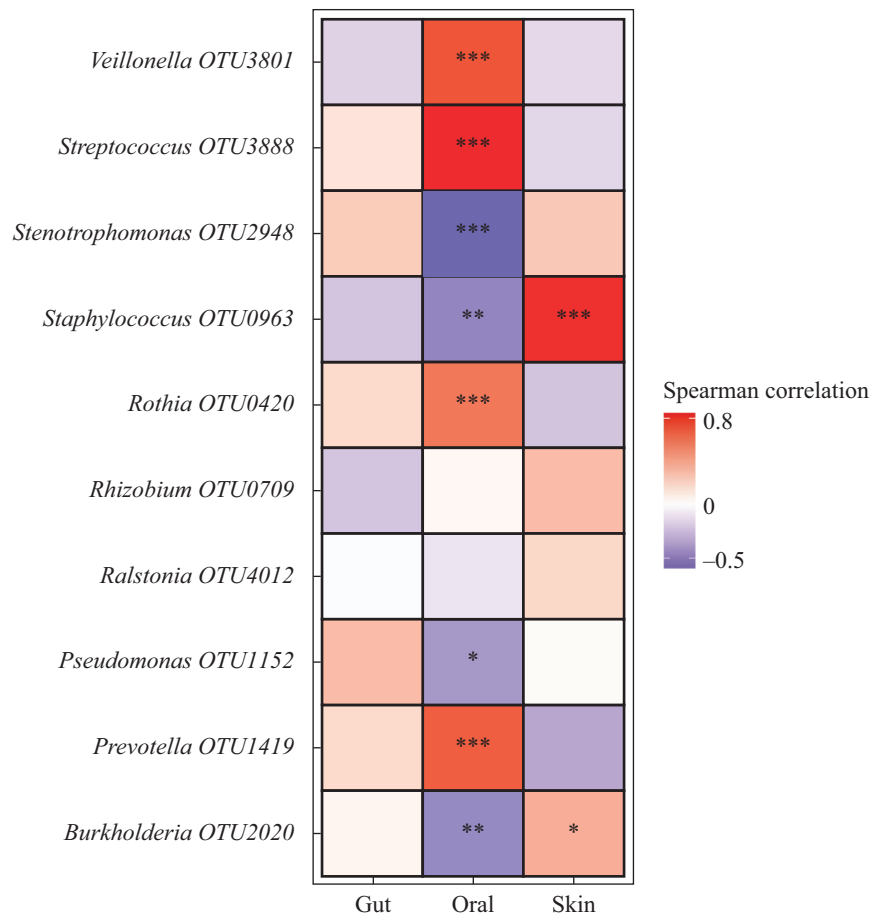


Figure 3. Correlations between abundance of top contaminant phylotypes and contribution to contamination from three sources: (1) human gut microbiota; (2) human oral microbiota; and (3) human skin microbiota. Phylotypes are defined as operational taxonomic units (OTUs) with the genus assignment for each OTU shown next to the OTU number. Data shown are Spearman correlation coefficients. Significant correlations are shown with asterisks: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

online supplementary material), efforts were made to characterize the impact of medication on the profiles of microbial contaminants on the aerosol facemasks.

A close examination of the medications prescribed for the subjects found 17 different medications for 28 subjects (Table S2, see online supplementary material). Medications used by five or more subjects included methylprednisolone, doxycycline, levofloxacin, azithromycin and vancomycin. Many of these medications were used simultaneously for individual subjects, resulting in complex medication regimens. In order to characterize the differences in medication regimens amongst the subjects, principal coordinate analysis was conducted for the medications used by the subjects (Figure S3, see online supplementary material). Levofloxacin, methylprednisolone, vancomycin and doxycycline were the only medications (of the 17 used by the subjects in this study) that correlated significantly with the ordination of medication regimens of individual subjects (Figure S3, see online supplementary material). As three of these four medications (i.e. levofloxacin, vancomycin and doxycycline) are antibiotics [42–46], it is likely that these medications would influence human microbiota and, subsequently, human contamination of aerosol facemasks. Indeed, further analysis with point-biserial correlation found significant negative correlation between levofloxacin treatment and microbial contamination of aerosol facemasks by oral

microbiota (Table S3, see online supplementary material), indicative of the significant impact of certain antibiotics on the microbial contaminant profile of aerosol facemasks.

Impact of levofloxacin treatment on microbial contamination

Given that common constituents of the human oral microbiota, such as *Streptococcus*, have been shown to be susceptible to levofloxacin [47], it could be postulated that levofloxacin treatment would potentially suppress human oral microbiota and, subsequently, reduce microbial contamination of aerosol facemasks by the human oral microbiota.

Previous analysis of microbial contaminants on aerosol facemask identified *Prevotella* OTU1419, *Streptococcus* OTU3888, *Veillonella* OTU3801 and *Rothia* OTU0420 as bacterial phylotypes representative of human oral microbiota (Figure 3). Comparison of microbial contaminant profiles between subjects treated with or without levofloxacin showed significant reductions in the abundance of these oral-sourced phylotypes as a result of levofloxacin treatment, with the only exception being *Rothia* OTU0420 which experienced a similarly substantial reduction but lacked significance (Figure 4). Accordingly, levofloxacin treatment led to a significantly smaller fraction of the microbial contamination attributable to the human oral

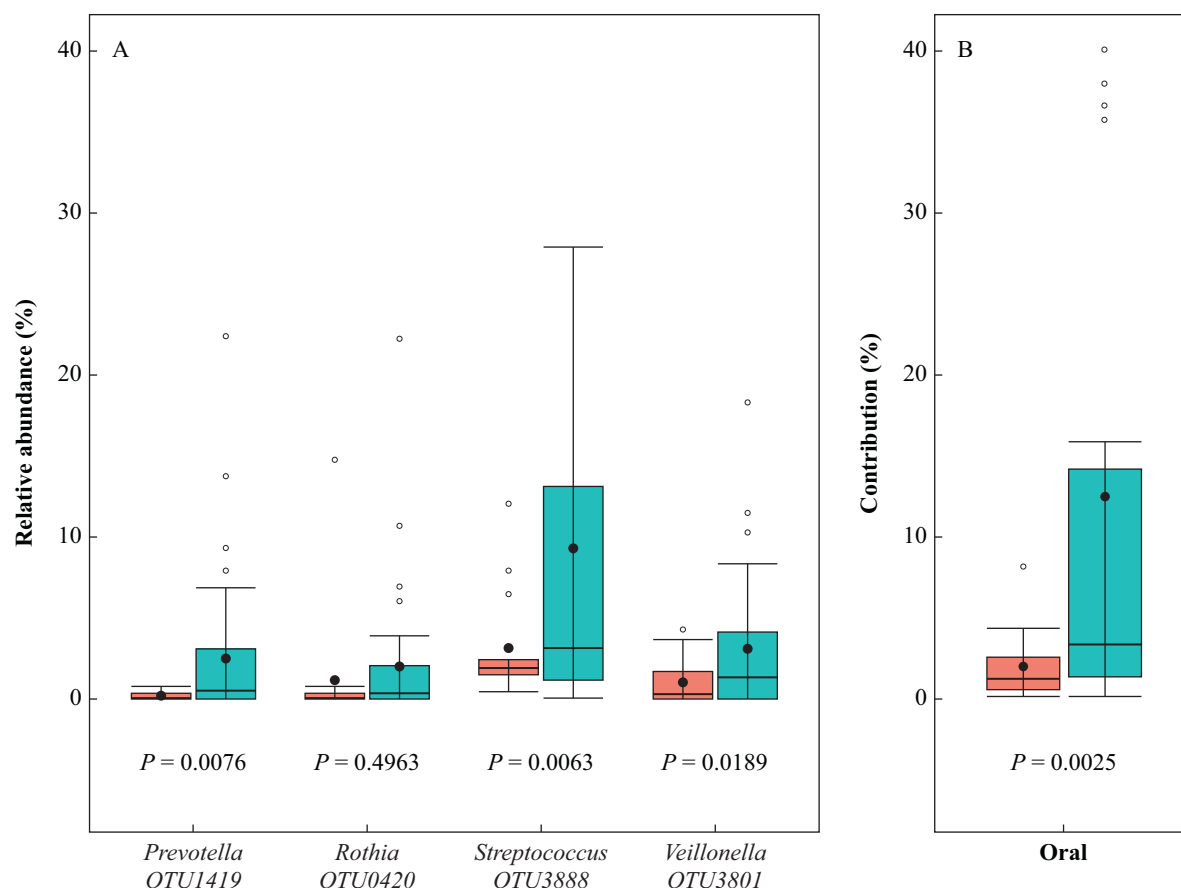


Figure 4. Impact of levofloxacin treatment on microbial contamination of aerosol facemasks. (A) Impact of levofloxacin treatment on relative abundance of human oral phylotypes in the facemask contaminant profile. (B) Impact of levofloxacin treatment on the contribution of human oral microbiota to microbial contamination in facemasks. Data shown are averages calculated for facemasks used by subjects with (red bars) or without (blue bars) levofloxacin treatment. Statistical significance is indicated by *P*-values according to Student's *t*-test.

microbiota (Figure 4), providing evidence that levofloxacin as an antibiotic can influence human oral microbiota [40,48] and, subsequently, the contamination of aerosol facemasks by the human oral microbiota.

Discussion

Nosocomial infections are major concerns in healthcare facilities. Microbial contamination of medical devices is considered to be a primary cause, prompting the need to characterize microbial contaminants in medical devices such as nebulizers. Previous studies of nebulizers using culture-dependent methods identified the occurrence of potential pathogens including *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and *Burkholderia cepacia* [7–9]. However, limitations of culture-dependent methods have prevented the acquisition of complete profiles of microbial contaminants. These limitations include the inability to detect viable but unculturable microbial populations, which typically account for a significant proportion of the microbial contaminants [6,10,12]. In this study, the use of culture-independent 16S rRNA amplicon sequencing technology enabled the acquisition of microbiome-scale comprehensive profiles of microbial contaminants, both culturable and non-culturable, in aerosol facemasks (Figure 1 and Figure S2, see

online supplementary material). The availability of comprehensive microbial profiles makes it possible to perform phylogenetic analysis, which is not readily feasible with culture-dependent methods. Indeed, analysis of microbiome-scale profiles of microbial contaminants on aerosol facemasks identified bacterial phylotypes which likely originated from the skin and oral microbiota of human subjects (Figure 3), including *Staphylococcus*, *Streptococcus*, *Veillonella*, *Prevotella* and *Rothia* [40,41].

Furthermore, the availability of microbiome-scale profiles of microbial contaminants allowed the use of a Bayesian source identification tool, SourceTracker, to trace the origins of microbial contamination on the aerosol facemasks [23]. It was found that a significant proportion of the microbial contaminants could be attributed to human skin and oral microbiota (Figure 2), which is important for the development of effective strategies for the prevention and mitigation of microbial contamination, such as the elimination of contamination from potential sources and targeted inactivation of specific pathogens.

Moreover, microbiome-scale analysis uncovered previously unknown factors impacting the composition of microbial contaminants on aerosol facemasks. By correlating medication regimens with human oral phylotypes present on aerosol

facemasks identified by microbiome-scale analysis, antibiotic treatment with levofloxacin was found to be linked to reduced contamination of the facemasks by oral microbiota (Figure 4), likely due to the susceptibility of oral bacteria, such as *Streptococcus*, to levofloxacin [47]. This observation represents the first report on the linkage between antibiotic treatment and microbial contamination of medical devices, which provides new perspectives into the dynamics of nosocomial infection risks for patients subjected to various antibiotic treatment regimens.

Given the significance of antibiotic treatment identified in this study, further investigation could be focused on the occurrence of antibiotic resistance and its linkage to the presence of specific microbial contaminants on facemasks. It is recognized that shotgun metagenomic sequencing, instead of 16S rRNA gene amplicon sequencing, is capable of identifying the functional repertoire of microbiomes, such as antibiotic resistance genes. Sequencing of 16S rRNA gene amplicons is a widely used technique for microbiome profiling. This technique typically targets specific hypervariable regions of the 16S rRNA gene (e.g. the V4 region in this study) which may lead to potential under-representation of certain microbial populations in derived microbiome profiles [49]. This technical issue needs to be taken into consideration during the analysis of microbiome data achieved by sequencing of 16S rRNA gene amplicons. It should be noted that microbiome profiling in this study was performed with DNA sequencing, which does not distinguish dead cells from viable cells. Therefore, caution should be taken in the interpretation of DNA-based microbiome data for the understanding of microbial activities.

In conclusion, culture-independent high-throughput sequencing was demonstrated for the capacity to acquire microbiome-scale profiles of microbial contaminants on aerosol facemasks. Microbial source identification enabled by the microbiome-scale profiles linked microbial contamination on aerosol facemasks to the human skin and oral microbiota. Antibiotic treatment with levofloxacin was found to reduce contamination of the facemasks by oral microbiota, representing the first report on the impact of antibiotic treatment on microbial contamination of medical devices.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhin.2023.01.008>.

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