Received: 13 April 2020 | Revised: 11 September 2020 | Accepted: 15 September 2020

DOI: 10.1111/ina.12750

ORIGINAL ARTICLE

WILEY

The mycobiomes and bacteriomes of sputum, saliva, and home dust

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Funding information
National Institute for Occupational Safety and Health, Grant/Award Number: T42OH008432; National Center for Advancing Translational Sciences, Grant/Award Number: 5UL1TR001425-04; National Institute of Environmental Health Sciences, Grant/Award Number: R21ES024807

Abstract

Respiratory microbiome is an understudied area of research compared to other microbiomes of the human body. The respiratory tract is exposed to an array of environmental pollutants, including microbes. Yet, we know very little about the relationship between environmental and respiratory microbiome. The primary aim of our study was to compare the mycobiomes and bacteriomes between three sample types from the same participants, including home dust, saliva, and sputum. Samples were collected from 40 adolescents in a longitudinal cohort. We analyzed the samples using 16s bacterial rDNA and ITS fungal rDNA gene sequencing, as well as quantitative PCR with universal fungal and bacterial primers. Results showed that home dust had the greatest alpha diversity between the three sample types for both bacteria and fungi. Dust had the highest total fungal load and the lowest total bacterial load. Sputum had greater bacterial diversity than saliva, but saliva had greater fungal diversity than sputum. The distribution of major bacterial phyla differed between all sample types. However, the distribution of major fungal classes differed only between sputum and saliva. Future research should examine the biological significance of the taxa found in each sample type based on microbial ecology and associations with health effects.

KEYWORDS

childhood exposure, home dust, indoor microbiome, induced sputum, respiratory microbiome, traffic-related air pollution

1 | INTRODUCTION

Respiratory microbiome is a significantly understudied area of research compared to other microbiomes of the human body. With the recent development of new molecular-based microbial measurement and identification techniques, it has been demonstrated that bacteria and fungi can be found in the lower respiratory tract.[1] The respiratory fungal microbiome (mycobiome) is particularly under investigated, despite evidence that fungal colonization may be involved in adverse respiratory health outcomes. Navarro et al[2] found that *Aspergillus* spp. in the clinical respiratory tract samples of cystic fibrosis (CF) patients was associated with 5%-10% decreased lung function. Carpagnano et al[3] also found that 70% of asthmatics sampled had fungal colonization in their exhaled breath condensate, compared to 0% of non-asthmatics.

The respiratory tract has constant exposure to an array of environmental pollutants, particles, and both pathogenic and non-pathogenic microbes, with each breath, yet we know very little about the relationship between environmental exposures and the respiratory microbiome. Though the primary source of

microbial migration for bacteria into the lower respiratory tract is believed to be microaspiration of saliva,[4] there is evidence that the environment has an impact on the respiratory microbiome. In a previous study, comparison of sputum of CF patients in the United Kingdom and the United States showed that the samples clustered by geographic region.[5] There is also evidence that abiotic environmental exposures, such as traffic-related air pollution (TRAP) or other chemicals, may impact the respiratory microbiome.[6,7] The environment impacts the microbiomes of other parts of the human body as well. Preliminary research indicates that immediate changes in the gut microbiota may occur when a person migrates from one country to another, such as when Thai immigrants settled in the United States[8] or when Latin Americans immigrated into the United States.[9] The skin microbiome affects the indoor microbiome, and the external environment impacts the skin microbiome.[10,11] The skin may also contribute to microbial colonization of the gut.[12] Pathogenic microorganisms in indoor environments may influence harboring more pathogens in children's gut microbiome. In humans, the composition of the gastrointestinal microbiome is first established during birth. Birth by Cesarean section or vaginal delivery also influences the gut's microbial composition. Babies born through the vaginal canal have non-pathogenic, beneficial gut microbiota similar to those found in the mother. However, the gut microbiota of babies delivered by C-section harbors more pathogenic bacteria such as Escherichia coli and Staphylococcus and it takes longer to develop non-pathogenic, beneficial gut microbiota.[13] The microbiome of the gut is understood to play an important role in immune system development.[14] However, it is not well understood how the respiratory microbiome may impact immune system development, especially the respiratory mycobiome.

As it is estimated that the United States population spends 87% of the time indoors, there is much potential for microbial exposure from home dust. It has also been shown that low fungal diversity in house dust is associated with childhood asthma development, indicating that the home dust may be an important source of exposure related to respiratory health.[15] A recent study also identified certain fungal and bacterial taxa in indoor dust that may be protective for asthma severity.[16] However, to our knowledge, there have not been any studies comparing the home dust microbiome to the lower respiratory tract microbiome in the same cohort of participants.

In our previous study, we examined the association between childhood exposure to TRAP and the microflora in the lower respiratory tract of children. Our findings indicate that exposure to TRAP in early childhood and adolescence may be associated with greater bacterial diversity in sputum samples.[6] The primary aim of this pilot study was to compare environmental and respiratory microbiomes. We compared mycobiomes and bacteriomes between three sample types from the same participants, including home dust, saliva, and sputum. A secondary aim of our study was to compare the home dust microbiome between the high and low TRAP exposure groups.

Practical Implications

- The primary aim of this study was to compare the bacterial and fungal communities of the lower respiratory tract and home dust.
- Induced sputum samples and dust wipes from the top of the bedroom door frame were collected from a cohort of adolescents.
- Home dust had a very low bacterial load and a very high fungal load compared to the lower respiratory tract.
- Home dust appears to be a more significant reservoir for fungi than for bacteria.
- The association between environmental and respiratory microbiome was most clearly seen between mycobiomes in dust and saliva.

2 | METHODS

2.1 | Recruitment

We recruited adolescents enrolled in the Cincinnati Childhood Allergy and Air Pollution Study (CCAAPS) cohort to participate in this study[17] based on their TRAP exposure, as described in Niemeier-Walsh et al.[6] Briefly, a land-use regression (LUR) model to estimate individual exposure to TRAP was developed as part of the CCAAPS cohort study.[18,19] A time-weighted average (TWA) daily exposure of elemental carbon attributable to traffic (ECAT), a surrogate of TRAP, was calculated for each child. Participants were categorized into the high or low TRAP exposure group, determined by whether their average exposure from birth through age 12 was above or below the overall cohort median exposure (0.33 mg/m^3 ECAT), respectively. Asthma status was determined by parent report of a physician diagnosis of asthma at age 12. Exclusion criteria for this study included having exposure to environmental tobacco smoke at either age 12 CCAAPS clinic visit or at the time of sputum sampling (~14 y), or having an upper or lower respiratory infection within 4 weeks prior to sputum sampling, following the protocol by Tunney et al.[20]

2.2 | Sample collection and preparation

Sample collection, DNA isolation, bacterial qPCR, and bacterial 16s rDNA sequencing were performed as described in Niemeier-Walsh et al.[6] We received informed parental consent and participant assent prior to sampling. Children were asked to rinse their mouth with nuclease-free water immediately before sample collection. Saliva and then induced sputum were collected from each participant with the Norgen Saliva and Sputum DNA Collection Kits (Norgen BioTek Corp., Thorold, ON, Canada) according to the manufacturer's instructions. After adding the Norgen Collection Kit preservative, which preserves the samples for up to five years at room temperature,

sputum and saliva samples were stored at room temperature until DNA extraction.

At the sputum induction appointment, the parents of the participants were given a home dust collection kit, which included nitrile gloves, a dust cloth (Swiffer, Procter & Gamble Company, Cincinnati, OH), a Stomacher rollbag (Seward Limited, Worthing, West Sussex, UK), and shipping materials to return the dust cloth to the lab. Parents were instructed to use the dust cloth to wipe the tops of doors and doorways in their child's bedroom while wearing the gloves until the cloth was visibly gray. They were then instructed to place the cloth in the rollbag, mark the date of collection, and place the bag in a sealed envelope to mail to the laboratory. Upon receipt of the dust wipe, the cloths were kept in the rollbag and stored at a −20°C freezer until extraction.

To extract the home dust from the dust cloths prior to DNA isolation, we used a modified version of the rigorous extraction protocol outlined by Adams et al.[21] A solution of 50 mL nuclease-free water + 0.05% Tween 20 in nuclease-free water was added to the rollbag with the dust cloth. The rollbag was then placed in the Stomacher 400 Circulator, a paddle blender originally developed for food science, and run at 230 rpm for 10 minutes. The extract was collected into a sterile 50-mL tube. This cycle was repeated for a total of 100 mL of extraction fluid collected from each dust cloth. The two 50-mL tubes were centrifuged at 6000*g* for 15 minutes at 4°C, and then, 35 mL of supernatant was removed from each tube. The two 50-mL tubes, each containing 15 mL of extract, were vortexed and combined into one 50-mL tube. The extract was centrifuged once more at 6000*g* for 15 minutes at 4°C, and 28 mL of supernatant was removed using a pipettor. The 2 mL of extract remaining that contained the dust pellet was vortexed and transferred to a sterile tube for storage in a −20°C freezer until DNA isolation.

2.3 | DNA isolation

DNA from saliva and sputum was isolated using the Norgen Saliva and Sputum DNA Isolation Kits (Norgen BioTek Corp., Thorold, ON, Canada) as described in Niemeier-Walsh et al.[6] The dust samples were also isolated using the Norgen Sputum DNA Isolation Kit to ensure that the sputum and dust samples would be comparable. We used the manufacturer's protocol with a single modification. The sample was incubated in an ultrasonic water bath at 65°C for 30 minutes upon adding the protein kinase K and lysozyme to the sample. This change was made to improve the DNA yield as we anticipated low concentrations, similar to those of biological aerosol samples from indoor air.[22] Reagent blanks were made for sputum, saliva, and dust samples.

2.4 | Real-time quantitative PCR (qPCR)

For bacteria, the universal bacterial primers UniBacteria_F and UniBacteria_R, and probe, UniBacteria_P1, for the amplification of the 16s bacterial ribosomal DNA (rDNA) were used.[23] DNA extracted from a known concentration of *Bacillus atrophaeus* cells was used as the standard. For fungi, the universal fungal primers 5.8FI and 5.8RI, and probe, 5.8PI, for the amplification of the internal transcribed spacer (ITS) regions of fungal rDNA were used.[24] Extracted DNA from a solution of *Aspergillus versicolor* with a known concentration of spores was used as the standard. We spiked a set of reaction mixtures with a known concentration of DNA to test for inhibition, and a serial dilution was included in the well plate as an internal standard to check for pipetting errors. The TaqMan system on Applied Biosystems StepOnePlus Real-Time PCR System (Thermo Fisher Scientific Inc, Waltham, MA) was used for amplification. All qPCR reactions were replicated three times per sample, and the reported value is the mean of the triplicates.

2.5 | Metagenomics sequencing

For bacterial sequencing, we amplified the V4 region of 16s bacterial rDNA (primer set 515F-806R). Bacterial PCR amplification and sequencing were performed by the Cincinnati Children's Hospital and Medical Center DNA Core as described by Niemeier-Walsh et al.[6] For fungal sequencing, we used the ITS1F-ITS2aR primers for the amplification of the ITS1 region. The ITS fungal rDNA region was selected over the 18s rDNA region because it has been found to be more precise in fungal community analysis.[25] The ITS1 region was selected over the ITS2 region because the pair of primers for the amplification of ITS1 has been found to be more selective, producing fewer non-fungal sequences, in addition to producing a higher number of sequences, than the primer pair for the amplification of the ITS2 region.[26] Fungal PCR and sequencing were performed by RTL Genomics, a division of Research and Testing Laboratories.

We removed the primer sequences using `cutadapt` v1.16.[27] We used the open-source R package, DADA2 v1.8, to process reads and for error correction[28] with the default parameters for quality filtering, error modeling, dereplication, denoising, and merging of paired-end reads. For bacteria, forward reads were truncated at 210, and reverse reads were truncated at 160 nucleotides. For fungi, reads with less than 50 base pairs were removed to get rid of suspicious very low-length reads. Reads with a quality score ≤ 2, with a maximum expected error rate for the forward or reverse read ≥ 2, or with a forward or reverse read that contained an ambiguous base, were removed. After error correction, the forward and reverse reads were merged to form an amplicon sequence variant (ASV) table. The DADA2 function `removeBimeraDenovo` was used for chimera removal. For bacteria, we used Silva version 132 as the reference database.[29] For fungi, the UNITE ITS database was used as the reference database.[30] Sequences were aligned using the `AlignSeqs` function in the DECIPHER package v2.12.0.[31] For bacteria, the phanghorn package v2.5.5 was used to generate a de novo phylogenetic tree.[32] For fungi, a de novo tree was created using agglomerative clustering of the sequence distance matrix using USEARCH.[33] Phyloseq v1.28 was used to integrate the sample metadata, ASV table, phylogenetic

tree, and taxonomic assignments for statistical analyses for both bacteria and fungi.[34]

2.6 | Data analysis

We used the qPCR data to account for differences in sequencing depth by multiplying the relative abundance of each bacterial and fungal ASV by the total bacterial and fungal load in each sample, respectively.[35,36] For bacteria, sputum and saliva samples were removed if they did not amplify during qPCR, did not amplify during PCR prior to sequencing, or had <5000 reads after sequencing. Due to low bacterial abundance in dust, dust samples were included if they amplified and had >100 reads. For fungi, dust and saliva samples were not included if they did not amplify or had <3000 reads after sequencing. Due to low fungal abundance in sputum, sputum samples were included if they amplified and had >400 reads. We created rarefaction curves for both bacteria and fungi. As a sensitivity analysis, we compared the alpha diversity and relative abundance results with a read cutoff of <2000 reads and with the aforementioned cutoffs for each sample. We used the phyloseq function `estimate_richness` to calculate the total number of observed ASVs and Shannon alpha diversity measures for the bacterial and fungal communities in each sample. The function `pd` in the picante R package (v1.8.0) was used to calculate Faith's phylogenetic diversity for the bacterial and fungal communities in each sample.[37]

The total bacterial load and total fungal load as measured by qPCR were compared between sample type using the Wilcoxon rank sum test. The Wilcoxon rank sum test was also used to compare the alpha diversity indices of bacteria and fungi in home dust between TRAP exposure groups, between genders, and between asthma status groups. The Kruskal-Wallis rank sum test was used to compare the alpha diversity indices across all three sample types. Pairwise Wilcoxon rank sum tests were also performed to compare the alpha diversity between sample types. The Adonis function in the vegan package (2.5.6) was used to implement a permutational multivariate analysis of variance to test for differences in beta diversity between sample types. A dispersion test for the homogeneity of variance across sample type was also conducted using the `vegan::betadisper` and `vegan::permutest` functions, as differences in variance may confound the Adonis test. The Human Microbiome Project (HMP) R package (v2.0.1) function `xdc.sevsample`, a likelihood ratio test for the multiple sample Dirichlet-Multinomial parameter test, was used to assess the distribution of major phyla between sample type.[38] Specifically, the bacterial ASV table was agglomerated to the phylum level and the five most abundant phyla, along with all other less abundant phyla grouped into one "other" category. For fungi, the same protocol was followed, but the ASV table was agglomerated to the class level instead of phylum. We also compared the *Basidiomycota*-to-*Ascomycota* ratio between the three types of samples, as described by Sokol et al.[39]

Heatmaps were constructed employing the R package for heatmap generation, *heatmap.plus*, with the custom distance measure library *amap*. The distance measure employed was Spearman correlation, and the clustering method was average linkage. The data were log 10 scaled, and three column color bars were constructed, namely (a) asthma status (asthmatic or non-asthmatic), (b) TRAP exposure (high or low), and (c) sample type (saliva, sputum, or dust). With these parameters, the heatmap was constructed following the normal protocol. Venn diagrams comparing the number of overlapping bacterial and fungal genera by sample type, using the absolute abundance of the five most abundant genera in each sample type, were created using `RAM::venn.diagram` (v 1.2.1.7).

3 | RESULTS

3.1 | Characteristics of study participants

Samples were included in the analyses and results if they amplified using both the qPCR universal primers and the primers used for 16s or ITS sequencing (Table 1). For the 10 sputum samples included in the fungal results, 60% of participants were female, 50% were in the high TRAP exposure group, 50% were asthmatic, and 90% had mothers with education beyond high school. For the 8 saliva samples included in the results, 50% of the participants were female, 50% were in the high TRAP exposure group, 37.5% were asthmatic, and 100% had mothers with education beyond high school. For the 32 dust samples included in the results, 47% of the participants were female, 47% were in the high TRAP exposure group, 28% were asthmatic, and 94% had mothers with education beyond high school. Twelve dust samples were included in the bacterial analyses because the majority of dust samples did not amplify during qPCR with the universal bacterial primers, or during PCR prior to sequencing with the V4 region primers. However, a substantial amount of DNA was measured in the dust samples using a Qubit fluorometer (Invitrogen, Carlsbad, CA), indicating that most of the DNA in these home dust samples did not originate from bacteria. For the 12 dust samples included in the bacterial

TABLE 1 Number of samples included in each sample type for both bacteria and fungi

Sample Type	Total number of samples collected	Number of samples included in bacterial analyses	Number of samples included in fungal analyses
Sputum	40	34	10
Saliva	40	36	8
Dust	33	12	32

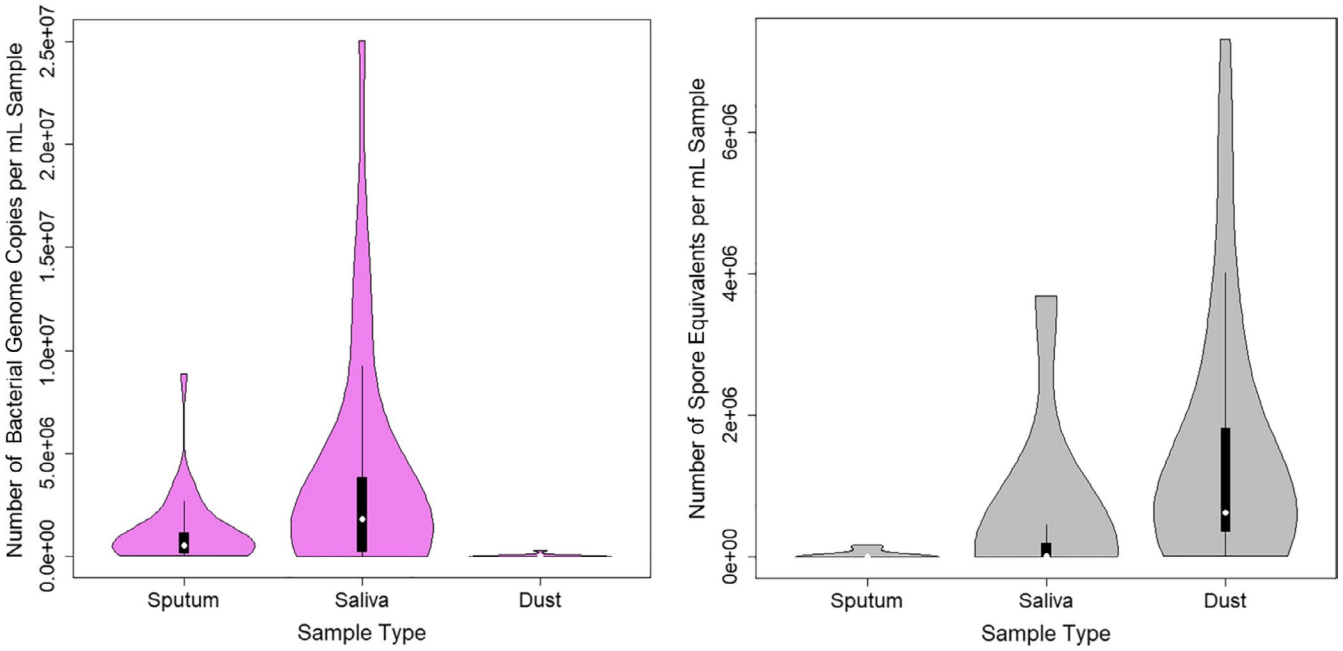

FIGURE 1 Violin plots demonstrating the total bacterial (left) and fungal (right) load of each sample type

analyses, 50% were in the high TRAP exposure group, and 42% were from asthmatic participants.

3.2 | Total bacterial and fungal load

Saliva had the highest bacterial load out of the three sample types (Figure 1 and Table S1). Dust had the highest fungal load out of the three sample types. When comparing the ratio of bacteria to fungi in each sample type, sputum had the highest, 35:1, and dust had the lowest, 1:41 (Table S1). The difference between the fungal and bacterial loads in saliva was smaller than in the other two sample types.

3.3 | Alpha diversity

For both bacteria and fungi, dust had the greatest median alpha diversity out of the three sample types using all three diversity measures (Figure 2). For bacteria, sputum had greater alpha diversity than saliva using all three alpha diversity measures (Figure 2, Table S2). However, saliva had greater fungal alpha diversity than sputum using all three diversity measures (Figure 2, Table S3). For bacteria, the Kruskal-Wallis rank sum test showed that there were not significant differences in the number of observed ASVs ($P = .46$) nor phylogenetic diversity ($P = .12$), but there was a significant difference in Shannon diversity ($P < .001$) between all three sample types. Results of the pairwise Wilcoxon rank sum test comparisons for bacteria are shown in Table S4. For bacteria, there was only one statistically significant pairwise comparison, Shannon diversity for sputum and saliva ($P < .001$). For fungi, the Kruskal-Wallis rank sum test showed that there were significant differences in the number of observed ASVs ($P < .001$), Shannon diversity ($P < .001$), and phylogenetic diversity ($P < .001$). Results of the pairwise Wilcoxon rank sum test comparisons for fungi are shown in Table S5. All pairwise comparisons were statistically significant for fungi.

When the three sample types were placed on a scatterplot with fungal alpha diversity as the *y*-axis and bacterial alpha diversity as the *x*-axis, each sample type clustered distinctly using all three alpha diversity measures (Figure 3). Dust had the most disperse cluster, with a wider range in diversity between individual samples. Saliva and sputum clustered more closely to one another.

3.4 | Beta diversity

Our previous work showed that the bacterial community composition in sputum and saliva were distinct from one another using the Bray-Curtis beta diversity measure.[6] When adding the dust samples to the non-metric multidimensional scaling (NMDS) Bray-Curtis plot, the bacterial community composition in sputum and saliva appear to overlap, while the dust is distinct from both (Figure 4). For fungi, the communities of all three sample types overlap (Figure 4). The Adonis test indicated that 23% of the variance between sample types could be attributed to sample type ($P = .001$). However, the homogeneity of dispersion test determined that the assumption of homogenous dispersion for the Adonis test was not met ($P = .004$). This indicates that the Adonis result may have been confounded by heterogeneous dispersions. For bacteria, the Adonis indicated that 14% of the variance between sample types could be attributed to sample type ($P = .002$). The homogeneity of dispersion test indicated that there were no significant differences in dispersion that may have confounded the Adonis results ($P = .08$).

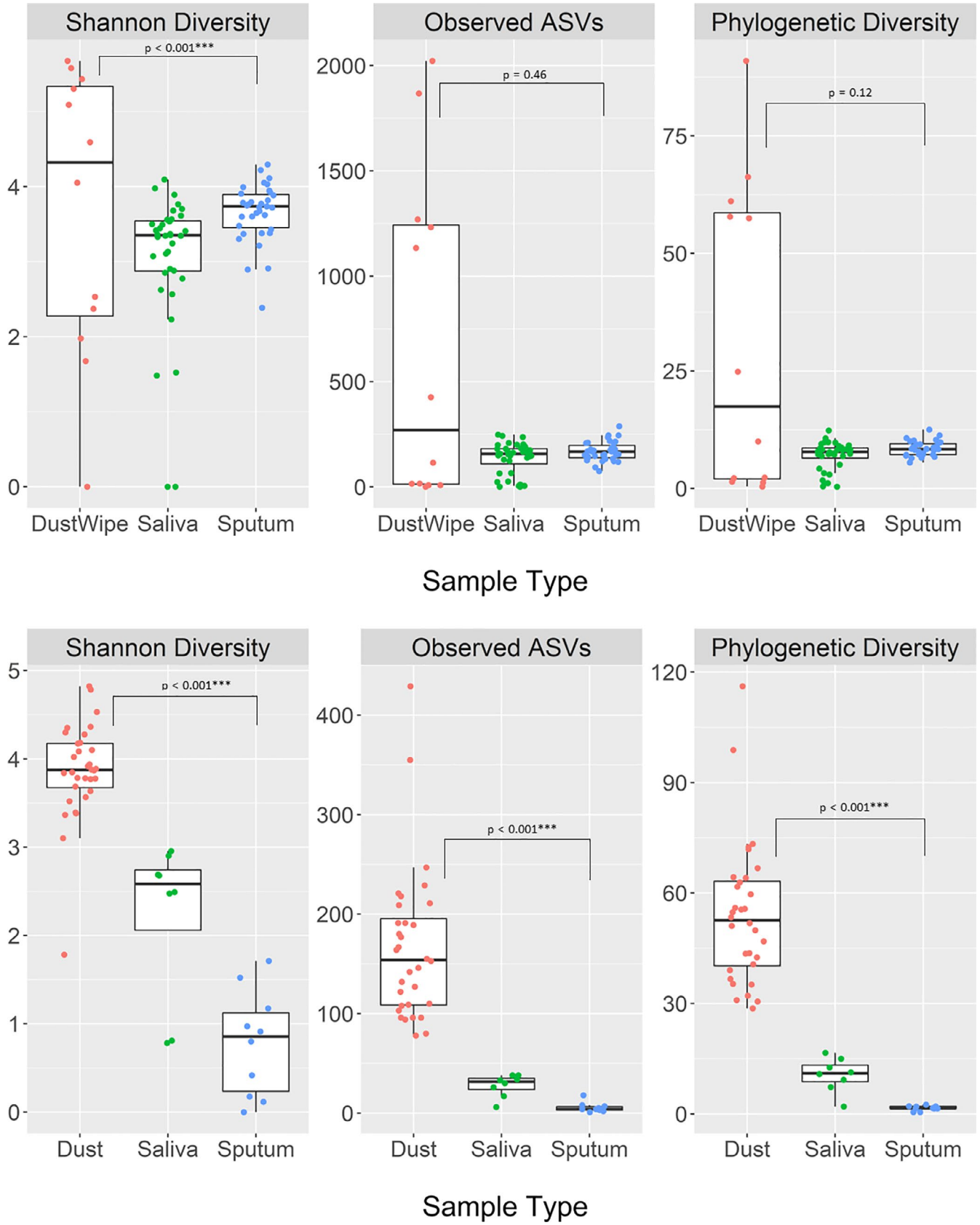

FIGURE 2 Box plots comparing the alpha diversity in the bacterial communities (top graphs) and fungal communities (lower graphs) in each sample type including Shannon diversity, the number of observed ASVs, and Faith's phylogenetic diversity, and Kruskal-Wallis rank sum test *P*-values for each

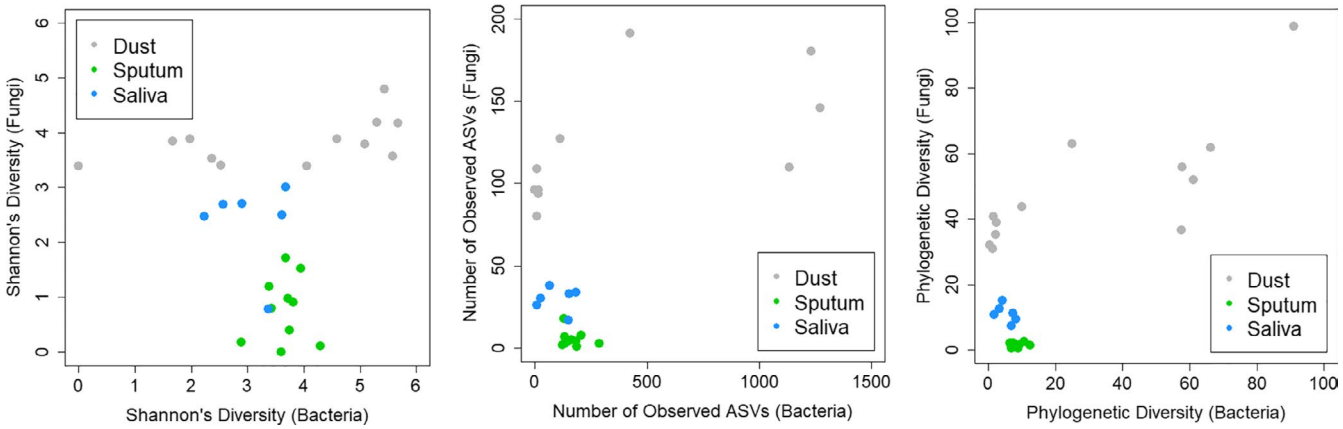

FIGURE 3 Scatterplots comparing the alpha diversity indices of the fungal communities (*y*-axis) to the alpha diversity of the bacterial communities (*x*-axis) in each sample type

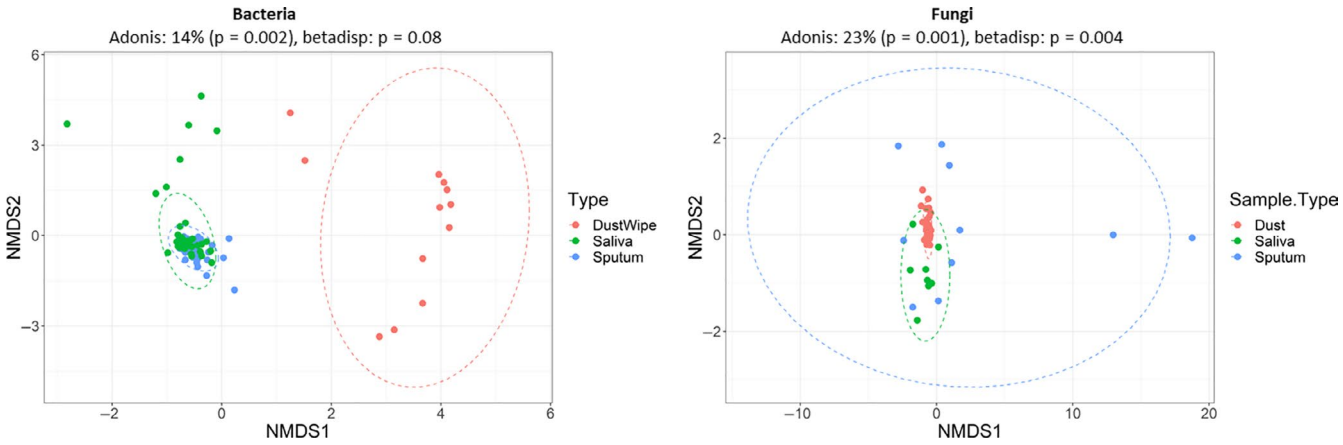

FIGURE 4 Bray-Curtis non-metric multidimensional scaling (NMDS) plot of the bacterial communities in each sample type (left) and the fungal communities in each sample type (right)

3.5 | Absolute abundance

The heatmap of the absolute abundance of bacterial phyla in each sample showed that the samples clustered strongest by sample type (Figure S1). The dust samples clustered close to one another. Bacterial phyla that dominated the dust samples, including *Acidobacteria*, *Chlorofexi*, *Planctomycetes*, *Verrucomicrobia*, *Armatimonadetes*, *Deinococcus-Thermus*, *Gemmatimonadetes*, and *Chlamydiae*, were much less prevalent in the sputum and saliva samples. The heatmap of the absolute abundance of fungal classes also shows that samples clustered strongest by sample type (Figure S2). All of the observed fungal classes were more abundant in dust than in sputum or saliva. The sputum and dust samples clustered distinctly from one another. The saliva samples were mixed in with both the sputum and dust samples.

Figures S3 and S4 show the overlapping bacterial and fungal genera by sample type, only including the five most abundant genera in each sample type. For bacteria, sputum and saliva had the same five most abundant genera, *Prevotella*, *Streptococcus*, *Neisseria*, *Veillonella*, and *Haemophilus*. However, the order of most to least abundant among those five genera differed between sputum and saliva. *Prevotella* was the most abundant bacterial genera in both sputum and saliva, but *Streptococcus* was more abundant in sputum than in saliva. Of the five most abundant bacterial genera in each sample type, *Streptococcus* was the only one that was common among all three sample types. For the five most abundant fungal genera in each sample type, *Candida*, *Cyberlindnera*, and *Fusarium* overlapped for sputum and saliva. Of the five most abundant fungal genera in dust, none overlapped between sputum or saliva.

3.6 | Relative abundance

For bacteria, the xdc.sevsample test showed that there was a significant difference in the distribution of major bacterial phyla between all three sample types ($P < .001$), between sputum and saliva ($P < .001$), between sputum and dust ($P < .001$), and between saliva and dust ($P < .001$). Dust had the highest relative abundance of *Proteobacteria* and *Actinobacteria*, and the lowest relative abundance of *Bacteroidetes*, out of the three sample types (Figure 5). The least abundant bacterial phyla, labeled "other" in Figure 5, only appeared

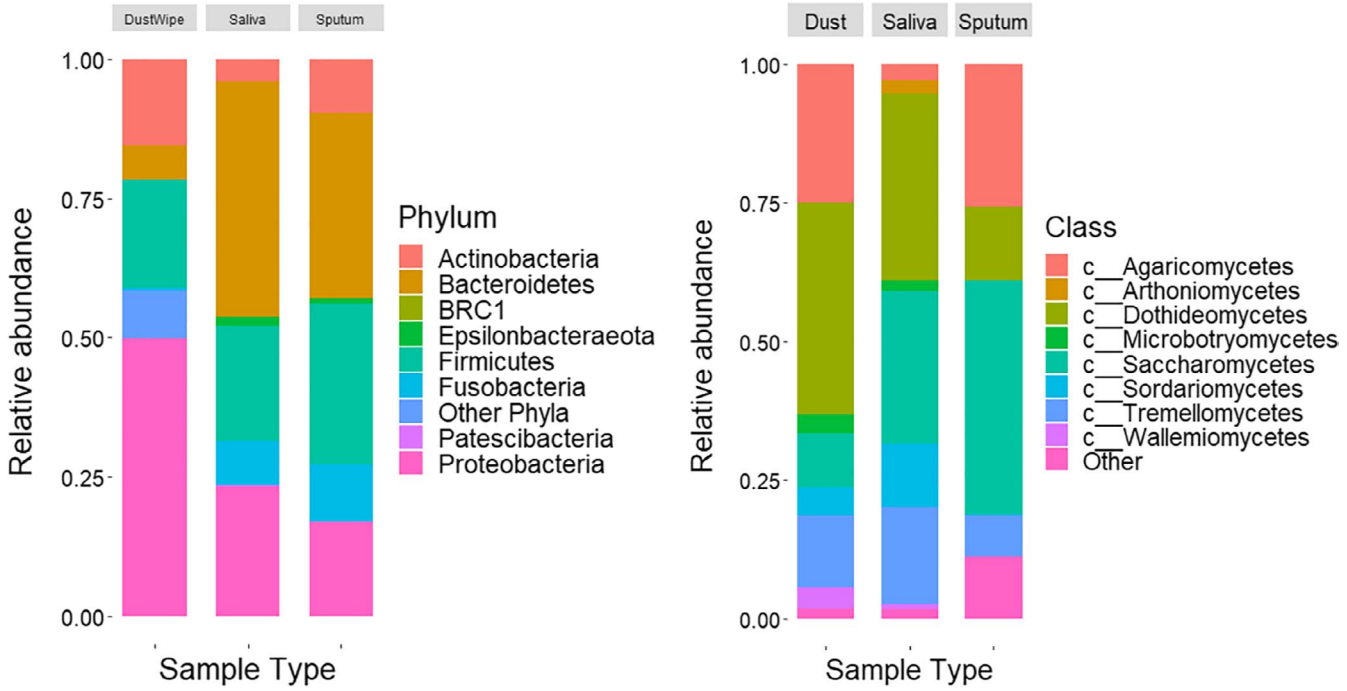

FIGURE 5 Relative abundance bar plots of the major bacterial phyla (left) and fungal classes (right) in each sample type

in dust. *Epsilonbacteraeota* and *Fusobacteria* only appeared in sputum and saliva. Saliva had greater relative abundance of *Bacteroidetes* and *Proteobacteria* than sputum.

According to the xdc.sevsample test, the distribution of major fungal classes differed significantly between sample type ($P < .001$). When comparing only sputum and saliva with xdc.sevsample, the distribution of major fungal classes differed significantly ($P < .001$). The distribution of major fungal classes also differed significantly when comparing sputum and dust ($P < .001$). When comparing saliva and dust, the distribution of major fungal classes did not differ significantly ($P = .43$). Looking at the relative abundance plots, *Microbotryomycetes* and *Saccharomycetes* appeared in saliva and dust, but not in sputum (Figure 5). Saliva and dust also appear to have similar relative abundance of *Dothideomycetes*. *Arthoniomycetes* only appeared in saliva and *Wallemiomycetes* only appeared in dust. Sputum and dust had similar relative abundance of *Agaricomycetes*. Sputum had the highest relative abundance of the least abundant fungal classes, labeled as "other" on the bar plot.

3.7 | Basidiomycota-to-ascomycota abundance ratio

The ratio of Basidiomycota:Ascomycota was 1:54 for sputum, 1:8.7 for saliva, and 1.1:1 for home dust.

3.8 | Sensitivity analysis for the number of reads per sample

The rarefaction curves showed that 2000 reads was an appropriate cutoff for all samples for both bacteria and fungi (Figure S5). However, many samples would have been excluded if we removed all samples with fewer than 2000 reads, further reducing our sample size. To ensure that our results would not be strongly impacted by including low-read samples, we compared the alpha diversity and relative abundance plots using the <2000 read cutoff and the plots created using the cutoff values for each sample type as described in the methods. When comparing the bacterial alpha diversity indices between the two read cutoffs, the median alpha diversity for dust increased when removing the low-read samples, but overall, the main findings did not change (Figures 2 and S6). The relative abundance results also did not change overall for bacteria when including the low-read samples (Figure S7). The results were similar for fungi. As the sputum samples already had such low values for fungal alpha diversity, removing the low-read samples did not change the overall picture (Figures 2 and S8). For relative abundance of fungal taxa, the sputum samples were all very different, with many samples dominated by just one or two fungal classes (Figure S9). As the main findings were not affected by including low-read samples, we decided to include them in the reported results.

3.9 | Traffic pollution and the home dust microbiome

We compared the dust mycobiome of participants between TRAP exposure groups, between genders, and between asthma status groups. There were no significant differences in the alpha diversity indices between TRAP exposure groups, between genders, nor between asthma status groups (Table S6). No significant differences in the distribution of major fungal classes were found in the dust samples between TRAP exposure groups ($P = .55$), between genders ($P = 1.0$), nor between asthma status groups ($P = 1.0$) (data not shown). We also compared the dust bacteriome between groups. No significant differences in alpha diversity were found between any of the groups (Table S7).

4 | DISCUSSION

4.1 | Mycobiomes and bacteriomes of dust, sputum, and saliva

Overall, we found major differences in both the bacterial and fungal microbiota in sputum, saliva, and home dust. Home dust had a high fungal load, but low bacterial load. Sputum had a higher bacterial load, but lower fungal load. For both bacteria and fungi, dust had the most diverse microbiota. We also identified the most abundant taxa in each sample type. We encountered some challenges in this study due to the low bacterial load in home dust and low fungal load in sputum.

Dust had the lowest total bacterial load and the highest fungal load out of the three sample types. This can be explained by the growth conditions of the home environment. The home environment is not as warm and moist as inside the human body. Though some bacteria may live up to weeks or years outside the body, most may only survive for a few hours or days.[40] In contrast, fungi are capable of surviving dry and frigid conditions for longer periods of time. Sputum had the lowest total fungal load out of the three sample types. This is likely because there are very few fungi that are able to survive and proliferate in the human body due to the higher temperature and the defense mechanisms of the immune system.[41] Saliva had the highest total bacterial load of the three sample types and a higher total fungal load than sputum. The oral cavity has the necessary growth conditions, as well as more nutrients available for microbiota than the lower respiratory tract. The oral cavity also has more unfiltered exposure to the environment than the lower respiratory tract.

Dust had a higher relative abundance of the bacterial phyla *Proteobacteria* than sputum or saliva. Dust also had a higher relative abundance of *Actinobacteria* than the other two sample types. *Actinobacteria* are most commonly associated with soil and fresh water sources, which would explain their greater relative abundance in dust. For fungi, the two most abundant classes were *Agaricomycetes* and *Dothideomycetes*. For both bacteria and fungi, the relative abundances of taxa were similar to those observed in previous studies of the home dust microbiome.[42] Additionally, the most abundant genus in the home dust wipes was *Epicoccum*, which was also found to be abundant in a previous study where an electrostatic dust cloth was used to collect home dust and analyzed with Illumina MiSeq after amplification of the ITS2 region.[43] *Epicoccum* is not typically found in culture-based analyses of home dust, but our study is consistent with previous findings where it is found to be abundant when using metagenomics sequencing methods.[43] In a recent study examining the microbiome of dust collected from classrooms in Malaysia, the presence of *Sphingobium* was identified as potentially protective of asthma severity.[16] We identified *Sphingobium* in both our sputum and home dust samples, though with very low abundance, 11 reads and 259 reads, respectively. However, it was not found in our saliva samples. The taxa found in sputum in our study were similar to those found in the respiratory tract in previous studies.[44,45] *Candida* was one of the most abundant fungal genera in both sputum and saliva, but not dust. *Candida* is a common cause of human fungal infections, though many species are commensals.[46] High relative abundance of *Candida* in the lung and oral cavity has been found in previous studies.[44]

Dust had the greatest median bacterial and fungal alpha diversity out of the three sample types with all three alpha diversity measures. This is reasonable as home dust contains microbes from both human and environmental sources. The number of fungal ASVs observed in our study was similar to previously reported values.[15] Sputum had a greater median bacterial alpha diversity than saliva, but saliva had a greater median fungal alpha diversity than sputum. These differences in alpha diversity and fungal load also appear to be consistent with the xdc.sevsample results, where the distribution of the major fungal classes in sputum was found to be significantly different from dust, but saliva and dust did not differ significantly. While the sputum and saliva DNA isolation kits used were from the same manufacturer, the kits were not identical, and therefore, we cannot rule out that they may have impacted the results. As the oral cavity has more direct exposure to the environment, it is plausible that saliva would be more similar to dust than sputum would be to dust. For beta diversity, the Bray-Curtis plots showed that there was an overlap of bacterial community composition between sputum and saliva, but not dust. The results of the plot and the Adonis test indicate that there were differences in bacterial community composition between sample types for bacteria. For fungal beta diversity, all three sample types overlapped on the Bray-Curtis plot, but the dust and saliva clustered more closely within the broader, more disperse sputum cluster. The Adonis test did indicate that there was a difference in the variance of fungal community composition that could be attributed to sample type; however, the assumption of homogeneity of dispersion was not met, so it is possible that it could have confounded the Adonis results for fungi. The heatmaps are in agreement with the alpha diversity and xdc.sevsample results. Dust clustered distinctly for bacteria, with very little overlap taxonomically with sputum or saliva. For fungi, dust and sputum were separate while saliva was somewhat scattered between the two. There was not

an obvious taxonomical divide between sample types for fungi like there was for bacteria.

There is evidence that the ratio of abundance of Basidiomycota-to-Ascomycota may be biologically relevant as it has been shown that patients with inflammatory bowel disease (IBD) have higher Basidiomycota-to-Ascomycota ratios in their fecal microbiota than healthy subjects.[39] However, IBD patients in remission had ratios closer to healthy subjects, indicating that the ratio may be related to inflammation. Therefore, we compared the Basidiomycota-to-Ascomycota ratios of each sample type. Home dust had the highest ratio, whereas sputum had the lowest ratio. Of all the genera identified in *Ascomycota* that decreased in IBD patients, *Saccharomyces* had the strongest signal.[39] Interestingly, *Saccharomyces* was the third most abundant genera in sputum in our study. It was not as abundant in saliva or dust, which both also had higher Basidiomycota-to-Ascomycota ratios than sputum. As *Saccharomyces* is associated with a decreased inflammatory response in mouse models and in Crohn's disease patients,[47,48] future studies should examine the biological relevance of this taxa as it relates to the respiratory and the environmental microbiomes.

We also examined the difference in dust mycobiomes and bacteriomes between high and low TRAP-exposed participants as there is evidence that there is an association between higher concentrations of biological aerosols and traffic pollution, possibly due to resuspension.[49,50] However, no significant differences were observed between the dust mycobiomes or bacteriomes of the two TRAP exposure groups.

4.2 | Strengths and limitations

The main weakness of this study was the small sample size due to the lack of amplification of samples. This indicates that there may be a need for much larger sample size for the initial recruitment of respiratory mycobiome and home dust bacteriome studies, as only about 25% of the sputum and saliva amplified for fungi and only 36% of the dust samples amplified for bacteria. This could also indicate that the marker gene sequencing method may not be the best approach for these samples and that an alternative method, such as shotgun metagenomic sequencing, should be explored.

When comparing the dust sample mycobiomes between TRAP exposure groups, we used ECAT to classify the participants into the high or low exposure groups. ECAT is a cumulative TRAP exposure estimation, whereas our dust samples were collected at a single time point. Though the settled dust collected reflects cumulative exposure, it does not reflect the same length of exposure time as the ECAT values. It would have been better to have indoor and outdoor traffic pollution measurements, such as PM2.5 or black carbon, as well as indoor and outdoor bioaerosol measurements at each house on the day of dust collection.

The greatest strength of our study is that it includes three sample types from the same cohort of children and that it examines both the bacteriomes and mycobiomes in the three sample types. We also have a very well-characterized exposure history for this cohort. Additionally, most respiratory microbiome research has been done with cystic fibrosis patients, which does not represent the overall population as broadly as our study.

5 | CONCLUSIONS

We observed differences in the mycobiomes and bacteriomes of sputum, saliva, and dust samples, including differences in the total fungal and bacterial load, alpha diversity, and abundance of taxa. Our greatest challenge in this study was the lack of amplification of dust samples for bacterial analysis and the lack of sputum and saliva for fungal analysis. This further demonstrates the need for larger sample size for initial recruitment in anticipation of many samples not being included in the analyses. This study was the first to examine both the bacterial and fungal communities in sputum, saliva, and dust samples taken from the same participants. We found that home dust had a very low bacterial load and a very high fungal load compared to the lower respiratory tract. Therefore, when considering the exposure pathways, home dust appears to be a more significant reservoir for fungi than for bacteria. The association between environmental and respiratory microbiome was most clearly seen between mycobiomes in dust and saliva. Future research should examine the biological significance of the taxa found in each sample type based on microbial ecology and associations with health effects. Housing characteristics should also be considered in future research.

ACKNOWLEDGMENTS

Support for this research was provided by a grant from the National Institute of Environmental Health Sciences, National Institutes of Health, grant #R21ES024807. Support was also provided by a grant from the National Institute for Occupational Safety and Health, Pilot Research Project Training Program, University of Cincinnati Education and Research Center Grant (#T42OH008432). The induced sputum samples were collected with the assistance of the Schubert Research Clinic at Cincinnati Children's Hospital Medical Center. The project described was also supported by the National Center for Advancing Translational Sciences of the National Institutes of Health, under Award Number 5UL1TR001425-04. The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding institutions. James Reigle assisted with the heatmaps.

CONFLICT OF INTEREST

The authors do not have any conflicts of interest.

AUTHOR CONTRIBUTIONS

Christine Niemeier-Walsh: Data curation (lead); Formal analysis (lead); Funding acquisition (supporting); Investigation (lead); Methodology (equal); Visualization (equal); Writing-original draft (lead); Writing-review & editing (equal). **Patrick H. Ryan:** Conceptualization (supporting); Data curation (supporting); Formal analysis (supporting); Funding acquisition (supporting); Investigation

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PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/ina.12750.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Niemeier-Walsh C, Ryan PH, Meller J, et al. The mycobiomes and bacteriomes of sputum, saliva, and home dust. *Indoor Air*. 2021;31:357–368. https://doi.org/10.1111/ina.12750