Environmental Research 231 (2023) 116063

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Childhood lower respiratory tract infections linked to residential airborne bacterial and fungal microbiota

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ARTICLE INFO

Handling Editor: Jose L Domingo

Keywords:
Indoor microbiota
Lower respiratory tract infections
Under-five children
High-throughput sequencing
Sub-saharan africa

ABSTRACT

Residential microbial composition likely contributes to the development of lower respiratory tract infections (LRTI) among children, but the association is poorly understood. We aimed to study the relationship between the indoor airborne dust bacterial and fungal microbiota and childhood LRTI in Ibadan, Nigeria.

Ninety-eight children under the age of five years hospitalized with LRTI were recruited and matched by age (±3 months), sex, and geographical location to 99 community-based controls without LRTI. Participants' homes were visited and sampled over a 14-day period for airborne house dust using electrostatic dustfall collectors (EDC). In airborne dust samples, the composition of bacterial and fungal communities was characterized by a meta-barcoding approach using amplicons targeting simultaneously the bacterial 16S rRNA gene and the internal-transcribed-spacer (ITS) region-1 of fungi in association with the SILVA and UNITE database respectively.

A 100-unit change in house dust bacterial, but not fungal, richness (OR 1.06; 95%CI 1.03–1.10) and a 1-unit change in Shannon diversity (OR 1.92; 95%CI 1.28–3.01) were both independently associated with childhood LRTI after adjusting for other indoor environmental risk factors. Beta-diversity analysis showed that bacterial (PERMANOVA $p < 0.001$, $R^2 = 0.036$) and fungal (PERMANOVA $p < 0.001$, $R^2 = 0.028$) community composition differed significantly between homes of cases and controls. Pair-wise differential abundance analysis using both DESEq2 and MaAsLin2 consistently identified the bacterial phyla *Deinococcota* (Benjamini-Hochberg (BH) adjusted p-value <0.001) and *Bacteriodota* (BH-adjusted p-value = 0.004) to be negatively associated with LRTI. Within the fungal microbiota, phylum *Ascomycota* abundance (BH adjusted p-value <0.001) was observed to be directly associated with LRTI, while *Basidiomycota* abundance (BH adjusted p-value <0.001) was negatively associated with LRTI.

Our study suggests that early-life exposure to certain airborne bacterial and fungal communities is associated with LRTI among children under the age of five years.

1. Introduction

Globally, lower respiratory tract infections (LRTIs) are the leading cause of morbidity and mortality particularly among children under the age of five years (Troeger et al., 2017). Annually, more than 2 million children under-five die due to LRTI in developing countries and 43% of these deaths occur in India, Nigeria, Democratic Republic Congo and Ethiopia (UNICEF/WHO, 2006). In 2017, LRTI was the single largest cause of under-five mortality, accounting for 140,520 (19%) deaths in Nigeria (Chao et al., 2018). Despite reports from several studies on the risk factors, LRTI remains a major public health issue that requires a concerted effort including indoor air quality improvement to reduce the burden of LRTI in sub-Saharan Africa.

Several studies have reported associations of house dust microbial exposures with adverse respiratory health effects including LRTI (Fakunle et al., 2021; Kanchongkittiphon et al., 2015). Indoor

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https://doi.org/10.1016/j.envres.2023.116063
Received 7 March 2023; Received in revised form 19 April 2023; Accepted 4 May 2023
Available online 6 May 2023

microbiological factors that contribute to observed health effects include fungi and bacteria, cell fragments, and secondary metabolites (Kanchongkittiphon et al., 2015). Determinants of indoor microbial composition and proliferation include human activities such as talking, cleaning and sanitation, presence of animals, geographical and climate factors, and structural failures such as roof leakages, poor ventilation and inadequate insulation (Kumar et al., 2021).

Children especially those under the age of five years spend more than 90% of their time at home indoors (National Academies of Sciences EaMN, 2017; Nazaroff, 2019). Moreover, children under five are considered to be a vulnerable sub-population, being more susceptible to respiratory health effects resulting from indoor microbial exposures due to their developing immune and respiratory system (Zeldin et al., 2006). Still, few studies have examined house dust microbial exposures in the context of LRTI among children under the age of five years (Fakunle et al., 2020a). The quest to discover the causative agents in house dust that explain associations with respiratory health in children is ongoing (Kanchongkittiphon et al., 2015; Adams et al., 2021). Exposure to high microbial diversity in homes in traditional farm areas were reported to be protective against childhood asthma (Ege et al., 2011; Dannemiller et al., 2014), but data on its association in residential homes with LRTI among children under five is scarce.

Current home microbiota studies have mainly utilized reservoir house dust samples from floor and/or matresses (Ege et al., 2011; Dannemiller et al., 2014; Kirjavainen et al., 2019). Dust that settles on a standard sampler surface (such as the electrostatic dustfall collectors) located above floor level appears to be a closer representation of the actual airborne exposure than reservoir house dust (Adams et al., 2015). The microbes identified in reservoir house dust (sampled by methods such as vacuuming) are unlikely to adequately represent microbial exposures from indoor airborne dusts. Furthermore, most current studies were conducted in middle- and high-income countries such as in Europe and the United States (Fakunle et al., 2020a, 2021). The composition of indoor microbial communities is geographically patterned, and significant variations has been observed in different climates, latitudes, and geographical regions (Barberán et al., 2015; Amend et al., 2010). Thus, microbiome diversity and composition observed in Europe and North America is unlikely to provide an adequate approximation of house dust microbial exposures in less developed countries with a tropical climate like Nigeria. In contrast to studies that focused mainly on childhood asthma (Adams et al., 2021; Kirjavainen et al., 2019), to our knowledge, no study has explored the complex relationship between indoor air microbiota and LRTI among children under the age of 5 years (Fig. 1) using culture-independent high-throughput sequencing (Fakunle et al., 2020a).

In this case-control study, we compared bacterial and fungal microbiota diversity and community structure in airborne dust collected in homes of children under-five years of age with and without LRTI in Ibadan, Nigeria.

2. Materials and methods

2.1. Study population

2.1.1. Identification and recruitment of cases and controls

Details of the study protocol and recruitment procedures have been published elsewhere (Fakunle et al., 2020b). Briefly, at the selected hospitals in Ibadan, Nigeria, all children younger than five years of age with LRTI confirmed by a pediatrician and admitted to the children's ward or emergency room were invited to participate in the study through their caregivers. Diagnosis of LRTI by a pediatrician, was mostly based on chest radiography. Chest radiography features included the presence of one or more features of patchy, segmental, or lobar consolidation; ± a positive air bronchogram; and ± pleural effusion. Controls (under-five children without evidence of LRTI i.e., absence of fever, cough and/or difficulty in breathing or other respiratory symptoms) were also screened by a pediatrician. Controls were not subjected to chest radiography. For every case of LRTI recruited from the hospitals and visited at home for indoor assessment, a control was identified, tested, and confirmed to not have LRTI and/or any of the respiratory signs and symptoms. Each control was primarily recruited from the same community where the cases reside in the same season and matched to cases by age (±3 months) and sex. A total of 200 children (100 case-control pairs) were contacted, and 98 cases and 99 controls agreed to participate in this study.

2.1.1.1. Collection of health data. A clinical proforma was developed using information from literature and administered by trained public health professionals to obtain vital health information about the child including breastfeeding status/duration, immunization status/vaccine received.

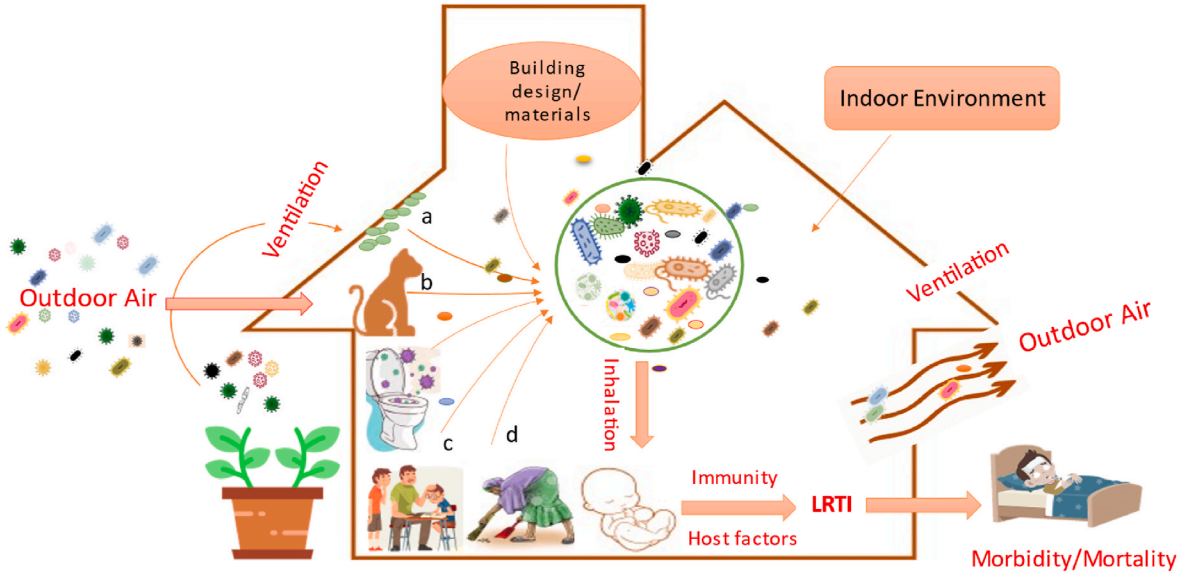

Fig. 1. Proposed schematic delineation of the interaction between childhood exposure to indoor microbiota and lower respiratory tract infections (LRTI) among children under-five years of age. Some of the factors that contribute to microbial growth within the indoor environment include **a**-dampness and mold growth; **b**-presence of pets; **c**-occupancy; **d**-indoor activities such as household cleaning.

2.1.1.2. Assessment of households using walkthrough inspection. A total of 197 homes of under-five children with and without LRTI were sampled. Indoor environmental characteristics and airborne dust samples were collected in each home between March 2019 and February 2020. A modified version of the walkthrough checklist instrument used in the South Durban Health Study (Jafta et al., 2017) was employed. The instrument was used by trained inspectors to document real-time observations on housing conditions (including type of house, material used in the construction of roof, walls and floor, visible mold growth and dampness or moisture on surfaces in the child's sleeping area) and household characteristics (such as number of rooms, occupancy, and keeping pets). Consented cases and controls were followed home within 24 h after recruitment to ensure that they were not prepared (by sweeping the floor, washing the toilets or cleaning the child's sleeping area) for the visit.

2.1.2. Airborne microbial sampling

Airborne microbial samples were collected in homes of cases and controls using an electrostatic dustfall collector (EDC) (Fakunle et al., 2020b; Noss et al., 2008). Each EDC sampler contained two electrostatic cloths (Albert Heijn, Zaandam, the Netherlands) that were rendered pyrogen-free before use by heating overnight at 200 °C. EDCs were assembled following a standard protocol: electrostatic cloths were fastened into polypropylene folders that had been cleaned with 70% alcohol under a sterile condition and kept in airtight clean ziplock bags until usage. During sampling, each EDC was opened and placed horizontally on a clean surface not less than 1.50 m above the floor in the child's sleeping/playing area for 14 days. In addition, blank samples were obtained from randomly selected homes of cases and controls by opening the EDC sampler briefly and immediately returned into the airtight lock plastic bag. At the end of the sampling period, each EDC was retrieved by the field assistant, closed, restored in the airtight plastic bag and transferred to the laboratory. There, sampled EDC cloths were carefully removed from each of the folders, placed in a sterile plastic lock bag, and stored at −80 °C freezer before processing.

2.2. DNA extraction and high-throughput sequencing

All EDC samples were shipped on dry-ice to the Institute for Risk Assessment Sciences (IRAS), Utrecht University, The Netherlands where they were stored at −80 °C until DNA extraction took place. To avoid any potential DNA isolation batch effect randomized EDCs of both cases and controls were processed at the same time during extraction. Total nucleic acids were isolated using a modified LGC (LGC Genomics GmbH, BERLIN, Germany) isolation procedure (Biesbroek et al., 2012). Briefly, EDC cloth was transferred in 50 ml tube containing 10 ml lysis buffer at room temperature, shaken for 10 min on a end-over-end shaker, and centrifuged at 4000 rpm for 15 min. After centrifugation, 800 μl aliquot of the supernatant was pipetted into a 2 ml tube, incubated at 95 °C for 10 min, and then allowed to cool to room temperature. About 650 μl of the sample was added to 1300 μl of binding buffer mixed with 10 μl of magnetic beads. The mixture was incubated for 30 min at room temperature, shaken at 800 rpm, and placed on a magnetic stand to remove the supernatant. After removing the supernatant, 200 μl wash buffer I was added to the precipitate, incubated shaken at 800 rpm at room temperature for 5 min, and spin down. The process was repeated with wash buffer II after which the tubes were opened to dry the beads at 55 °C. The DNA was then eluted with 50 μl elution buffer and stored at −20 °C before amplicon-sequencing.

2.3. Sequencing and taxonomic annotation

Bacterial community composition was assessed by sequencing the combined V5–V6 hypervariable region of the 16S rRNA gene. This is due to its superior phylogenetic resolution for bacterial phyla (Yang et al., 2016) and the possibility of using a multiplex procedure simultaneously for fungi on ITS. Briefly, the 16S hyper-variable region V5–V6 was first amplified by 25 cycles of 3 parallel and later pooled PCR reactions to maximise detected diversity using the primers 5′-ATTAGATACCCTGGTAGTCC-3′ and 5′-TCACRRCACGAGCTGACGACA-3′ for bacteria and in a separate set of PCR reactions using the primers 5′-CTTGGTCATTTAGAGGAAGTAA-3′ and 5′-GCTGCGTTCTTCATCGATGC-3′ targeting ITS-1 for fungi (Mbareche et al., 2020). PCR products were checked on a 2200 Tape station and all amplicons were subsequently equimolar pooled, and a barcode was added per sample prior to sequencing. Sequencing was performed using a version 3 paired-end 300 bp sequencing kit on a MiSeq sequencer (Illumina Inc., San Diego, CA, United States). Negative controls were included in DNA extractions and PCRs to identify potential (cross) contamination between samples during processing and to confirm the sterility of reagents, while a mock community (ZymoBIOMICS™ Microbial Community Standard containing bacterial and yeasts community) was included in the sequencing run as a positive and specificity control.

Raw sequence data processing and statistical analyses were performed in R 4.0.2 (R Core Team, 2014). The amplicon sequences were demultiplexed and 16S amplicons were separated from ITS amplicons while simultaneously trimming primer-derived sequences using cutadapt version 2.8 (Martin, 2011). Raw sequencing data per amplicon per sample is deposited at NCBI short read archive under BioProject accession PRJNA930885. Raw trimmed sequences were subsequently filtered, trimmed, error-corrected, dereplicated, chimera-checked, and merged using the pseudo-pooling options from the DaDa2 package (v.1.16.0)(Callahan et al., 2016). By using the standard parameters with a setting of TruncLength=(180, 175), trimLeft=(0,0), maxEE = 2, and minOverlap = 10 *for bacteria and* TruncLength=(180, 175), trimLeft=(0, 0), maxEE = 2, and minOverlap = 10 *for fungi*. Reads were classified using the naïve Bayesian classifier implemented in DaDa2 against the bacterial SILVA (v128 database) (Quast et al., 2013) and fungal UNITE (version 7.2 database) (Nilsson et al., 2019) respectively. BLASTnt was used to identify the most likely species name belonging to identified genera using majority voting for the longest sequence if they were having equal identity scores.

2.4. Data analysis

Statistical analyses were performed with the phyloseq (McMurdie and Holmes, 2013) and vegan (Oksanen et al., 2013) R packages using R Statistical Analysis Software version 4.1. The sequenced samples for bacteria and fungi had a range of 208–327,258 and 58–610,604 reads per sample respectively. The final dataset contained 13,944,447 and 1, 751,006 reads for bacteria and fungi respectively and 107,487 different amplicon sequence variants (ASVs) for bacteria and 8565 for fungi. The phyloseq object is deposited at zenodo.org (http://doi.org/10.5281/zenodo.7599214). All statistical tests used p-values adjusted for false discovery rate as described by Benjamini and Hochberg, with an adjusted p-value less than 0.05 considered significant (Benjamini and Hochberg, 1995). We investigated three aspects of the microbiota data for relationships with LRTI: alpha diversity, beta diversity and the mean relative taxa abundance in homes of cases and controls.

Alpha diversity matrix (richness, Shannon and inverse Simpson's diversity) in each sample representing bacterial and fungal taxonomic diversity within samples were calculated based on uniformly rarefied data at 30,000 reads for bacteria and 1000 reads for fungi after inspection of the bacterial and fungal rarefaction plot (Figure S1, A and B). We also examined the relationship between bacterial and fungal diversities, environmental exposure measures and LRTI case-control status as described below (multivariable regression modelling).

Differences in the microbial community composition between homes of cases and controls (beta-diversity) were analyzed using relative abundance data at genus level. Beta-diversity was measured by Bray-Curtis dissimilarity and visualized by Principal Coordinates Analysis (PCoA) and tested using permutational multivariate analysis of variance (PERMANOVA) using the adonis and betadisper functions from Vegan R

package (Oksanen et al., 2013). Procrustes non-randomized and randomized analysis using the PROTEST function (Jackson, 1995) was performed to determine the relationship between the bacterial and fungal ordination in samples of cases and controls. Pair-wise differential abundance analysis using DESeq2 (version 1.28) and MaAsLin2 was performed at ASV, genus, family and phylum level using prevalence filtered taxonomic data at 0.1% relative abundance in more than 25% of the samples to identify the significant different taxa that contributes to the overall differences in the microbial community assemblage in homes of cases versus homes of controls. The differential abundance analyses were done adjusting for the covariates included in the case-control matching (sex, age and geographical location) and others identified in the directed acyclic graph (DAG). Similar microbiota analysis had been employed in a previous community-based case-control study among patients with and without chronic obstructive pulmonary disease (COPD) (van Kersen et al., 2022) and in a matched case-control study to determine whether the broiler litter microbiota composition was associated with *Campylobacter* isolation within the broiler house (Valeris-Chacin et al., 2021).

2.5. Multivariable regression modelling

Figure S2 presents a directed acyclic graph (DAG) detailing the causal relationships potentially affecting the association between exposure to indoor microbiome diversity and LRTI. The individual circle connotes an individual exposure (node) of theoretical relevance; each node is interrelated by directional arrows that represent theoretical associations based on the researchers' assessment of prior literature and determination of biological plausibility. The association of interest, therefore, is the link represented by the green arrow connecting the exposure and outcome. Age, gender, immunization/breastfeeding status (blue nodes) are theoretically causally associated with the outcome alone (ancestors of outcome). The other exposure (red node) is in theory causally associated with both the exposure and the outcome. To adjust for potential confounders in the association of interest, it is necessary to block all alternate routes between the exposure and outcome (red nodes). Identified potential confounders were introduced in multivariable models. Multivariable logistic regression analysis was used to determine the associations between respectively the fungal and the bacterial alpha diversity measures with LRTI adjusting for potential confounders (see Figure S2) including season, environmental tobacco smoke, visible dampness and molds, presence of pets and occupant density. The same variables were included in the beta-diversity model analysis using PERMANOVA.

2.6. Descriptive analysis of residential air microbiota

Positive controls were added to the qPCR runs to verify that the method can amplify the target nucleic acid while field blanks and negative controls were included to observe mass contamination in the amplification process. All positive controls were excluded from the dataset before analysis of the reads. Sequencing bacterial 16S rRNA gene amplicons resulted in a total of annotated 13,944,447 read-pairs with an average of 70,783 read-pairs per sample, ranging from 26 to 201,987 reads for all 197 samples. Processing the raw data in DaDa2 resulted in 140,881 amplicon sequence variants (ASVs). Contaminant ASVs were identified and removed using the decontam package (version 1.8.0) which uses data from the blanks and negative controls. These contaminants were mainly microbial genera that have previously been described as laboratory contaminants (Salter et al., 2014). Bacterial compositionality analyses were performed using 107,487 taxa after removing 33,394 taxa that did not reach a confident level of detection (prevalence >0.1% abundant in at least two samples) with 59 phyla and 607 genera. Sequencing fungal-derived ITS amplicons generated 1,751,006 annotated read-pairs with an average of 8934 read-pairs per sample (ranging from 4 to 120, 084 read-pairs). The raw data analysis resulted in 131,145 amplicon sequence variants (ASVs) with 9 phyla and 840 genera. Fungal compositional analyses were performed using 8565 taxa after removing 122,580 taxa that did not pass the low abundant and minimal sample occurring prevalence filtering criteria. The proportion of unassigned taxa observed at different taxonomic levels is presented in Table S1.

2.7. Ethical consideration

The study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (Ref No: BE545/17), the University of Ibadan/University College Hospital Ethics Committee (Ref No.: UI/EC/17/0077), and the Oyo State Research Ethical Review committee (Ref. No.: 13 CE/479/462). Informed consent to visit the homes was obtained from caregivers of these children before participation in the study.

3. Results

3.1. Child and household characteristics

Detailed characteristics of the children and their households included in the study can be found in Table 1. The majority of control children (84.8%) were exclusively breastfed compared to children with LRTI (71.4%) ($p = 0.02$). Also, more cases (17.3%) had a previous history of LRTI than controls (8.1%).

A mini-flat was the dominant form of residence for participants, regardless of case-control status (cases: 49.0% *vs* controls: 47.5%). The mean floor space per occupant was significantly higher in homes of controls (44.7 ± 14.5 m^2/person) than cases (32.0 ± 13.5 m^2/person). Notably, the presence of visible dampness (21.4% vs 2.0%), visible molds (11.2% vs 2.0%) and pets in the house (38.8% *vs* 20.2%) in homes of cases versus controls differed significantly. Our previous report also showed an exposure-response relationship between residential dampness and severe LRTI among children under the age of five years (Fakunle et al., 2023).

Table 1
Description of child and household characteristics among LRTI cases and community-based age and sex-matched controls.

Characteristics	Cases; N = 98	Controls; N = 99	*P*
Age (months); mean (SD)	7.11 (1.19)	6.92 (1.02)	0.85
Sex; Males	62 (63.3%)	64 (64.7%)	0.84
Breastfeeding; exclusive	70 (71.4%)	84 (84.8%)	**0.02**
Immunization; completed	81 (82.7%)	84 (84.8%)	0.68
History of LRTI	17 (17.3%)	8 (8.1%)	0.05
Home Characteristics			
Type of House			
A Room Apartment	36 (36.7%)	25 (25.3%)	-
Mini Flat	48 (49.0%)	47 (47.5%)	0.30
Apartment Building/Flat	6 (6.1%)	14 (14.1%)	0.31
Bungalow	8 (8.2%)	13 (13.1%)	0.10
Season			
Wet	70 (71.4%)	62 (62.6%)	0.14
Dry	28 (28.6%)	37 (37.4%)	
Occupant density (m^2/person); mean (SD)	32.04 (13.50)	44.67 (14.52)	**<0.001**
Mud House Wall Construction (n (%))	16 (16.5%)	7 (7.2%)	**0.04**
Visible Dampness (n (%))	21 (21.4%)	2 (2.0%)	**<0.001**
Environmental tobacco smoke (n (%))	10 (10.2%)	5 (5.1%)	0.17
Visible Mold; (n (%))	11 (11.2%)	2 (2.0%)	**0.009**
Pets; (n (%))	38 (38.8%)	20 (20.2%)	**0.004**

[†]p-value obtained using Mann-Whitney *U* test; SD- Standard deviation; IR; interquartile range; dampness was defined as the presence/absence of moisture/mold growth on the walls or roof in the child's sleeping area; pets was defined as presence/absence of any pets in the house.

3.2. Residential air microbiome composition

An overview of the taxonomic composition at phylum level can be found in Fig. 2. For bacteria, relative abundance was dominated by *Proteobacteria* which accounted for an average of 43% and 45% of all phyla in homes of cases and controls, followed by *Actinobacteriota* (34% *vs* 30%) and *Firmicutes* (18% *vs* 19%) in homes of cases and controls respectively. At genus level, relative abundance was dominated by *Acinetobacter* (14% vs 11%) followed by *Pseudomonas* (9% vs 10%) and *Corynebacterium* (8% vs 8%) in homes of cases and controls respectively (Figure S3A). The mycobiota was dominated by the phylum *Ascomycota* (75% *vs* 64%) and *Basidiomycota* (20% *vs* 30%) in homes of cases and controls respectively. *Aspergillus* (45% vs 39%) followed by *Cladosporium* (18% vs 24%) and *Trametes* (11% vs 11%) were the dominant fungal genera (Figure S3B). The relative abundance plot for the bacterial and fungal microbiota at phylum level can be found in Figure S4 (A and B).

3.3. Alpha-diversity and association with LRTI

We tested for differences in within-subject alpha-diversity using data rarefied at 30,000 and 1000 sequences per sample for the bacterial and fungal ASVs respectively, maintaining about 90% of all samples (excluding negative controls and field blanks). The alpha-diversity measures for bacterial and fungal microbiota at ASV level in homes of cases and controls are presented in Fig. 3. For the bacterial microbiota, the median (range) observed richness was higher in homes of cases [2281 (181–6072)] than controls [1168 (136–5641)], $p < 0.001$. Similarly, Shannon diversity was higher in homes of cases 6.14 (3.59–8.08) than in controls 5.87 (2.42–7.73), $p = 0.016$. For the mycobiota, the median (range) observed richness was slightly higher in homes of cases [105 (25–249)] than controls [97 (39–314)] but not statistically significant ($p = 0.42$). A similar trend in the bacterial and fungal observed richness at genus level was recorded in homes of cases and controls (Figure S5 A and B)

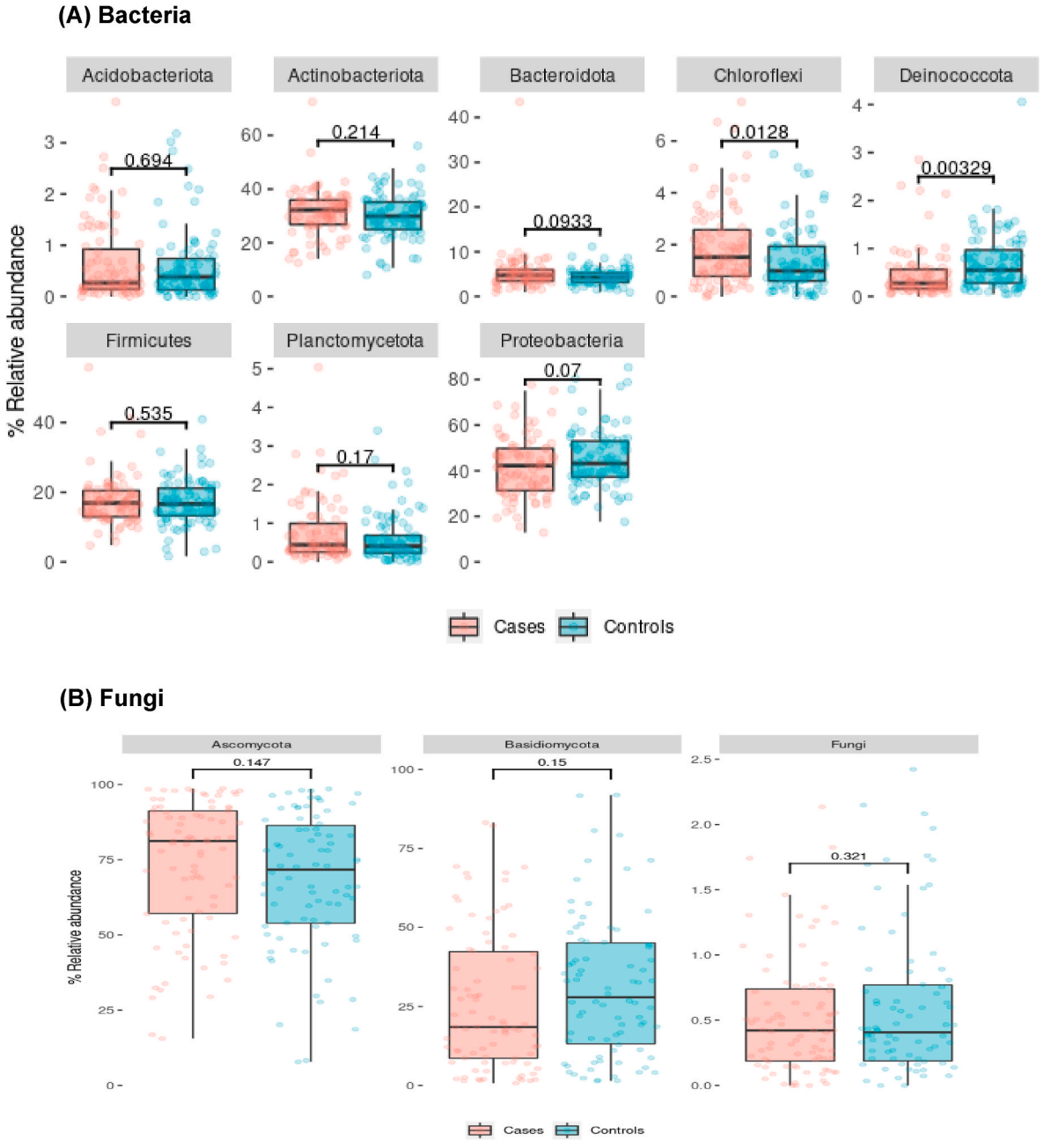

Fig. 2. Relative abundances at Phylum level, showing taxa accounting for at least 0.5% (mean relative abundance) of the indoor air microbiome for (A) Bacteria; (B) Fungi; using independent sample *t*-test.

After adjusting for season, environmental tobacco smoke (ETS), visible dampness and mold, presence of pets, and occupant density, each 10-unit increase in bacterial richness was associated with a 1% increase in having an additional LRTI (OR 1.01; 95%CI 1.00–1.02). Also, aone-unit increase in bacterial Shannon diversity was associated with a 92% increase in having an additional LRTI (OR 1.92; 95%CI 1.28–3.01) (Table 2). In contrast, fungal alpha-diversity did not show any statistically significant association with LRTI among under-five children (OR 0.72; 95%CI 0.42–1.20). Other indoor environmental covariates found to be statistically significantly associated with a higher risk of LRTI were season, visible dampness, presence of pets, and a higher occupant density (fewer m^2 per person).

3.4. Beta-diversity

We found that the bacterial (PERMANOVA $p < 0.001$, $R^2 = 0.036$, homogeneity (of dispersion) $p < 0.001$) and fungal (PERMANOVA $p < 0.001$, $R^2 = 0.028$, homogeneity $p = 0.003$) community composition and dispersion differed in homes of cases and controls (Fig. 4) even after adjusting for season, visible dampness and molds, environmental tobacco smoke and pets, and occupant density. Season also contributed to these differences for bacterial (PERMANOVA $p = 0.01$, $R^2 = 0.010$) and fungal (PERMANOVA $p = 0.01$, $R^2 = 0.012$) communities (Table 3). Therefore, the most important determinant for bacterial and fungal compositional differences between cases and controls based on the multivariable PERMANOVA was season. To gain more insight as to whether the microbial community differences are similar for bacteria and fungi, we employed Procrustes analysis using maximum diversity containing PCoA Bray-Curtis ordination data of axis 1 and 2. Procrustes analysis showed a low but significant correlation between the bacterial and fungal community structure of 196 samples for which both microbiota diversities where available ($r = 0.22$; $p = 0.0002$; Fig S6A), while correlation was lost in an analysis with randomized sample identifiers (Fig S6B).

3.5. Differential abundance analysis

Fig. 5A contains a list of bacterial genera that were significantly different after adjusting for selected covariates, based on differential abundance between cases and controls (log 2-fold change (LFC) increased by 2.5 or decreased by LFC of 0.5 as identified by DESeq2). Overall, at genus level DESeq2 identified 29 bacterial genera belonging to the phyla *Proteobacteria* (LFC = 2.88; adjusted p-value = 0.009), *Firmicutes* (LFC = 2.82; adjusted p-value = 0.001), *Actinobacteriota* (LFC = 2.76; adjusted p-value = 0.009), and *Chloroflexi* (LFC = 1.48; adjusted p-value = 0.001) to be significantly higher in homes of cases than controls. In contrast, 14 genera belonging to the phyla *Deinococcota* (LFC = −0.54; adjusted p-value =<0.001), and *Bacteriodota* (LFC = −2.00; adjusted p-value=<0.001) were significantly higher in homes of controls than cases (Table S3A). A Pearson correlation analysis revealed a low to moderate correlation between the taxa, with pearson correlation coefficients ranging from −0.25 to 0.63. Highest correlation was observed for *Proteobacteria* with *Deinococcota* (rho = 0.63; $p < 0.05$) Figure S9 (Figure S7). Also, a total of 41 bacterial species including, *Corynebactrium kroppenstedtii* (LFC = −1.98; adjusted p-value=<0.001), *Pseudomonas azotoformans* (LFC = −0.55; adjusted p-value = 0.001), *Pseudomonas protegens* (LFC = −5.96; adjusted p-value=<0.001) and *Staphylococcus capitis* (LFC = −1.93; adjusted p-value=<0.001) were found to be significantly higher in homes of controls than cases (Table S3A). In comparison to DESeq2, MaAsLin2 identified 23 bacterial

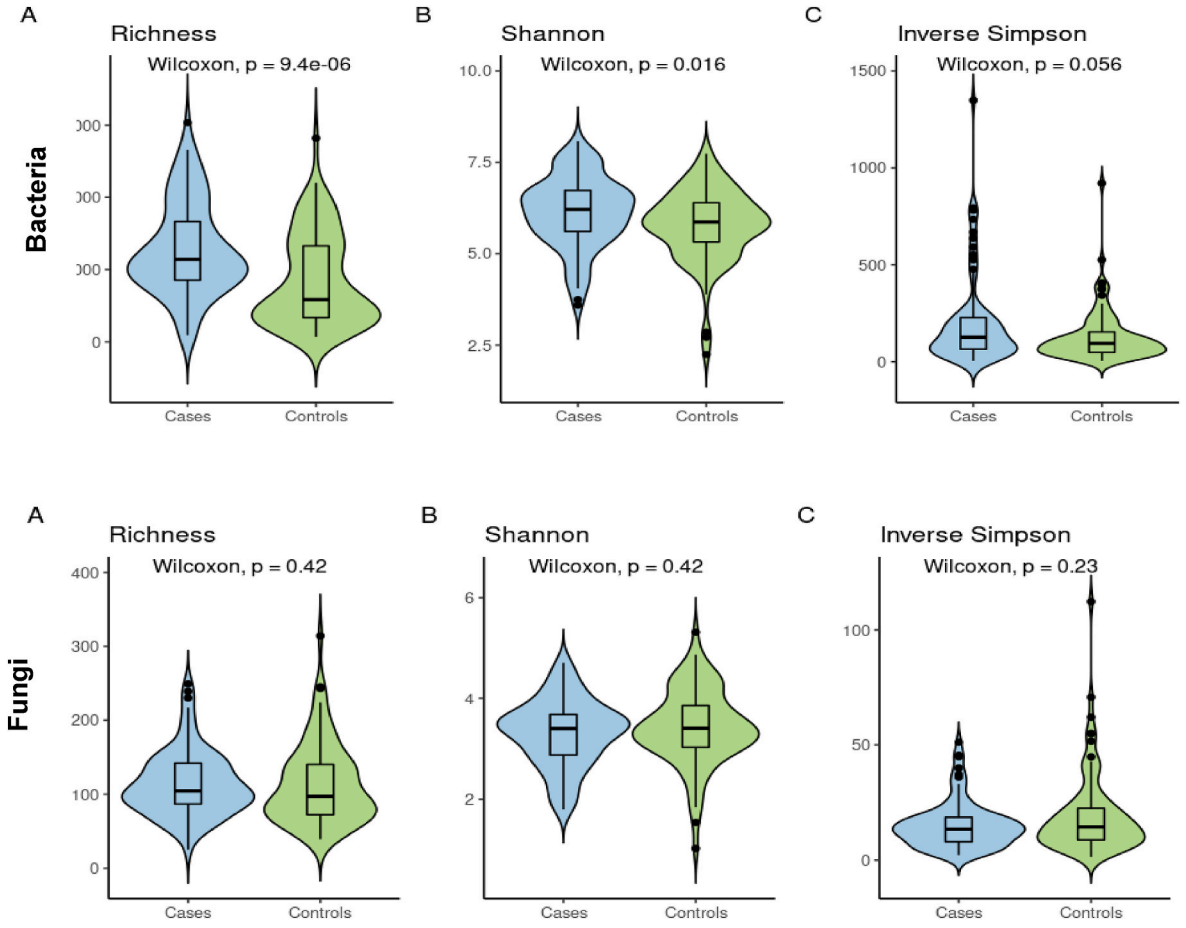

Fig. 3. Bacterial and Fungal Richness, Shannon and Simpson indices in homes of cases versus controls at ASV level.

Table 2
Adjusted multivariable regression model for the association between richness or Shannon diversity of bacterial and fungal microbiome and LRTI among under-five children.

Model	Bacteria *Cases vs Controls*		Fungi *Cases vs Controls*	
	Unadjusted OR (95% CI)	*aOR (95%CI)*	Unadjusted OR (95% CI)	*aOR (95%CI)*
Model I: Richness (100 unit increase)	**1.06 (1.03–1.08)**	**1.06 (1.03–1.10)**	1.03 (0.56–1.92)	0.96 (0.45–2.02)
Season; Wet vs dry	**1.58 (1.15–2.95)**	**3.37 (1.388.86)**	**2.08 (1.10–3.98)**	**2.78 (1.25–6.41)**
Smoker in house; yes vs no	2.76 (0.88–10.39)	3.92 (0.87–10.42)	2.59 (0.83–9.78)	2.64 (0.70–11.45)
Mold; yes vs no	**1.73 (1.43–37.49)**	5.91 (0.32–122.35)	**5.93 (1.53–39.11)**	4.21 (0.51–49.25)
Dampness; yes vs no	**12.98 (3.62–23.13)**	**10.67 (2.28–81.34)**	**13.01 (3.64–33.16)**	**10.92 (2.46–80.51)**
Pets; yes vs no	**2.41 (1.24–4.81)**	**3.16 (1.30–8.14)**	**3.13 (1.59–6.36)**	**3.83 (1.64–9.45)**
Occupant density	**0.93 (0.91–0.95)**	**0.91 (0.88–0.94)**	**0.94 (0.91–0.96)**	**0.93 (0.89–0.95)**
Model II: Shannon diversity (1 unit increase)	**1.46 (1.08–2.04)**	**1.92 (1.28–3.01)**	0.80 (0.52–1.23)	0.72 (0.42–1.20)
Season; Wet vs dry	**1.58 (1.15–2.95)**	**3.56 (1.60–8.35)**	**2.08 (1.10–3.98)**	**2.96 (1.33–6.86)**
Smoker in house; yes vs no	2.76 (0.88–10.39)	2.96 (0.80–12.01)	2.59 (0.83–9.78)	2.04 (0.56–8.11)
Mold; yes vs no	**1.73 (1.43–37.49)**	5.57 (0.44–104.92)	**5.93 (1.53–39.11)**	5.47 (0.62–64.43)
Dampness; yes vs no	**12.98 (3.62–23.13)**	**16.37 (3.42–126.66)**	**13.01 (3.64–33.16)**	**11.94 (2.64–89.00)**
Pets; yes vs no	**2.41 (1.24–4.81)**	**3.16 (1.41–7.42)**	**3.13 (1.59–6.36)**	**4.04 (1.7210.11)**
Occupant density	**0.93 (0.91–0.95)**	**0.92 (0.89–0.95)**	**0.94 (0.91–0.96)**	**0.92 (0.89–0.95)**

OR – Odds ratio; aOR – adjusted Odds ratio; CI – Confidence interval; odds ratios indicate the fold change in odds of LRTI for a 100-unit change in microbial richness and one-unit change in Shannon diversity, e.g., a 6% increase in LRTI odds for a 100-unit increase in bacterial richness or a 92% increase in LRTI odds for a one-unit increase in bacterial Shannon diversity. Model was adjusted for season, environmental tobacco smoke, mold, dampness, pets, and occupant density; separate models were run for richness and Shannon diversity.

genera belonging to the phyla *Deinococcota* ($\beta = -1.29$, Benjamini-Hochberg (BH)-adjusted $p < 0.001$), and *Bacteriodota* ($\beta = -1.23$, BH-adjusted $p = 0.04$) to be negatively associated with LRTI (Fig. 6) while *Proteobacteria* ($\beta = 0.12$, BH-adjusted $p = 0.19$), and *Firmicutes* ($\beta = 0.23$, BH-adjusted $p = 0.14$) were not significantly associated with LRTI (Table S4A).

Also, Fig. 5B contains a list of fungal genera that were significantly different, based on differential abundance between cases and controls. DESeq2 identified 4 fungal genera belonging to the phylum *Ascomycota* (LFC = 4.69; adjusted p-value = 0.009) that were positively associated with LRTI while another 4 genera belonging to *Basidiomycota* (LFC = −2.15; adjusted p-value = 0.002) were negatively associated with LRTI among children under the age of five years (Table S3B). The differential abundance at species level is as presented in Table S3B. To support findings from DESEq2, MaAsLin2 showed similar trend and identified 34 genera belonging to the phylum *Ascomycota* ($\beta = 2.24$, BH adjusted $p < 0.001$) and 2 genera belonging to *Basidiomycota* ($\beta = -1.12$, BH adjusted $p < 0.001$) to be significantly associated with LRTI among children under the age of five years (Fig. 6 and Table S4B).

4. Discussion

In this case-control study, we showed a significantly higher bacterial alpha-diversity in airborne dust of homes of under-five children with LRTI than those without LRTI. Also, we found that the bacterial and fungal community differed in homes of under-five children with and without LRTI after adjusting for covariates such as season and indoor environmental characteristics. Furthermore, we found evidence suggesting that 43 bacterial and 8 fungal genera in house dust were significantly associated with LRTI among under-five children.

The home environment has its ownmicrobial ecology dominated by the occupants and the outdoor microbiota; therefore, different homes are likely to have different microbiota (Fahimipour et al., 2018; Adams et al., 2013). Nevertheless, the direct environments coupled with heritability, and lifestyle are known to shape the human microbiome and facilitate the development of microbiome-targeted therapies (Gacesa et al., 2022; Lopera-Maya et al., 2022). Our multivariable logistic regression model adjusting for season and home characteristics found a positive association between bacterial alpha-diversity and LRTI among under-five children. This finding is consistent with the few available studies on early childhood respiratory infection (Stark et al., 2003; Grant et al., 2012). Results of studies on asthma also reported an association between high levels of bacterial richness in homes and asthma symptoms of children (Dannemiller et al., 2016) and slightly higher bacterial richness in house dust of patients with asthma than those without asthma ($p > 0.05$) in an adult farming population in the United States (Lee et al., 2021). In contrast, Fu et al. (2020a) found that overall bacterial richness in settled dust was not significantly associated with respiratory tract infections among students living in school dormitories in Northern China (OR 1.00; 95% CI 0.83–1.21). Another study by Adams et al. (2021) revealed several positive associations between microbial indicators such as richness in settled dust samples and respiratory symptom score among primary school pupils in Finland, while in the Netherlands, the associations was mostly inverse and statistically non-significant. The lack of association observed between fungal alpha-diversity and LRTI among under-five children in our current study could be a result of the lower sequencing depth for fungi as compared to bacteria. The lower sequencing depth for fungi is likely the result of a lower yield from the DNA isolation method used during extraction and the multiplexed sequencing approach. Rarefaction curves as shown in Figure S1 Bi indicates that in general the sequencing depth was enough to allow reaching a plateau of the number of detected taxa. Furthermore, the method has been benchmarked for bacterial microbiota using known samples and community controls but has not been extensively used to profile the mycobiome."

Our study using PERMANOVA analysis and test for homogeneity found a high degree of dispersion in the bacterial and fungal community composition in house dust samples from homes of under-five children with and without LRTI indicating a high degree of differences. A similar study among asthmatic patients found a significant difference in the overall bacterial composition in house dust samples from homes of asthmatic patients and non-asthmatic subjects (Karvonen et al., 2019) while Cox et al. (2022), were unable to detect any significant differences in beta-diversity of bacterial and fungal microbiota in floor dust samples between the absence and presence of asthma, wheeze, aeroallergens and rhinitis. Also, we found that the bacterial and fungal community structure was associated with season suggesting a different bacterial and fungal profile between the dry and rainy seasons.

Our study mirrored conclusions from other studies that aimed to link the indoor environmental microbiome to health effects that reported *Proteobacteria*, *Actinobacteria* and *Firmicutes* (Fu et al., 2020a; Hewitt et al., 2012) as the main indoor air bacterial phyla in terms of relative abundance. By DESEq2, we found abundance of 29 bacterial genera belonging to 4 phyla: *Proteobacteria*, *Firmicutes*, *Actinobacteriota*, and *Chloroflexi* to be positively associated with LRTI, while abundance of 14 genera belonging to 2 phyla: *Deinococcota*, and *Bacteriodota* was

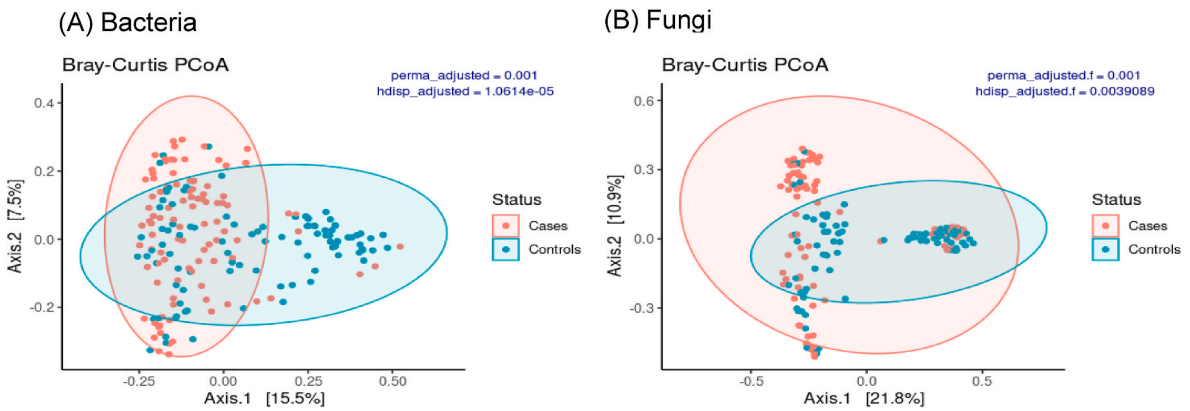

Fig. 4. Beta-diversity PCoA (Bray-Curtis) by case-control status (excluding negative/positive/field controls) for (A) Bacterial and (B) Fungal microbiome. Points represent the indoor air microbiome in homes of 98 LRTI cases and 99 controls after transforming read counts to relative abundances. PERMANOVA analysis was adjusted for season, visible dampness and molds, environmental tobacco smoke and pets, and occupant density. PERMANOVA adjusted p-value suggests a significant difference in the bacterial and fungal community structure between homes of cases and controls.

Table 3
Multivariable PERMANOVA comparing indoor air microbiome composition (PCoA Bray-Curtis dissimilarity) in homes of under-five children with and without LRTI.

Model	Bacteria				Fungi			
	Univariate		Multivariate (Interactive)		Univariate		Multivariate (Interactive)	
	PERMANOVA		PERMANOVA		PERMANOVA		PERMANOVA	
	P	R2	P	R2	P	R2	P	R2
Case/control status	**<0.001**	**0.036**	**<0.001**	**0.036**	**<0.001**	**0.029**	**<0.001**	**0.028**
Season; Wet vs dry	**0.02**	**0.009**	**0.01**	**0.009**	**0.03**	**0.010**	**0.01**	**0.012**
Smoker in house; yes vs no	0.72	0.004	0.61	0.004	0.81	0.004	0.80	0.003
Mold; yes vs no	0.20	0.006	0.51	0.004	0.15	0.007	0.43	0.005
Dampness; yes vs no	**0.01**	**0.009**	0.24	0.006	0.04	0.009	0.31	0.005
Pets; yes vs no	0.11	0.006	0.65	0.004	0.09	0.008	0.34	0.005
Occupant density	0.09	0.007	0.17	0.006	**<0.001**	**0.018**	**0.004**	**0.013**

PERMANOVA – permutational Multivariate analysis of variance adjusting for season, visible dampness and molds, presence of any smoker and pets, and occupant density while comparing bacterial and fungal composition between homes of cases and controls.

negatively associated with LRTI among under-five children. Of note, it is unusual to see the phylum *Deinococcota Thermus,* an extremophile bacteria known to occur in environments such as thermophilic composting conditions (Theodorakopoulos et al., 2013), in the home environment. Although some findings on the differential abundance were not consistent between two different differential abundance detection algorithms, DESEq2 and MaAsLin2, the identified taxa followed similar directions (more/less in A versus B) using both methods. The risk estimate for the association between the phylum *Proteobacteria* and LRTI among under-five children is consistent with previous human health and microbiome studies (Fu et al., 2020a). A recent cross-sectional study of nasopharyngeal microbiota showed that several *Proteobacteria* species, including *Klebsiella pneumoniae* and *Moraxella* spp., were associated with respiratory syncytial virus severity (de Steenhuijsen Piters et al., 2016). These bacterial genera can also exert their adverse effect by reducing the host immune defense, leading to susceptibility to other pathogens, such as viral infections (Man et al., 2019; Hanada et al., 2018; de Koff et al., 2021). Therefore, more functional metagenomics, transcriptomic, and metabolomics studies in humans linking indoor microbiota composition differences with host-disease phenotypes, are needed to disentangle the complex interactions between bacterial and viral pathogens in respiratory infections, especially among children under the age of five years. According to Fu et al. (2020a), the bacterial phylum *Actinobacteriota* was found to be protectively associated with respiratory infections among students. A few other studies also reported that taxa richness and abundance of *Actinobacteria* were protectively associated with childhood asthma (Karvonen et al., 2019; O'Connor et al., 2018) and asthma symptoms among university students (Fu et al., 2020b). This contradicts our findings as we found *Actinobacteria* to be positively associated with increased risk of LRTI among under-five children. Whether the *Actinobacteria* found in Asia and Africa differ and pose different health effects is unclear. Thus, a need for further investigation of the significance on *Actinobacteria* in the epidemiology of LRTI among under-five children. To corroborate our claim, a few genera of the *Actinobacteria*; *Corynebacteria* and *Micrococcus* have been reported to cause pneumonia (Yang et al., 2018a). More interestingly, there has been greater interest in several *Corynebacteria* subspecies as these organisms have been cited being emerging pathogens (Yang et al., 2018b). Another interesting finding of this study is the potential protective effect of the phylum *Bacteroidota* against LRTI in under-five children which was consistent by DESEq2 and MaAsLin2. A murine model of aspergillosis stated that an increased risk of fungal pneumonia was associated with loss of protective anaerobes such as *Clostridiales* and *Bacteroidota* (Nunzi et al., 2021). However, the mechanisms behind their protective ability as well as how they integrate with the immune system during the response to infection is yet to be investigated.

In this study, all the LRTI-related fungal genera belonged to the *Ascomycota*, a large fungal phylum with high abundance and diversity in most indoor and outdoor environments (Adams et al., 2021; Nunzi et al., 2021). The genus *Aspergillus* is known to be the most common of the *Ascomycota* and can be harmful to humans (Seyedmousavi et al., 2015). Aspergillus fumigatus is the most common and life-threatening airborne opportunistic fungal pathogen, which is particularly important among immunocompromised hosts and under-five children with immature respiratory system (Patterson and Strek, 2010). To the best of our knowledge, this is the first-time other genera of the *Ascomycota*; *Hortaea,*

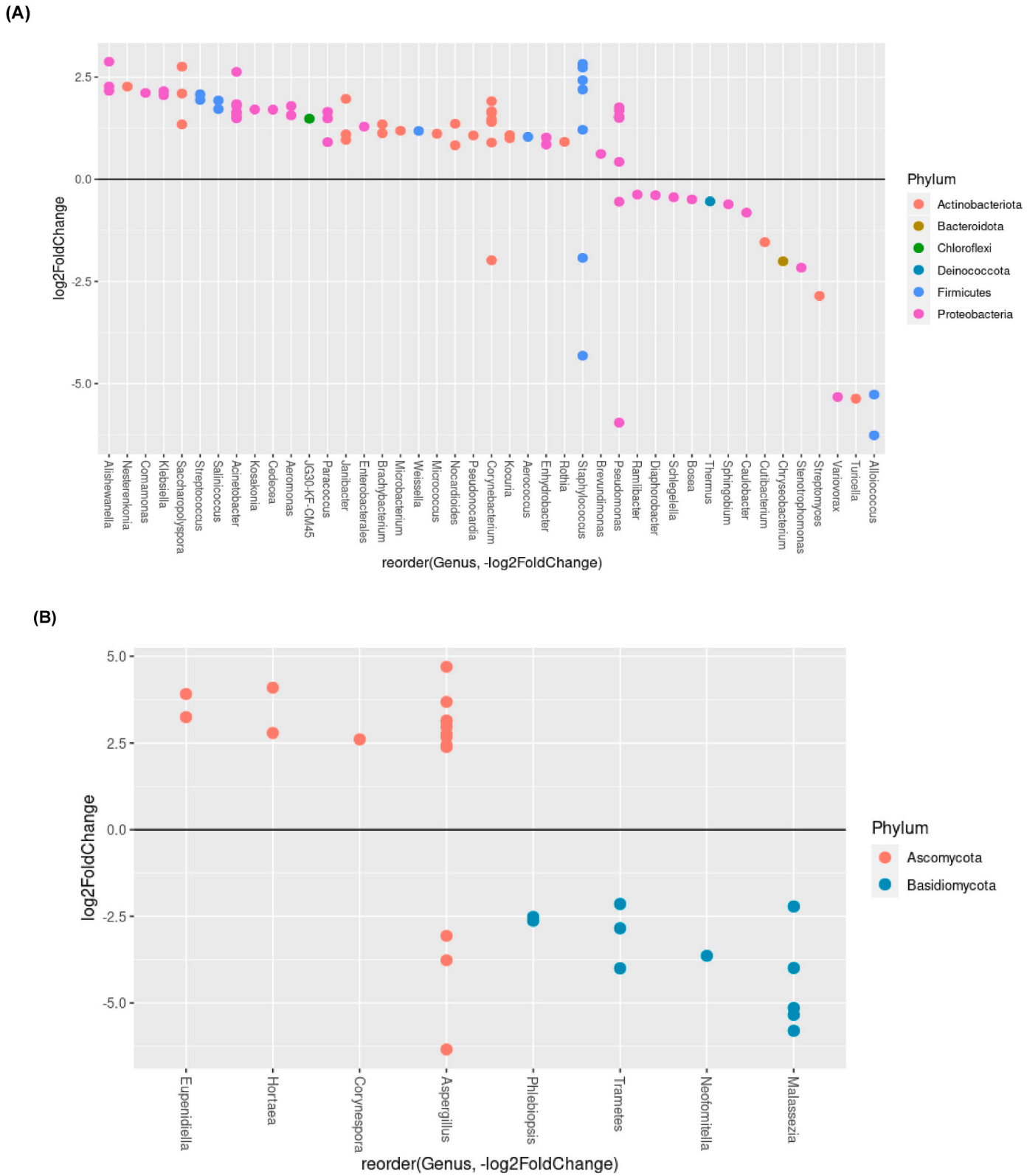

Fig. 5. DESeq2 results showing the log2 fold change value (y-axis) of **(A)** Bacterial and **(B)** Fungal in the indoor air samples between under-five children with and without LRTI. Each line on the x-axis indicates the genus, each point represents an individual ASV within the genus, the colour of the points indicates the phylum.

Eupenidiella, and *Corynespora,* are implicated in the risk of LRTI among under-five children. In support of our findings, recent molecular studies show a significant presence of the *Basidiomycota* in the indoor environment (Green et al., 2017; Green, 2018). Another interesting finding from our study is that all genera of the phylum *Basidiomycota*; *Trametes*, *Malassezia*, *Phlebiopsis*, and *Neofomitella,* were negatively associated with LRTI among under-five children. The protective mechanism is unclear but potentially explained by antioxidant properties of members of the genus *Basidiomycota* (Zmitrovich et al., 2012). For example, *Trametes* are known to contain a variety of powerful antioxidants and

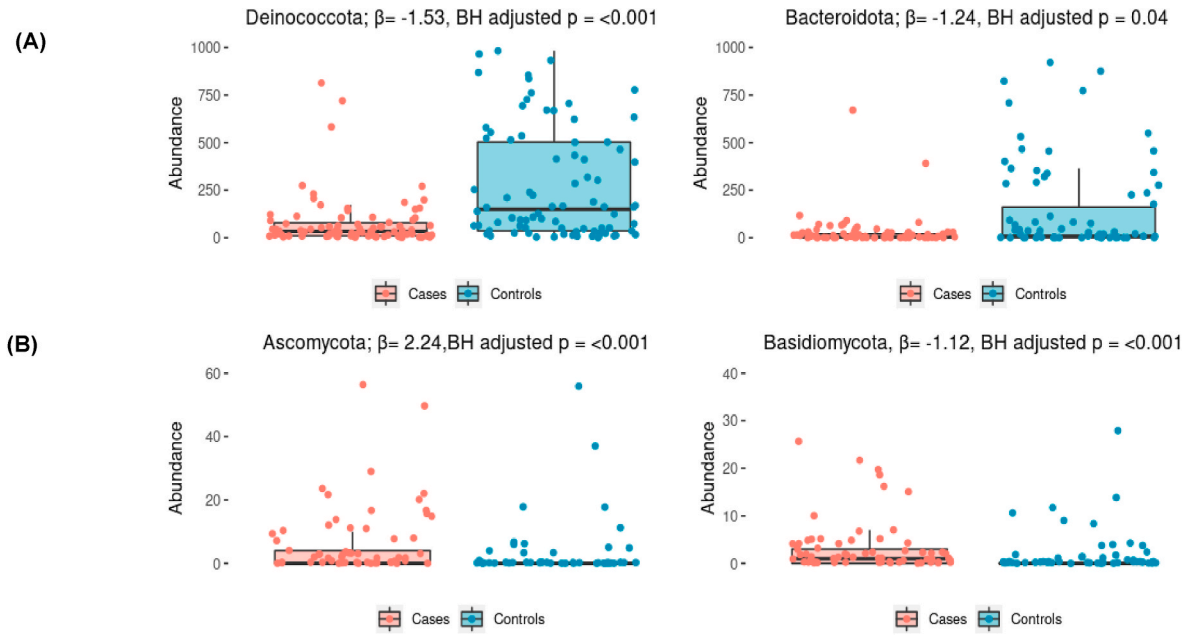

Fig. 6. MaAsLin2 results showing the abundance (y-axis) of **(A)** Bacterial and **(B)** Fungal phyla in the indoor air samples of under-five children with and without LRTI. Each boxplot represents Phylum that were significantly associated with LRTI among under-five children.

other compounds that may help boost the immune system to fight certain infections (Zmitrovich et al., 2012). Also, extracts of the *Trametes* are considered as strong antigenotoxic agents able to stimulate a genome-protective response of cells (Knežević et al., 2015).

It is important to note that microbiome studies are a relatively new field of research and therefore the number of indoor microbiome studies in relation to childhood respiratory infections are still relatively small. Also, geographical and seasonal influences might differ between studies. While Nigeria is among the countries with the highest childhood LRTI burden of morbidity and mortality (Walker et al., 2013), studies on indoor air exposures in Nigeria or other Sub-Saharan African countries are few. In addition, methodological differences and variations in study population makes comparisons very difficult. Nevertheless, it is a very promising research field that requires more longitudinal and/or multi-omics designs to clarify the exact role of the microbiota in the development of LRTI among under-five children. Our data is infact generalizable to the entire Nigerian population of under-five children as access to the admission beds is not dependent on socioeconomic status, ethnic group, or family background. All children visiting the health centres for LRTI are admitted upon the doctor's recommendation for treatment. Novel molecular research methods might enable us to design innovative ways of prevention and treatment of diseases, because by altering the microbiota of the built environment and/or individuals it might be possible to intervene in pathways leading to disease.

A major strength of our study is the application of a culture-independent high-throughput approach to characterize bacterial and fungal taxonomic composition in the residential environment. These powerful molecular methods provide a detailed description of the microbiota in environmental samples, revealing difficult to culture taxa, with high level of precision, accuracy and sensitivity compared culture-based method. Also, several studies have made use of active airborne sampling (Oluwakemi Omolola, 2018; Setlhare et al., 2014) and passive airborne sampling using petri dishes (Ana et al., 2013; Sule et al., 2017), but the current study is the first to apply the electrostatic dust collectors (EDCs), that better accounts for the extended exposures occurring at home, for microbiome analysis in relation to LRTI among under-five children. Studies assessing different airborne sampling methods demonstrated that dust that settles on a standard sampler surface located at a significant level above the floor (such as the EDC) is a closer representative of the actual airborne exposure (Frankel et al., 2012; Wurtz et al., 2005) than house dust. In addition, the clinical outcomes used in the study, particularly for the case definition, with the diagnosis of LRTI based on chest radiography was a major strength as this help minimize the risk of misclassification.

There are also some limitations in our study. We used amplicon sequencing to characterize microbial taxonomic composition in airborne dust samples. Due to technical limitation of amplicon sequencing and potential variation in marker-gene copy-number, we can only characterize and quantify the microbiota reliably and comparable to genus level, rather than more taxonomically resolved species and strain levels. To address this, a less robust system for species identification was employed based on majority voting in BLAST to NCBI NR database. It is common that species within a genus or even strains from the same species could have different virulence factors thus posing a different human health risk. More taxonomically resolved techniques, such as shotgun metagenomics, sequencing could improve the identification accuracy for future indoor microbiome surveys, but environmental (non-microbial) DNA presence may require very deep sequencing at already intrinsically higher costs per sample. Another limitation of our study is the use of respiratory signs and symptoms to define LRTI among community controls which could have introduced some outcome definition bias. This was minimized by actively engaging a pediatrician in the assessment of the controls. Our inability to investigate the viral composition in the samples was an additional limitation. Also, there are other important confounders such as age of the building, daycare attendance, ventilation, indoor temperature and relative humidity etc. that were not included in the multivariable adjustment analyses which could have led to residual confounding. We hope that future studies would close this gap and provide epidemiological evidence of the contribution to LRTI of airborne viruses in homes of children under the age of five years.

5. Conclusions

Our study suggests that childhood exposure to certain indoor airborne bacterial and fungal communities is associated with LRTI among children under the age of five years. Both bacterial and fungal taxonomic abundance as well as community structure differed

significantly between homes of under-five children with and without LRTI. The bacterial phyla; *Deinococcota*, and *Bacteriodota,* and fungal phyla*; Basidiomycota* are indicative for a consistent protective effect on LRTI while the fungi; *Ascomycota* stood out as an independent risk factor for LRTI among children under the age of five years.

Credit authors statement

Conceptualization, Writing – original drafted preparation, statistical analysis - A.G.F.; L.A.M.S, R.N.N, and N.J. participated in study design and conceptualization; L.A.M.S, A.B, I.M.W and W.V.K reviewed the study instruments, guided the wetlab and bioinformatic statistical analysis; L.A.M.S, R.N.N, A.B, I.M.W and N.J provided extensive review of the drafts. All authors have read and agreed to the final version of the manuscript.

Support statement

The study was supported by the European Respiratory Society – ERS Short-term Research Fellowship April 2020 (STRF April 2020)-STRTF202004-00799. Also, we acknowledge the support received from the College of Health (CHS) PhD Scholarship of the University of KwaZulu-Natal, South Africa and the Institute of Risk Assessment, Utrecht University The Netherlands.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgements

We acknowledge the support received from the College of Health (CHS) PhD Scholarship of the University of KwaZulu-Natal, South Africa and the Institute of Risk Assessment, Utrecht University, The Netherlands. We also appreciate the contributions of the IRAS laboratory technicians (Gijs van Riel, Hadassa van Hoorn, Peter Scherpenisse, Jack Spithoven and Monique Tersteeg - Zijderveld) who assisted with DNA extractions and Jack Spithoven for providing assistance with the transportation of the EDCs.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envres.2023.116063.

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