

Next-generation DNA sequencing reveals that low fungal diversity in house dust is associated with childhood asthma development

Abstract Dampness and visible mold in homes are associated with asthma development, but causal mechanisms remain unclear. The goal of this research was to explore associations among measured dampness, fungal exposure, and childhood asthma development without the bias of culture-based microbial analysis. In the low-income, Latino CHAMACOS birth cohort, house dust was collected at age 12 months, and asthma status was determined at age 7 years. The current analysis included 13 asthma cases and 28 controls. Next-generation DNA sequencing methods quantified fungal taxa and diversity. Lower fungal diversity (number of fungal operational taxonomic units) was significantly associated with increased risk of asthma development: unadjusted odds ratio (OR) 4.80 (95% confidence interval (CI) 1.04–22.1). Control for potential confounders strengthened this relationship. Decreased diversity within the genus *Cryptococcus* was significantly associated with increased asthma risk (OR 21.0, 95% CI 2.16–204). No fungal taxon (species, genus, class) was significantly positively associated with asthma development, and one was significantly negatively associated. Elevated moisture was associated with increased fungal diversity, and moisture/mold indicators were associated with four fungal taxa. Next-generation DNA sequencing provided comprehensive estimates of fungal identity and diversity, demonstrating significant associations between low fungal diversity and childhood asthma development in this community.

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Practical Implications

Early life exposure to low fungal diversity in house dust was associated with increased risk for later asthma development in this low-income, immigrant community. No individual fungal taxon (species, genus, or class) was associated with asthma development, although exposure to low diversity within the genus *Cryptococcus* was associated with asthma development. Future asthma development studies should incorporate fungal diversity measurements, in addition to measuring individual fungal taxa. These results represent a step toward identifying the aspect(s) of indoor microbial populations that are associated with asthma development and suggest that understanding the factors that control diversity in the indoor environment may lead to public health recommendations for asthma prevention in the future.

Introduction

Growing evidence suggests that the development of asthma is associated with microbial exposures (Heederik and Von Mutius, 2012; Mendell et al., 2011). Although the specific microbial exposure routes and causal agents are not clear (Tischer and Heinrich, 2013), qualitative assessments of dampness and visible

mold in homes have been documented as consistently associated with asthma development (Mendell et al., 2011; Pekkanen et al., 2007; Quansah et al., 2012). Dampness can also contribute to changes in the indoor microbial population (Hyvärinen et al., 2002; Pitkäranta et al., 2011). The traditional fungal measurement technique of culturing has not identified consistent associations between microbial populations and asthma and

severely underestimates the quantity and diversity of microorganisms (Bridge and Spooner, 2001; Douwes and Pearce, 2003). The lack of adequate quantitative fungal sampling and analysis techniques for use in homes has been cited as a limitation to linking indoor fungal exposure with respiratory outcomes (Douwes and Pearce, 2003; Hamilos, 2010), and this lack of techniques prevents the development of thresholds to define health-related microbial exposure limits and identify effective interventions.

Taxonomic analysis via next-generation DNA sequencing of ribosomal RNA-encoding genes represents an underutilized opportunity to quantitatively study fungal populations in the residential environment in relation to asthma development and dampness or visible mold growth. Sequencing of the internal transcribed spacer (ITS) region of ribosomal fungal DNA allows identification down to the species level (Schoch et al., 2012). DNA sequencing of the fungal ITS region provides accurate, quantitative information about the diversity of a fungal community not available from culture-based analysis, or even quantitative polymerase chain reaction (qPCR) assays. Decreased microbial diversity has previously been associated with asthma development through culturing (Ege et al., 2011), although an ITS DNA sequence-based study of fungal diversity and asthma development has not yet been conducted.

In this study, next-generation DNA sequencing of fungal ITS regions was utilized to describe fungal diversity and species relative abundances in house dust collected at 12 months of age of children later determined to be asthmatic or non-asthmatic. Dust samples and data on home environmental characteristics and health outcomes used in this nested case-control study were obtained from the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) prospective birth cohort study (Bradman et al., 2005; Eskenazi et al., 2003). Statistical analyses examined relationships between fungal diversity/species abundance and childhood asthma development, as well as between fungal diversity/species abundance and dampness/mold in homes.

Methods

This method section provides an overview of the study cohort, indoor dust sampling, fungal analyses, and statistical methods to determine associations among microbial populations, asthma development, and moisture. Detailed methods are available online in the Supporting Information at [http://onlinelibrary.wiley.com/journal/10.1111/\(ISSN\)1600-0668](http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1600-0668).

Study population

The CHAMACOS birth cohort study enrolled 601 predominantly low-income, Latina pregnant women

living in the Salinas Valley, California, USA. Of the 601 enrolled, 526 (88%) delivered surviving singletons. By the 7-year visit, children were lost to follow-up for the following reasons: 72 moved, 59 refused, 24 could not be traced, 21 could not schedule a visit, and two became deceased (Bouchard et al., 2011). Of the remaining 348 participants, 292 (84%) had available dust collected from the 12-month home visit for possible inclusion in this analysis.

For this analysis, asthma cases and controls were selected based on asthma status at age 7 years. Children were defined as having asthma if a maternal interview indicated current asthma symptoms and either currently prescribed asthma medication or prior physician asthma diagnosis. All 13 asthma cases with available dust from the 12-month home visit were included, plus 28 randomly selected controls without asthma, frequency matched by sex (Table 1). Chi-square analysis revealed that the 28 representative control homes did not differ significantly from the original cohort of singleton births based on maternal education, poverty category, or mother's birth country. However, the chi-square test for mother's birth country may not be valid due to too few homes in some categories. The institutional review board at University of California, Berkeley, approved the study, and written informed consent was obtained.

Home visit and dust collection

The home visit at enrollment and 12-months postpartum included maternal interview, environmental assessment, wall moisture readings, and dust collection. Dust was collected using a high volume surface sampler (HVS3) vacuum cleaner (Lewis et al., 1994) with a MediVac dust sampling head (Medivac Plc,

Table 1 Demographic information for the 41 children in this study

Characteristic	Case <i>n</i> (%)	Control <i>n</i> (%)	Total <i>n</i> (%)
Household income			
At or below US poverty line	11 (85)	20 (71)	31 (76)
Above US poverty line up to 200%	2 (15)	8 (29)	10 (24)
>200% US poverty line	0 (0)	0 (0)	0 (0)
Mother's country of birth			
Mexico	13 (100)	24 (86)	37 (90)
US	0 (0)	3 (11)	3 (7.3)
Other	0 (0)	1 (3.6)	1 (2.4)
Mother's education level			
Up to 6th grade	8 (62)	15 (54)	23 (56)
7th–12th grade	4 (31)	8 (29)	12 (29)
High school graduate or more	1 (7.7)	5 (18)	6 (15)
Child's gender			
Male	4 (31)	7 (25)	11 (27)
Female	9 (69)	21 (75)	30 (73)

US, United States.

Wilmslow, Cheshire, UK) from the main living area, child's sleeping area, and kitchen. Dust was collected from a 1 m² area vacuumed for at least 2 min using four double passes of the surface. After collection, samples were placed on ice with a desiccant and then transferred to the laboratory for storage at -80°C.

Moisture was quantitatively measured from the middle of three walls, a window corner, and any suspected damp areas in the living area and child's sleeping area using a CT100 pinless moisture meter (Electrophysics, Dutton, ON, Canada). Moisture meters from different manufacturers may have different absolute reading values on gypsum board (Harriman, 2008), and the moisture content readings on drywall or plaster using this device are on a relative scale. Thus, our analysis included a range of moisture value thresholds to demarcate damp walls, including readings of 17, 21, and 24. Homes with any reading above these thresholds were characterized as having damp walls and were analyzed for associations with microbial growth and asthma. Qualitative moisture indicators included visible mold growth, water damage, leaks under the kitchen sink, peeling paint, rotting wood, and musty odor. Other collected information included income, smoking indoors, indoor pets, other occupants, and season of visit (rainy/dry).

Fungal analysis

DNA was extracted (Yamamoto et al., 2012) from 10 mg of dust and amplified with multiplexed ITS1F and ITS4 primers (Larena et al., 1999; Manter and Vivanco, 2007). Unincorporated primers and PCR reagents were separated from PCR amplicons using the UltraClean 96 well PCR Clean-Up Kit (Mebio Laboratory, Carlsbad, CA, USA), and amplicons were quantified using the Quant-iT PicoGreen assay (Invitrogen, Carlsbad, CA, USA) prior to normalizing the concentrations in all samples and pooling. Pooled DNA was further purified with Angencourt Ampure beads (Beckman Coulter, Brea, CA, USA) and sequenced on 1/8 of a plate on the 454 GS FLX Titanium DNA sequencing platform (454 Life Sciences, Branford, CT, Brea, CA, USA) at the Duke University Genome Sequencing and Analysis Core Resource. PCR no-template controls and laboratory negative controls (no dust added) produced in parallel with sample preparation and DNA extraction did not amplify. No DNA was detected in these control samples when analyzed by the Quant-iT PicoGreen DNA assay or by gel electrophoresis. Sequences have been archived in the European Nucleotide Archive with accession number ERP002369.

Five allergenic fungal species (*Aspergillus fumigatus*, *Alternaria alternata*, *Cladosporium cladosporioides*, *Epicoecum nigrum*, and *Penicillium* spp.), total bacteria, total fungi, and shed human skin cells were

measured with qPCR, with additional details in the Supporting Information.

For diversity analyses, the bioinformatics analysis toolkit QIIME, version 1.5 (Caporaso et al., 2010) was used to process DNA sequencing data. Sequences were trimmed if the read length was less than 300 bp or if the read quality score was less than 20. All sequences containing ambiguous bases and sequences unassigned to a multiplex identifier (MID) were removed prior to denoising. After denoising (Quince et al., 2011), sequences were clustered using uclust (Edgar, 2010) at 97% similarity. For rarefaction curve production and α diversity (within-sample diversity) analysis, the operational taxonomic unit (OTU) table was trimmed to 450 reads per sample (three samples with <450 reads were excluded), and the number of observed species was determined for each sample (in addition to Fisher's α , Shannon diversity index, and Chao1 richness estimator). For β diversity (between sample diversity) and principal coordinate analysis (PCoA), all available quality-trimmed reads were utilized to calculate the Morisita Horn (Horn, 1966) (non-phylogenetic) distance. Results were assessed through PCoA plots and analysis of similarity (ANOSIM, available through QIIME) to determine the statistical significance of clustering.

For taxonomic assignment, the RDP pipeline initial process (Cole et al., 2009) was used to trim the raw sequence read file with the equivalent quality and length criteria specified above, and BLASTn-based annotation (Altschul et al., 1990) was performed against a database containing all fungal sequences identified to the rank of species (Nilsson et al., 2009). Multilevel taxonomic identification was made at all taxonomic ranks by FHiTINGS, version 1.1 (Dannemiller et al., 2013). The values at all taxonomic levels from the FHiTINGS files were used to calculate the relative abundance for each identification at the species, genus, or class rank. Also, to estimate the absolute concentration of each identified species per gram of dust, relative abundance values were multiplied by the total fungal spore quantities per mg of dust, as determined by qPCR with universal fungal primers, to produce absolute abundance values.

Diversity within genera with at least 10 species and classes was determined using the FHiTINGS output. Only samples with at least 1000 sequences per sample were included in this analysis for normalization. The number of different species identified by at least one sequence was determined within each genus and class.

Statistical analysis

SAS, version 9.2 (SAS Institute, Inc., Cary, NC, USA), was used for statistical analysis, with significance defined as $P < 0.05$. Fungal diversity differences were assessed with two sample t -tests. Odds ratios (ORs) for

associations between asthma status, fungal diversity, and household/demographic factors were calculated on dichotomous independent variables (continuous variables dichotomized at median value). Binary variables were used to avoid concerns with normality of the data distributions and also due to the semiquantitative nature of 454 pyrosequencing taxon abundance data (Amend et al., 2010). Potential confounding variables were selected for analysis *a priori* (Table 2).

Associations between fungal taxa and asthma or moisture were first analyzed by calculating ORs based on dichotomized variables. Next, these findings were adjusted for multiple comparisons using significance analysis of microarrays (SAMs) (Li and Tibshirani, 2011; Tusher et al., 2001), version 4.00a. While SAM was designed for gene expression analysis, here it was used to calculate the false discovery rate and *Q*-values for many taxonomic comparisons (species, genus, class) against case/control status and moisture indicators. A *Q*-value is similar to a *P*-value but is adjusted for multiple comparison testing. More details of this analysis are in the Supporting Information. Results were considered statistically significant after adjustment for multiple comparisons if *P* < 0.05 and *Q* < 0.05.

Results

Overview

Demographic information for the 13 asthma cases and 28 controls included in the study is shown in Table 1.

After quality trimming, 52 058 sequences total were included in the analysis, and the normalized samples contained 902 different operational taxonomic units (OTUs) combined. Fungal diversity was reported here as the number of species-level OTUs in samples normalized to 450 sequences and ranged from 29 to 142 fungal OTUs per sample with a median of 80.5. The coefficients of variation for the number of fungal OTUs of four replicates (different aliquots of the same dust samples) were 4%, 8%, 11%, and 13%, and the cumulative coefficient of variation was 9%. A total of 652 non-singleton species were identified by BLAST analysis, and 180 had at least 20 sequences total among all samples (Table S1). Replicate analysis for fungal taxonomic identifications appears in Figure S1 a–d.

Fungal diversity, asthma, and moisture

Lower fungal diversity in house dust was associated with increased risk of childhood asthma development by *t*-test (*P* = 0.04) (Figure 1a,b). The unadjusted OR of developing asthma with low fungal diversity (dichotomized at the median) was 4.80 (95% confidence interval (CI) 1.04–22.1) (Table 2). Other fungal α diversity (within-sample diversity) measures, including Fisher’s α , Shannon diversity index, and Chao1 richness estimator (Table S2), also demonstrated lower diversity measurements in case versus control homes. In addition to diversity for all fungi, species diversity within abundant fungal genera and classes were also examined (Table S3). Only decreased *Cryptococcus* genus diversity was

Table 2 Unadjusted and adjusted odds ratios for the association of potential risk factors with asthma development and with low fungal diversity, and for the association of low fungal diversity with asthma development

Potential Risk Factor ^a	n (%)	Unadjusted		Adjusted	
		Potential risk factor OR for low fungal diversity ^b OR (95% CI)	Potential risk factor OR for asthma development OR (95% CI)	Potential risk factor OR for asthma development, adjusted for low fungal diversity OR (95% CI)	Low fungal diversity and asthma development, adjusted for potential risk factor OR (95% CI)
Low fungal diversity	–	–	4.80 (1.04–22.1)	–	–
Rainy season ^c	21 (55)	1.24 (0.34–4.45)	0.45 (0.11–1.80)	0.36 (0.08–1.65)	5.52 (1.12–27.3)
Smoking inside home ^d	0 (0)	–	–	–	–
Pets ^e	5 (13)	1.59 (0.24–10.8)	1.53 (0.22–10.6)	1.35 (0.17–10.6)	4.74 (1.03–21.9)
Other children ^f	19 (50)	1.23 (0.35–4.41)	1.00 (0.25–3.93)	0.92 (0.21–3.94)	4.83 (1.04–22.3)
Below poverty line ^g	30 (79)	0.53 (0.11–2.60)	4.05 (0.44–37.4)	6.28 (0.6–65.3)	6.21 (1.25–31.0)
Moisture ^h (>17)	15 (39)	1.25 (0.34–4.59)	1.14 (0.28–4.60)	1.06 (0.24–4.68)	4.79 (1.04–22.1)
Moisture ^h (>21)	12 (32)	0.61 (0.15–2.44)	0.63 (0.14–2.93)	0.73 (0.14–3.70)	4.68 (1.01–21.7)
Moisture ^h (>24)	5 (13)	0.21 (0.02–2.07)	1.53 (0.22–10.6)	3.47 (0.35–34.1)	6.43 (1.15–35.8)
Visible mold ⁱ	11 (29)	0.77 (0.19–3.16)	0.75 (0.16–3.53)	0.8 (0.16–4.16)	4.76 (1.03–22.0)
≥2 moisture indicators ^j	13 (34)	0.79 (0.21–3.03)	0.53 (0.12–2.46)	0.54 (0.11–2.73)	4.77 (1.02–22.2)

^aStatistically significant (*P* < 0.05) odds ratios are in bold. ^bLow fungal diversity was defined by creating a dichotomous variable separated by the median (80.5 fungal OTUs). ^cRainy season was defined as sampling between November 1 – April 30. ^dSmoking was defined as at least one person smoking indoors as reported by the resident. While 2 (5.3%) case and 2 (5.3%) control children were exposed to smoke, there was no smoking inside any of the homes. ^ePets included birds or mammals living inside the home within one year, and no homes had cats. ^f‘Other children’ represents at least two other children under age 12 years living inside the home as reported by the resident. ^g‘Below poverty line’ represents homes with incomes below the US government-defined poverty line. ^h‘Moisture’ variables represent a dichotomous variable for any moisture meter reading anywhere in the living area or child’s sleeping area that exceeded the indicated threshold (17, 21, or 24). ⁱ‘Visible mold’ represents moderates or extensive growth. ^jThe sum of two or more moisture indicators represents qualitative moisture indicators including peeling paint, water damage, rotting wood, musty odor, water leak in the kitchen, or visible mold growth.

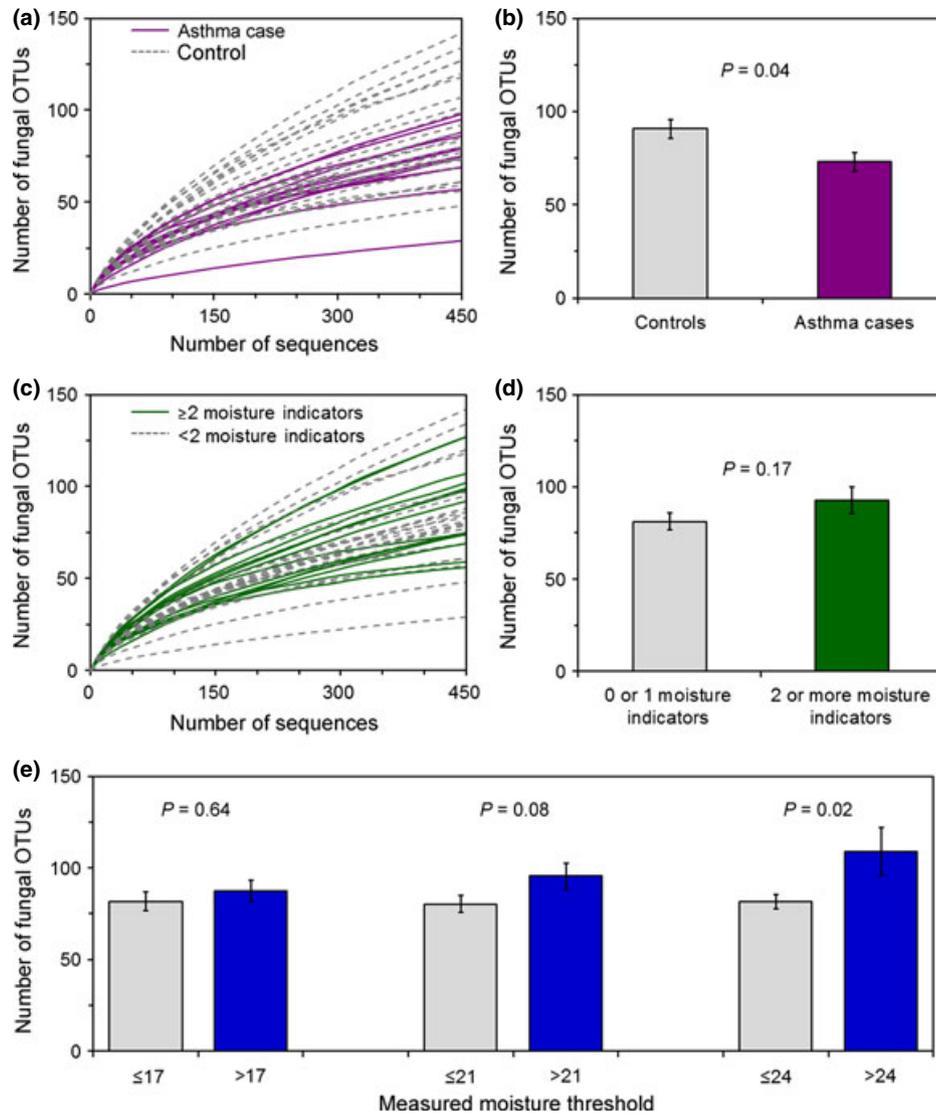


Fig. 1 Fungal diversity associations with asthma development and with the presence of moisture indicators. (a,b) Rarefaction analysis for fungi in case and control homes ($n = 12$ for asthma cases, $n = 26$ for controls), with values summarized in a bar graph. (c,d) Rarefaction analysis for fungi in houses with two or more qualitative moisture indicators ($n = 13$ for homes with two or more moisture indicators, $n = 25$ for homes with fewer than two moisture indicators), with values summarized in a bar graph. Moisture indicators included peeling paint, water damage, rotting wood, musty odor, water leak in the kitchen, or visible mold growth. (e) Bar graph summary of rarefaction analysis for measured moisture at three threshold values (17, 21, and 24). The maximum moisture reading anywhere in the living area or child's sleeping area was used. For the three thresholds of 17, 21, and 24, the respective numbers of homes with low moisture were $n = 23$, 26, and 33, and the respective numbers of homes with high moisture were $n = 15$, 12, and 5. Rarefaction curves appear in Figure S4. Error bars represent standard errors. All OTUs are defined at 97% similarity

statistically significantly associated with an increase in asthma risk (OR 21.0, 95% CI 2.16–204). Increased diversity within any genus or class was not associated with increased asthma risk.

Increases in some quantitative and qualitative measures of moisture were associated with increased fungal diversity. Homes with two or more of six qualitative moisture indicators showed a trend toward higher fungal diversity ($P = 0.17$) (Figure 1c, d). Water damage, rotting wood, and musty odor were present in few (<5) homes with fungal diversity values and were not statistically associated with fungal diversity. Among the variables visible mold

growth, water leak in the kitchen, and peeling paint (Figures S2 and S3), water leak in the kitchen was associated with increased fungal diversity ($P = 0.04$). Dust sampling during the rainy season did not affect fungal diversity (Figure S2). For quantitative wall moisture measurements, presence in a home of levels exceeding each of the three thresholds of 17, 21, and 24 was associated with increased fungal diversity, and this difference was significant for the most stringent threshold (>24 , $P = 0.02$) (Figures 1e and S4). Finally, neither moisture indicators nor sampling during the rainy season were associated with asthma status (Table S4).

Other asthma risk factors

Potential confounders of the relationship between fungal diversity and asthma, selected *a priori*, were all not significantly associated with either asthma development or fungal diversity in logistic models (Table 2). None of these variables showed positive confounding of the relationship between fungal diversity and asthma development. Rainy season, poverty, and measured moisture (>24) showed a tendency toward negative confounding (partially obscuring the relationship between asthma development and fungal diversity) (Table 2). Including these three variables individually in logistic regression models increased the OR for asthma and fungal diversity between 15–34%, and moved the OR for the potential confounder and asthma farther from the null. In the adjusted models, 95% CIs for low fungal diversity and asthma always excluded 1.0, while those for the potential confounding variables did not.

Visible mold growth and case status modified the relationship between moisture presence and fungal diversity. Figure 2 shows that in homes without visible mold growth, increased moisture content was associated with statistically significantly increased fungal diversity (Figure 2a). In homes with visible mold growth, increased moisture content was associated with non-significantly decreased fungal diversity (Figure 2b). In control homes, moisture content was

associated with statistically significantly increased fungal diversity (Figure 2c), whereas in case homes moisture content had no association with fungal diversity (Figure 2d). Similar trends were also present with *Cryptococcus* diversity (Figure S5), and *Cryptococcus* diversity was highly related to the total number of fungal OTUs (Figure S6). Overall, these results indicate that unknown factor(s) that partially obscure the relationship of increased diversity with increased moisture may be present in homes with visible mold and in asthma case homes.

Fungal taxa, asthma, and moisture

Quantitative PCR measurement of total fungal spore equivalents, bacterial genomes, human cells, and five allergenic fungal species was not significantly related to asthma (Table S5). By pyrosequencing, the most abundant (>1%) species were examined for differences in relative abundance between case and control homes (Figure S7a). While average differences were observed, such as for human-associated *Candida intermedia* and *Malassezia globosa*, none were statistically significant. Using total fungal qPCR results to transform data into absolute concentrations did not significantly alter the results (Figure S7b,c).

ORs for asthma and all individual species (Table S6), genera (Table S7), and classes (Table S8 and

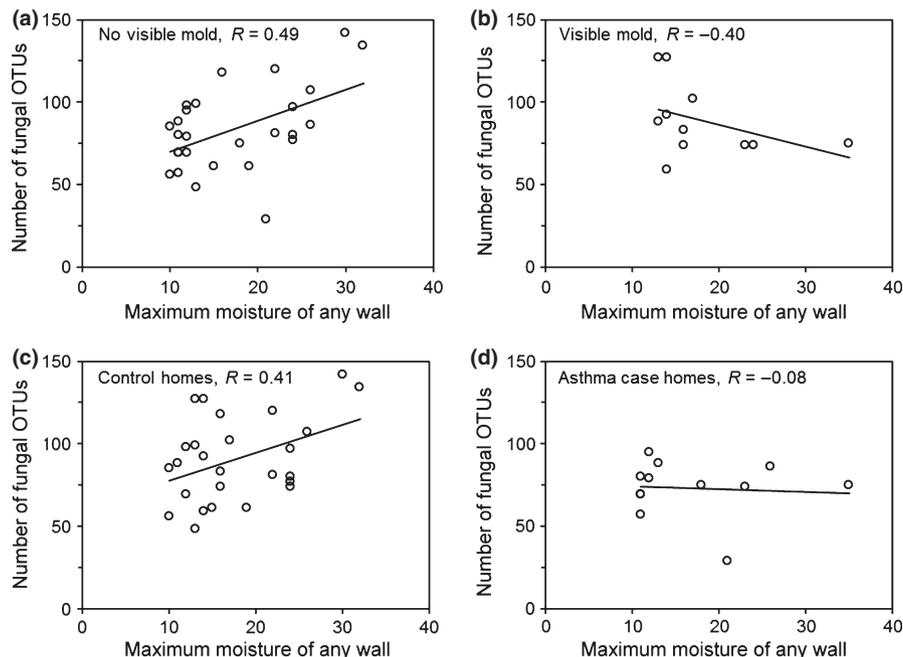


Fig. 2 Pearson correlation coefficients and graphs comparing maximum moisture content of any wall in the home compared with number of fungal OTUs in floor dust. Graphs are (a, b) stratified by homes without ($n = 27$) and with ($n = 11$) visible mold growth and (c, d) stratified by asthma control ($n = 26$) and case ($n = 12$) homes. All OTUs are defined at 97% similarity. Sequences were normalized to 450 sequences per sample. The respective Pearson correlation coefficient P -values were (a) 0.009, (b) 0.22, (c) 0.04, and (d) 0.81

Figure S8) showed no statistically significant positive associations between any specific fungal taxa and asthma risk. After adjustment for multiple comparisons, only *Aureobasidium pullulans* showed a statistically significant negative association (OR 0.19, 95% CI 0.04–0.87) (Table S9). Finally, analysis of fungal population β diversity (between samples) showed that fungal communities in asthma case and control homes shared many similarities and no clear, consistent differences (ANOSIM $P = 0.10$) (Figure S9a).

The species *Cryptococcus uzbekistanensis*, *Cryptococcus albidus*, *Coniosporium apollinis*, and the class Ustilaginomycetes were significantly positively associated with mold/moisture indicators after adjustment for multiple comparisons (Table S9). No differences in fungal population β diversity were found with these moisture or mold measures (Figures S9 and S10).

Discussion

This is the first study to our knowledge to examine fungal diversity in house dust and associations with later asthma development using next-generation DNA sequencing in a prospective, nested case–control study. In this population, lower number of fungal OTUs in house dust was strongly and significantly associated with increased risk of later asthma development, and this was strengthened with control for potential confounders. Previous studies have associated lower bacterial diversity with increased asthma risk (Ege et al., 2011), and one previous study examined nine fungal species by culturing and found a similar trend (Ege et al., 2011). This trend was seen even though culturing underestimates fungal diversity (Bridge and Spooner, 2001).

Moisture and visible mold

Moisture was associated with increased fungal diversity in house dust, as found previously (Pitkäranta et al., 2008). Moisture has also been positively associated with asthma (Mendell et al., 2011; Pekkanen et al., 2007). This relationship may be partially obscured by the possible negative confounding seen here among asthma development, moisture, and fungal diversity. Although high measured wall moisture (Figure 3) had a non-significant positive unadjusted association with asthma development, it was also inversely associated with low fungal diversity. In turn, low fungal diversity was strongly, positively associated with asthma development. However, when both measured wall moisture and low fungal diversity were included in the asthma model (Figure 3), the adjusted ORs for each increased by 127% and 34%, respectively, relative to the unadjusted ORs. Thus, there is a suggestion of negative confounding that may partially obscure both the relationship between low fungal

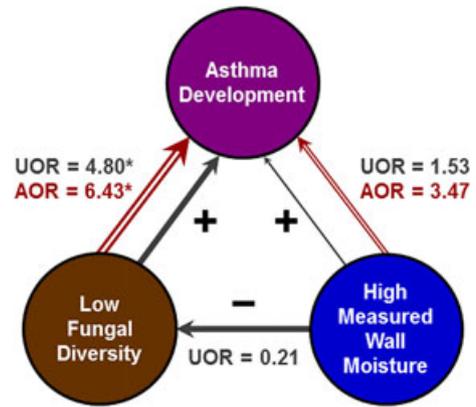


Fig. 3 Negative confounding of the relationship between low fungal diversity and asthma development by measured home moisture. The unadjusted models are represented in solid gray arrows, and the adjusted model is represented in open red arrows. Negative confounding by high measured wall moisture (>24) reduced the association seen in unadjusted models for low fungal diversity and asthma development. This was because measured wall moisture had a positive association with asthma development but a negative association with low fungal diversity. With adjustment for measured wall moisture, the association between low fungal diversity and asthma development increased by 34% and that between measured wall moisture and asthma development increased by 127%. UOR, unadjusted odds ratio (solid gray), AOR, adjusted odds ratio (open red), * $P < 0.05$

diversity and asthma, as well as the relationship between moisture and asthma.

Visible mold growth modified the relationship between moisture and fungal diversity. Potential hypotheses include that visible mold growth with high moisture releases antifungal metabolites (Schalchli et al., 2011) that reduce fungal diversity in house dust, or that the number of fungal OTUs decreases due to the dominance of one or more actively growing species (Adams et al., 2013a). In asthma case homes, there may be growth inhibitors present or diversity may be reduced for other yet unknown reasons. These results suggest that homes with visible mold growth and homes of asthma cases may be similar in some way related to the prevention of an increase in microbial diversity with an increase in moisture.

Fungal exposure and increased asthma risk

Previous studies have focused on associating the presence of detrimental fungal species with asthma risk (Agarwal and Gupta, 2011; Arbes et al., 2007; Reponen et al., 2012; Stark et al., 2005). While multiple mechanisms may be important, here we found similar fungal taxa in asthma case and control homes, with no detrimental and one potentially protective species. Similarities in fungal taxa in house dust may be due to contributions from common sources, such as outdoor air (Adams et al., 2013b; Goebes et al., 2010)

and human shedding (Park et al., 2012). Although abundance of no fungal taxon was identified as an asthma risk factor, reduced diversity within *Cryptococcus* was associated with increased asthma risk. *Cryptococcus* is a genus of yeasts associated with soil, humans, and animals, and some species are pathogens (Levitz, 1991). Here, *Cryptococcus* was the most prevalent genus, and diversity within *Cryptococcus* was associated with overall diversity. This genus is in the Basidiomycota phylum, which is a source of aeroallergens (Lehrer et al., 1994) but until recently lacked allergen research compared with Ascomycota due to culturing difficulties (Simon-Nobbe et al., 2008). Subclinical infection with *Cryptococcus neoformans* has been suggested to have a role in the pathogenesis of asthma (Goldman and Huffnagle, 2009; Goldman et al., 2006). Further research is needed to explore the association of this genus with asthma development.

Together, these findings advance knowledge about household factors associated with increased asthma risk. Fungal concentration and diversity are not necessarily related, and the dominance of a taxon within a population may affect human health due more to decreased diversity than the related increase in the taxon's absolute concentration. Our results point to the 'biodiversity hypothesis' (Von Hertzen et al., 2011) because a decrease in fungal diversity was the main factor consistently associated with increased asthma risk. Visible mold growth and household moisture may also increase risk of asthma development; if these two factors work through a reduction in fungal diversity as in Figure 2b, then perhaps both need to be present simultaneously to have an effect, as found previously (Gunnbjörnsdottir et al., 2003). Moisture/mold and asthma may also be linked through diversity of specific taxa (perhaps *Cryptococcus*, and/or others). These findings give new direction to the study of microbes in homes and, if validated in future studies, represent a step toward eventual public health recommendations to reduce asthma development. Further research aimed at understanding household factors that control diversity in the indoor environment will also be necessary.

Additional considerations

This study was conducted in a low-income, immigrant community of predominantly Mexican descent. The novel results should be confirmed in future studies with larger cohorts and in groups with different demographics. One characteristic of populations of Mexican descent is low overall asthma prevalence rates compared with other racial/ethnic groups (Arif et al., 2003; Lara et al., 2006). These asthma rates for those of Mexican descent are reported at approximately 5% in the United States (Akinbami et al., 2011, 2012), compared with the

asthma prevalence rate of those of Puerto Rican descent in the United States of 16.1% (Akinbami et al., 2012). In California, the childhood current asthma prevalence rate among all Hispanics is 5.1% (95% CI 3.6–7.1%) (CDC, 2010). Using data collected from Salinas, CA (Vogt et al., 2008), we also estimate the asthma prevalence of those of Mexican descent in the area to be 4.4%. The overall asthma prevalence rate in the CHAMACOS cohort of 292 was 4.5%, which is consistent with previously reported values.

Humans are exposed to house dust through inhalation, dermal, and oral exposure routes (Butte and Heinzow, 2002). Room occupants resuspend floor dust, which contributes to the microbial population in the air (Hospodsky et al., 2012). While much literature has focused on inhalation exposure for allergic asthma (Cockcroft et al., 2007), reduced gut microbial diversity is also associated with hypersensitivity disease (Bisgaard et al., 2011; Sjögren et al., 2009) and autoimmune disease (Markle et al., 2013), and skin contact may be an important exposure route for asthma development (Redlich, 2010). All three exposure routes may be additive (Marsella et al., 2006) and may contribute to the relationship between low fungal diversity exposure early in life and later asthma development, possibly through immune system training (Heederik and Von Mutius, 2012).

Strengths and limitations

Strengths of this study include the prospective, nested case-control design with early exposure measurements. The previous study estimating fungal diversity by culturing nine species was cross-sectional (Ege et al., 2011). The largely homogeneous, agricultural community of similar socioeconomic status in a defined geographic region reduced variability that might otherwise obscure associations. Previously, DNA-based techniques (qPCR) have found associations between fungal exposures and asthma development (Reponen et al., 2011, 2012). However, qPCR-based techniques require *a priori* selection of species or taxa of interest, even though many fungal species that bind human IgE have not yet been clinically recognized (Green et al., 2009) and the types of microorganisms that are responsible for immune system priming and development are not yet known. The thoroughness of a qPCR-based diversity analysis is also limited by the number of currently available assays. The 454 pyrosequencing method allows for comprehensive analysis of fungal communities, including diversity analysis and taxa-specific analysis without *a priori* selection of specific taxonomic groups. Given the large number of potential associations assessed, we also controlled for 'multiple testing' to reduce the likelihood of chance findings.

Limitations of this study include the small sample size, which limited statistical power and precision of estimates. However, the nested case-control design that included all available asthma cases in a larger prospective cohort enhances power and the ability to generate causal hypotheses. The relationship between fungal diversity and asthma was sufficiently strong to be detectable in this sample, although with wide confidence limits. The ability to explore confounding was also limited, but individual potential confounding variables appeared not to enhance this relationship, but actually to obscure it. Other potential confounders not addressed here may exist, and exposures to allergens or endotoxin should be considered in future studies. Larger sample sizes in future studies may result in increased ability to identify additional significant associations. This study was conducted in a homogeneous low-income community of Mexican descent, and future studies should also confirm the generalizability of these findings to other populations. Additionally, while pyrosequencing represents the most thorough fungal population analysis method reported to date, additional species at very low abundance may not have been detected. A previous study has raised concerns that differences in collected fungal biomass can artificially alter fungal diversity estimates (Adams et al., 2013a). However, in this study, fungal biomass determined by qPCR was not associated with the detected number of fungal OTUs. Floor dust sampling methods can affect the results of microbial measurements (Hyvärinen et al., 2006; Schram-Bijkerk et al., 2006), such that care should be taken with direct comparison of this study's quantitative results to other studies that may have used disparate sampling methods. Although these results require replication in a larger population with more rigorous confirmation of asthma status, these findings demonstrate novel relationships among childhood asthma development, fungal diversity, and moisture in homes.

Conclusions

Lower fungal diversity in homes of children at 12 months of age was associated with increased probability of later asthma development. While no individual fungal taxa were associated with increased risk of asthma development, low *Cryptococcus* diversity was associated with higher asthma risk. The association between high moisture and high fungal diversity has a tendency to partially obscure their true relationships with asthma development. Our results indicate that future research should expand from primarily exclusive study of individual fungal taxa in homes to also incorporate fungal diversity measurements. Improved understanding of household exposure to low fungal diversity and the associations with health outcomes may eventually inform preventive medicine and public health interventions

to reduce the burden of asthma on our society and especially on our children.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Reproducibility analysis for four duplicate sequence libraries, A, B, C, and D, and example Significance Analysis of Microarrays (SAM) plotsheet.

Figure S2. Rarefaction analyses for fungi in dust samples collected from homes during the rainy season and homes with and without visible mold growth.

Figure S3. Rarefaction analyses for fungi in dust samples collected from homes with and without a water leak and with and without peeling paint as moisture indicators.

Figure S4. Rarefaction analyses for fungi in dust samples collected from homes with and without the maximum moisture content of any wall recorded above a threshold.

Figure S5. Pearson correlation coefficients and graphs of maximum moisture content of any wall in the home compared to the number of *Cryptococcus* species in floor dust.

Figure S6. Pearson correlation coefficient and graph of the number of total fungal OTUs compared to the number of *Cryptococcus* species in floor dust.

Figure S7. The most abundant species (>1%) among all samples analyzed by average relative abundance in case and control homes.

Figure S8. Relative abundance of fungal sequences in case and control homes by rank of class.

Figure S9. Principal coordinate analysis graphs of fungal diversity in floor dust.

Figure S10. Principal coordinate analysis graphs of fungal diversity in floor dust.

Table S1. List of identified fungal species in all homes with at least 20 sequences total from all samples in order from most abundant to least abundant by number of sequences identified.

Table S2. Additional fungal α diversity measures calculated in QIIME with the mean values in asthma case and control homes and *P*-values.

Table S3. Odds ratios for low fungal diversity with classes and prevalent genera in asthma case versus control homes.

Table S4. Summary of moisture indicators found in homes and unadjusted odds ratios for moisture indicators and childhood asthma.

tors and childhood asthma.

Table S5. Odds ratios for qPCR measurements in 13 asthma case versus 28 control homes.

Table S6. Odds ratios for fungal species abundances in asthma case versus control homes.

Table S7. Odds ratios for fungal genus abundances in asthma case versus control homes.

Table S8. Odds ratios for fungal class abundances in asthma case versus control homes.

Table S9. Odds ratios for statistically significant fungal species, genera, or classes in homes with and without two or more qualitative moisture indicators, visible mold growth, measured moisture at three threshold values (17, 21, and 24) and case/control homes.

Data S1. Methods.

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