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Characteristics of annual mold variations and association with childhood allergic symptoms/diseases via combining surveys and home visit measurements

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Abstract
The presence of dampness and visible molds leads to concerns of poor indoor air quality which has been consistently linked with increased exacerbation and development of allergy and respiratory diseases. Due to the limitations of epidemiological surveys, the actual fungal exposure characteristics in residences has not been sufficiently understood. This study aims to characterize household fungal diversity and its' annual temporal and spatial variations. We developed combined cross-sectional survey, repeated air sampling around a year and DNA sequencing methods. The questionnaire survey was conducted in 2019 and 4943 valid cases were received from parents; a follow-up case-control study (11 cases and 12 controls) was designed, and onsite measurements of indoor environments were repeated in typical summer, transient season and winter; dust from floor and beddings in children' room were collected and ITS based DNA sequencing of totally 68 samples was conducted. Results from 3361 children without changes to their residences since birth verified the significant associations of indoor dampness/mold indicators and prevalence of children' reported diseases, with increased adjusted odd ratios (aORs) >1 for studied asthma, wheeze, allergic rhinitis and eczema. The airborne fungal concentrations from air sampling were higher than 1000 CFU/m^3 in summer, regardless of indoors and outdoors, indicating an intermediate pollution level. The DNA sequencing for dust showed the *Aspergillus* was the predominant at genus level and the *Aspergillus_penicillioides* was the most common at species level; while the fungal community and composition varied significantly in different homes and seasons, according to α and β diversity analyses. The comprehensive research methods contribute to a holistic understanding of indoor fungal exposure, including the concentrations, seasonal variations, community and diversity, and verifies the relations with children' adverse health outcomes. The study further elucidates the role of microbiome in human health, which helps setting health-protective thresholds and managing mold treatments in buildings, to promote indoor air quality and human well-beings.
Keywords
Residential environment; dampness and mold; airborne fungal concentration; DNA sequencing; Seasonal variation.
Practical Implications
This study reveals the indoor fungal exposure characteristics in residences through a combined cross-sectional survey, repeated air sampling around a year and DNA sequencing method. The findings reveal the seasonal variations of indoor fungal exposure and differences among homes, and further verify the associations with childhood allergic diseases/symptoms. This contributes to setting health-protective thresholds and managing mold treatments in buildings, and updating the air quality standards in future.

1 INTRODUCTION

Mold are ubiquitous microbial contaminants in buildings, and can grow once sufficient moistures and appropriate temperatures are present[1,2]. It is estimated that the proportion of buildings having indoor mold problems is ～45% in Europe, ～40% in the USA, ～30% in Canada and ～50% in Australia[3]. Recent requirements for more efficient use of energy in the operation and use of buildings have increased the thermal insulation and airtightness against air leakages, leading to a higher risk of mold presence in buildings[4-6].

People spend most of their time indoors, especially for children who are more susceptible to effects of environmental factors. It has been widely acknowledged that exposure to indoor mold is significantly associated with a wide range of adverse health effects, triggering from allergic reactions, simple irritations in the respiratory system and eyes, to more severe conditions such as asthma, hypersensitivity pneumonitis, allergic rhinitis and eczema, just to mention a few [7-10]. In US, it was estimated that 21% of the confirmed asthma cases were attributable to indoor mold [11]. Antova et al. [12] based on a PATY study from 12 countries (North America, Russia and ten Eastern and Western countries) among over 58000 children and reported that all countries had positive associations between respiratory diseases and household mold. Caliaud et al. [13] reviewed 61 recent publications regarding the visible mold or mold odor or quantitative assessment of culturable fungi or mold species; the results showed the significant associations between indoor mold exposure and the development and exacerbation of asthma and rhinitis in children. In addition, dampness, mold growth, and odor caused by the production of microbial volatile organic compounds (mVOC) can also have risks to cause multiple allergic and respiratory diseases [14,15]. Generally, the Institute of Medicine (IOM)[16], the World Health Organization (WHO)[17], as well as the enormous epidemiological research and meta-analyses [8,14,18,19], have provided clear and consistent evidences of associations of occupancy with damp or mold indoor environments and the manifestation of adverse respiratory and allergic symptoms such as development of new asthma, exacerbation of existing asthma, allergic rhinitis, and respiratory infections. However, majority of these studies are based on epidemiological surveys and the obtained results are referred to self-reported questions from questionnaires. As a result, the actual fungal exposure for children in homes and the solid evidences with adverse health outcomes are still insufficient, so that the safe exposure concentrations are not yet well established.

To respond to the limitations, the quantitative measures of observed mold and moisture damage in interior homes, concentrations of fungi in indoor air or dust have been explored, aiming to explore the exposure of microorganisms and/or their products and the relations with specific health effects. For example, some studies have provided the robust evidences of the close relationships between elevated environmental fungal spore count and asthma deterioration [20,21]. Through a meta-analysis, Sharpe et al. [8] found that exposure to elevated concentrations of *Aspergillus*, *Penicillium*, *Alternaria* and *Cladosporium* was associated with increased risk of asthma. Some panel studies [8,18,22,23] have also reported the positive associations between mold exposure (in particular exposure to *Penicillium*) in asthmatic children and an increased variability in peak expiratory flow rate, a higher asthma severity score, an increase in the number of days with symptoms or hospital visits. Using the quantitative EMRI (Environmental Relative Moldiness Index) values, Reponen et al. [24] showed that the adjusted odds ratio of developing asthma in children was 2.6 (95% CI 1.10–6.26) higher in homes with a high ERMI value (>5.2) than those with low ERMI value (<5.2). Vesper et al. [25] also found that children in homes with a higher average ERMI value (12.4) had much more severe asthma, which quantified the positive correlation between mold exposure and asthma occurrence and severity. Compared to cross-sectional surveys, these studies quantified the indoor mold exposure by home visit and measurements of related indices and provided the dose-response relationships between indoor mold exposure and the probability of adverse health effects. Whereas, it is known that the indoor fungi or mold exposures are affected by climate conditions (e.g., temperature, air movement and

humidity), outdoor bioaerosols [26-28], and change with space and time. Moreover, the building design functions (e.g. thermal insulation, airtightness), house characteristics (ages, locations, directions), and occupants' behavior that influence ventilation, heating and cooling, etc., all play important roles in affecting indoor fungal exposure [29-31]. In such cases, majority of the current onsite studies quantifying airborne fungal concentration are one-time sampling so that the stationary sampling may be significantly impacted by test conditions at that time [32]. Moreover, as only a small part (0.1%-10%) of the total airborne microorganisms could be cultivated [33] from air sampling, the culturable based method might possibly underestimate the actual concentrations in the air. Therefore, the representativeness for reflecting inhalation exposure characteristics for indoor fungi is disputable by short-term variability caused by indoor residents' activities and ventilation behavior. In addition, due to the limited repeatability, it has low validity for evaluating long-term exposure levels.

To overcome the aforementioned deficiencies of air sampling, some non-culture methods are developed. The high-throughput molecular techniques (e.g. 16S rDNA sequencing for bacteria, ITS sequencing for fungi, qRT-PCR, metagenomics) enable the provision of excellent insights into community structure, composition, and diversity of bioaerosols in indoor and outdoor environments [27,34-39].Taxonomic analysis via next-generation DNA sequencing of ribosomal RNA-encoding genes is therefore a good alternative to quantitatively study fungal populations [40]. In such context, this study developed a combined method of cross-sectional surveys based on questionnaires, repeated indoor air sampling in different seasons, and DNA sequencing from dust collection. The work is excepted to provide holistic understandings on the dampness/mold exposures in residence, its temporal and spatial variations, and thus evaluate the associations with children' targeted diseases, which are achieved by the following objectives:

1) to verify the associations between indoor dampness/mold exposure and children's specific diseases in residences based on questionnaire survey;

2) to reveal the quantitative dynamic variations of airborne fungal concentrations based on case-control designs and repeated onsite measurements in different seasons, as well as the fungal community and diversity through ITS based DNA sequencing;

3) to verify the significant associations between airborne fungal exposures and childhood allergic symptoms and diseases, depending on the employed comprehensive research method.

2 METHODS

The study was designed in two stage. A cross-sectional survey was conducted in homes with children in Chongqing at the first stage, to provide an overall understanding of household dampness and mold occurrence and children' health situations in residences. Given the limitations of qualitative understanding from the questionnaire-based investigation, a longitudinal survey was designed at the second stage. During this period, onsite measurements and inspections were conducted in investigated homes. The active air sampling and dust collections were employed to quantify domestic airborne fungal contaminations and composition, providing more illuminating understanding of bioaerosols in the residences. The design flow diagram is illustrated in Figure 1. The details for performing the study are explained in following parts.

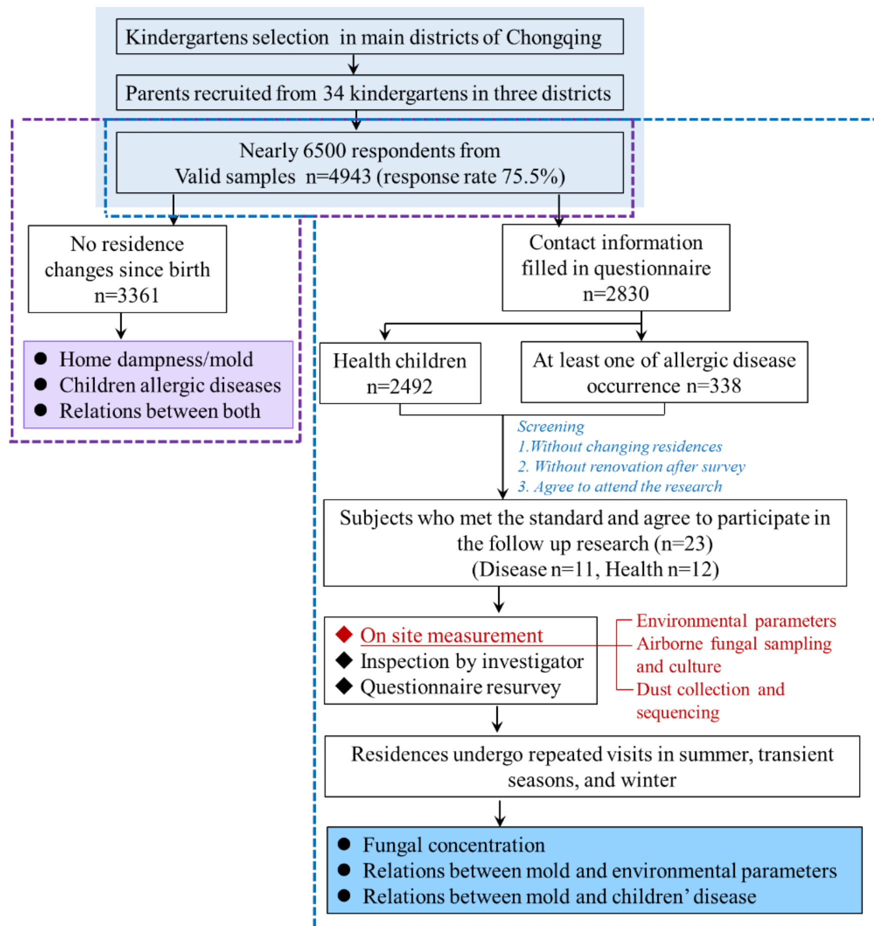


Figure 1. Flow diagram of questionnaires survey and follow-up measurement.

2.1 Cross-sectional survey in children' homes

Following up the large-scale survey of household environments and children health in residences during 2010-2011 (China Children Home Health program, CCHH)[41], a repeated questionnaire survey was conducted in Chongqing in 2019. Through the same multistage stratified random sampling process as the first period, 34 kindergarten schools from three districts (Shapingba District, Jiulongpo District, and Jiangbei District) of Chongqing were considered and nearly 6500 preschool children were surveyed. For ethics approval, before the survey, consent was sort from all parents/guardians of children involved. Details of the data collection method has been previously described in literature [42-44]. After data processing and coding, 4943 valid questionnaires were received from parents or guardians (a response rate of 75.5%). To note, this study focused on children who were permanent residents and did not change residences since birth. With these requirements, among the 4943 valid cases, 3361 children answered no changes of residences since birth and were adopted in this study, as seen in Figure 1.

To further explore the indoor dampness and mold situations in homes, six damp/mold indicators were designed in questionnaire, namely, visible mold spots, visible damp stains, condensation on windowpane, water damage, damp clothing, and moldy odor, which were derived from the Dampness in Buildings and Health (DBH) study in Sweden [45]. A "*yes*" answer defined that the child was exposed to the corresponding damp indicators. In addition, the information of health outcomes for children were collected in accordance with the International Study of Asthma and Allergies in Childhood (ISAAC) [46]. The target diseases and symptoms included wheeze, doctor-diagnosed asthma, allergic rhinitis and eczema (yes/no). Both exposure periods of lifetime ever and currently (in the last 12 months before the survey) were considered. A positive response for these questions was considered to indicate that the surveyed child had the corresponding disease

or symptom.

2.2 Longitudinal investigation in children' homes

2.2.1 Subjects selection

Based on the valid 4943 case enrolled in the first stage, only 2830 surveyed children had the contact phone information. Therefore, at the second stage for in-home visit, the measured homes/residences were recruited based on the questionnaire. Children who reported one or more respiratory and allergic symptoms/diseases in the questionnaire survey were regarded as unhealthy children, while the healthy children were no such symptoms. As a result, we firstly classified the 2830 cases into health group (2492 cases) and unhealthy group (338 cases) (Figure 1).

For the second-step selection, we focused on homes which met three requirements: 1) without changing residences; 2) without renovation after survey; 3) agree to attend the study. Homes with contact information were phoned by the research team, after assessing the conditions of building characteristics, damp situations and children' health status. After implementing the screening criteria, totally 23 standardized homes were willing to join in the experiments, which included 11 cases and 12 controls, as seen in Figure 1. All participants signed informed consent letter before participation.

Detailed information of home characteristics, indoor damp/mold situations and children' specific diseases has been listed in Table S1. Most of the buildings (52.2%) were constructed during 2000-2010. 39.1% children had reported lifetime ever doctor diagnosed eczema and 43.5% children reported allergic rhinitis during the past 12 months before survey. For these investigated homes, appropriately 50% had more than one dampness/mold indicator in the first year of child' lifetime, or during the past 12 months.

2.2.2 Home visit and onsite measurement

The home visit at enrollment included a repeated questionnaire interview by face-to-face, indoor dampness/mold inspection, air sampling and dust collection from children' sleeping area in bedroom. Before visiting, an appointment was made of visiting their homes within the following 2 days and performing the house sampling. The residents were asked to remain indoor in a daily state and keep daily activities, avoiding cleaning in the bedroom or living room.

Indoor temperature, relative humidity and CO_2 were measured and recorded by sensors during the sampling period (HOBO Data Logger, Onset, US, temperature: −10–60°C with 0.1°C accuracy, relative humidity: 0.1–99.9% with 1% accuracy, CO_2: 0–10000ppm with 50ppm accuracy). The tests lasted for an entire day duration and the instrument were collected on the next day.

Concurrently, the six-stage Andersen sampler characterized by the high collection efficiency >98%(FA-1, Kangjie Co., Liaoning, China), was employed for air sampling in living room and child's bedroom of each measured residence. Prior to each sampling, the samplers were sterilized with 75% ethanol. During the tests, six agar Petri dish were put in the sampler and the instrument was placed at the central location of each room and at a height of 1-1.2m above the floor, simulating aspiration from the children breathing zone. The sampling time was 5 minutes and the set flow rate was 28.3L/min. In addition, to determine the effect of outdoor surroundings, the atmospheric bioaerosols were simultaneously collected outside each studied building at the representative location. The air sample was collected for 10 min with a flow rate of 28.3L/min.

After samplings, all the petri dishes were quickly capped and tightly sealed using sealing tape. Then they were transported to incubator in laboratory and incubated under constant conditions of 28±1°C and 50±5%, strictly followed by GB/T 18204.3[47]. The cultivating process lasted 7 days and the researchers took high-definition photos of fungal growth using a camera from the third day. The number of fungal on growth media were counted after visual inspection at the end of the entire incubation period (when the fungal grew quickly, the counting was conducted in advance). The concentration for airborne fungi was expressed as colony forming units per cubic meter of air (i.e.

CFU/m^3).

All measurements were conducted in both living room and children' bedroom of each home. The measurements were repeatedly taken in summer (July-August), transient season (October-November) and winter (January-February) respectively. To minimize the errors, all instruments had been calibrated before each sampling and the same sets were used during the whole period.

2.2.3 Dust collections and DNA sequencing for fungal population

Compared to indoor air sampling , the dust on children' beds and floors cannot be disturbed by residents' activity and provides a reference for long term exposure evaluation[48]. Study from Frankel et al. [49] showed that settled dust can be useful as a proxy for inhalable microbial exposure. As a result, the study also adopted the settled dust sampling in parallel with air sample. Dust from children' beds and floors in bedrooms were collected using a high volume surface sampler vacuum cleaner, with a high-efficiency particle (HEPA) filter trap. The sampling was performed by vacuuming 1 m^2 area for at least 4 min. Immediately after each sample was taken, it was packaged in sterile bag and then transferred to the laboratory for storage at −80°C for subsequent DNA extraction and amplification.

To further understand the community structure, composition diversity of household fungi, the high-throughput sequencing technology was employed, and DNA was extracted from the archived dust samples. The ultra-high-throughput microbial community analysis (Illumina MiSeq) was performed, and the internal transcribed spacer (ITS) region of fungal ribosomal DNA was amplified by PCR with ITS1F/ITS2aR primers (Front primer: ITS1F-5'-CTTGGTCATTTAGAGGAAGTAA-3'; end primer: ITS2-5'-GCTGCGTTCTTCATCGATGC-3'). With the defined nucleic identity threshold (97% identity for an affiliation), the clustering step was performed to group similar sequences. The results refer to an open reference operational taxonomic unit (OTU) picking process and a complete-linkage method to generate groups of sequences or OTUs. When there are non-identified genera, they are defined as "other fungi".

2.3 Statistical analysis

For questionnaire survey data, descriptive statistics were conducted to evaluate the occurrence proportions for indoor dampness/mold indicators and the studied diseases of children. For home visit data, the non-parametric Mann-Whitney test was applied on seasonal and home variations of fungal concentrations and the Kruskal-Wallis test was conducted to compare concentration differences. The Pearson's correlation analysis was employed to demonstrate the relations of environmental parameters and airborne fungal concentrations. In addition, a binary logistic regression was used to explore the associations of household dampness/mold related indicators from cross-sectional survey, or the measured airborne fungal concentrations from home measurements, with children' targeted diseases. Here to explain, to better explore the independent effects of indoor dampness/mold or measured airborne fungi on children' allergic symptoms/diseases, four covariates were adjusted in logistic regression, including the children gender, ages, the residence location (urban/suburban/rural) and genetic disorders in the family. Thus, the results were expressed by the adjusted OR values, namely aOR, with 95% CI values. All the statistical analyses were performed using IBM SPSS 22.0. A p-value < 0.05 was considered to be statistically significant.

To determine the airborne fungal concentration, the cultural fungal concentration in the air was calculated by Equation (1) and expressed by CFU/m^3 of the air.

$$C_i = \frac{P_i * 1000}{F * t} \tag{1}$$

Where C_i is the concentration of fungal at stage i, CFU/m^3; P_i is the corrected counting number at stage i, i=1..6; F is the flow rate, in this study, it is 28.3L/min ; t is the sampling time for each sample, in this study, 5 min for indoors and 10 min for outdoors.

Considering the superposition of the fungal particles when the particles went through the same sieve pore [50], the number of colonies P_i was corrected by "positive-hole correction" proposed by Andersen[51]. The correction method was shown in Equation (2):

$$P_i = N(\frac{1}{N} + \frac{1}{N-1} + \frac{1}{N-2} + ... + \frac{1}{N-r+1}) \quad (2)$$

Where N is the hole number of stage i, in this study, it is a constant value of 400; r is the actual CFU of fungal by visual inspection at stage i during test.

To explore the relationship between airborne fungal concentrations and thermal environments, regression analysis was conducted in study. Given that the mold germination and spore spread are affected significantly by the coupled effects of environmental temperature and humidity, where the filamentous fungi growth rate would be accelerated in the range of 25-30°C[52] and most mold spores can germinate when the surface relative humidity exceeds 80% [17], a comprehensive Temperature-Humidity Index (THI) was adopted, which can be calculated by Equation (3).

$$THI = 0.8 * T_a + (RH * \frac{T_a}{500}) \quad (3)$$

Where THI is the temperature-humidity index, °C; T_a is the air temperature, °C; RH is the relative humidity, %.

For conducting ITS sequencing for collected dust, total 68 samples from three seasons from 23 homes were obtained, with one sample missing in summer. After quality trimming, the valid tags were in the range of 43194-73472, with mean length of 226.3-307.89bp. We in this study examined four metrics of the microbiome data: the operational taxonomic unit (OTU) comparisons, the top 15 samples at different fungal taxa, α diversity and β diversity. Among these indicators, the α-diversity and β-diversity were determined to identify changes in microbial community structures. The α diversity reflects the degrees of fungal taxa and includes the Rarefaction curves, Violinplot analysis, Specaccum Cumulative curve and Rank Abundance analysis. In this study, the Chao 1 estimator (species richness) was calculated to estimate the number of species in a community, based on OTUs with 97% sequence similarity. Compared to α diversity, the β diversity shows significant differences in the fungal communities between different groups, based on the OTUs, or the community structure. There are several methods of analyses for β diversity, including Distance matrix, PCA, PCoA, NMDS, UPGMA. The PCoA (principal co-ordinates analysis) provides the results based on various distance matrix and shows the differences of individuals and between groups. Therefore, this study adopted PCoA plot and analysis, which was based on the Bray-Curtis method, to determine the statistical significance of clustering.

3 RESULTS

3.1 Association of indoor dampness/mold indicators and children' diseases from questionnaire survey

Figure 2 exhibits the associations of children' specific diseases of asthma, wheeze, allergic rhinitis and eczema and the six damp-related indicators, with the adjusted four covariates of gender, sex, location and family genetic factors. The results are presented with aOR values in Figure 2 and the detailed values were provided in supplementary Table S2. From Figure 2, there were positive relations with the increased aORs>1 ($p<0.05$), and they were more significant for asthma and wheeze. For example, the associations were significant for asthma in the past 12 months and past 12-month visible mold spots (aOR=3.26, 95%CI:1.91-5.58), visible mold spots (aOR=4.23, 95%CI:2.26-7.94), mold odor (aOR=2.57, 95%CI:1.78-3.72), as well as lifetime ever wheeze and visible mold spots (aOR=5.39, 95%CI:2.92-9.93), visible damp stains (aOR=3.85, 95%CI:1.82-8.12), water damage (aOR=3.87, 95%CI:1.93-7.76), and condensation in windows (aOR=2.66,

95%CI:1.36-5.17), et al. In contrast, the aOR values between indoor dampness/mold indicators and children' allergic rhinitis and eczema were slightly lower but were still higher than 1, showing a risk factor for exposure. As the building construction ages have significant effects on building dampness [29,44], our analysis selected the residences without changes since the child was born so that the outcomes provide robust support for such positive correlations.

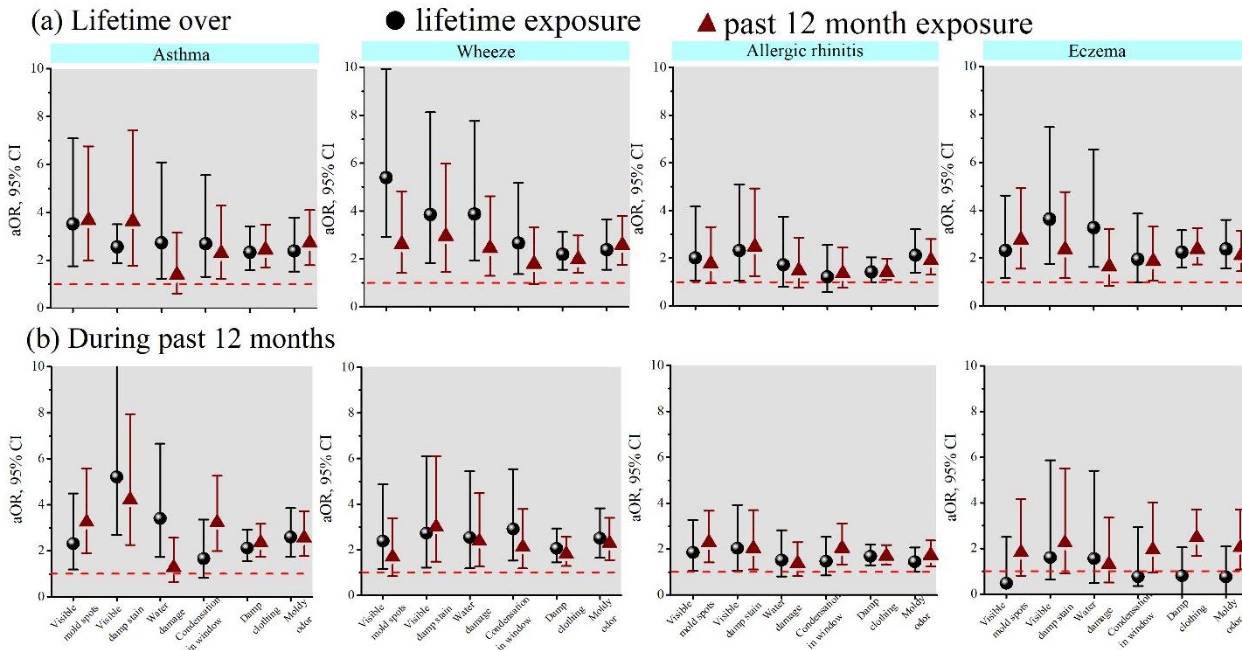


Figure 2 Associations of the childhood diseases with indoor dampness/mold indicators.

3.2 Household fungal exposure variations during onsite measurements

To assess the indoor mold contaminations in children' homes, the indoor airborne fungal concentrations were calculated from the air sampling and cultivation in lab. In addition, it has been acknowledged that the size distribution of bioaerosol particles would directly affect the depositions in human respiratory system[53,54], where the spores less than 10μm can easily reach the lower airways[55]. Therefore, the particle size characteristics were also analyzed in the following section.

3.2.1 Variations of airborne fungal concentrations in different seasons

Figure 3 exhibits the airborne fungal concentrations in different seasons. The average values are also marked with black lines in box, accompanied with maximum, minimum, 25th and 75th percentile values respectively. Regardless of the measured area of outdoor, indoor living room or bedroom, the concentrations of culturable airborne fungi were significantly higher in summer. This was followed by periods during the transient seasons and the concentrations were the lowest in winter. In particular, the mean concentration was about 1515 CFU/m^3 in the living room in summer, which was three times as high as that in winter (~462 CFU/m^3). According to American Conference of Governmental Industrial Hygienists (ACGIH)[56], it is regarded as intermediate pollution level when the limit value for airborne fungi is higher >1000 CFU/m^3 [57]. In this case, except for winter, the indoor fungal concentrations for majority of the children' homes were at medium pollution level. Moreover, Figure 3 also demonstrates the ratio of airborne fungal concentrations between indoor and outdoor. In summer and transient season, the I/O values were higher than 1, indicating the fungal contaminations were possible from indoor sources; while the indoor fungal concentration was affected by outdoor in winter, with I/O<1.

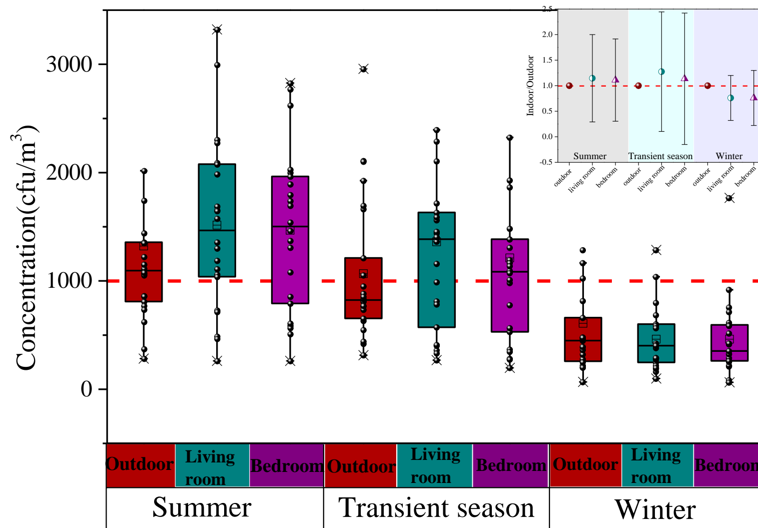


Figure 3 Fungal concentrations in measured residences in different seasons.

3.2.2 Size distributions of measured airborne fungi

Thanks to the Andersen impactor categorizing bioaerosol particles into six fractions according to the aerodynamic diameters, we counted the proportion distribution of different particle sizes at different levels. The results are plotted in Figure 4. Regardless of the different seasons, the highest proportion for fungal particle size were observed for aerodynamic diameters within the range of 2.1–3.3 μm. Particularly, the proportions for different aerodynamic diameters of fungal spores in different seasons were relatively stable for outdoors. For indoors, there were slight variations of the distributions, e.g., the proportions for particle diameter in the range of 0.65–1.1 μm (VI) were higher in transient season and in summer. This indicated that there would be much higher risks for fine particles penetrating into the terminal bronchioles and alveoli of the human body in these seasons.

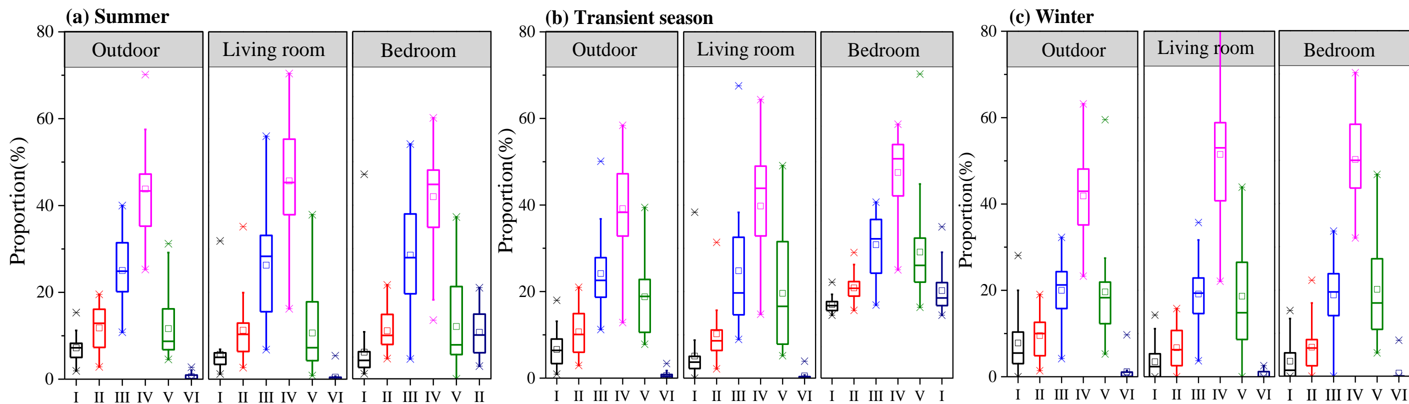


Figure 4 Size distributions of airborne fungi in different seasons
(I > 7.0 μm, II: 4.7~7.0 μm, III: 3.3~4.7 μm, IV: 2.1~3.3 μm, V:1.1~2.1 μm, VI: 0.65~1.1 μm).

3.3 Fungal characteristic for collected dusts based on DNA sequencing

The DNA sequencing of the fungal ITS region takes advantage to identify down to the species level and provide quantitative information about the diversity of fungal community [58]. Figure 5 demonstrates the OUT level bar for the collected 68 dust samples in children' bedroom from different seasons, where the total valid tags for each sample was counted at each taxa. Through identification, a total of 10 phylum, 39 class, 129 order, 314 family, 1055 genus and 2443 species were included in the fungal populations, except for the undefined fungal at each level. To provide details, the following section presents the main analyses regarding the community structure,

composition, the α diversity and β diversity.

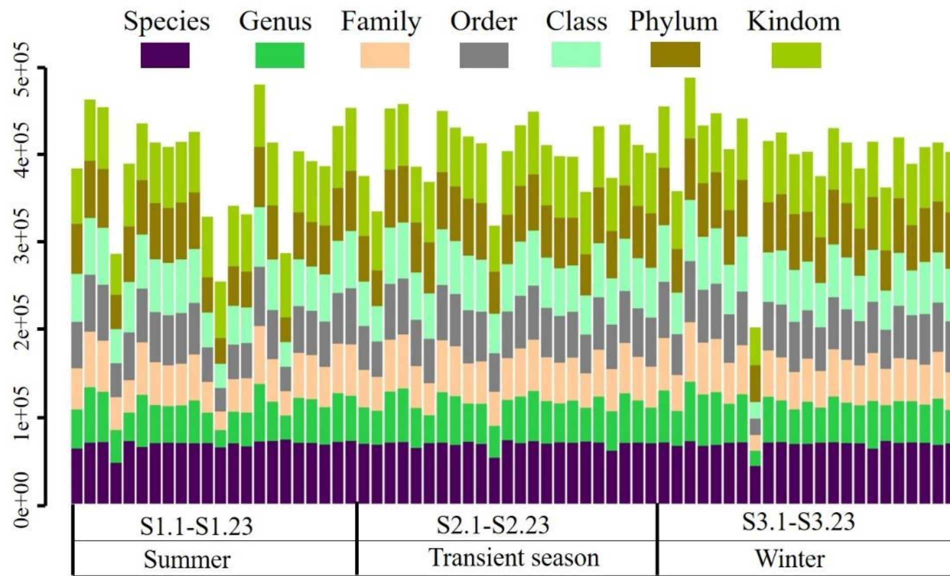
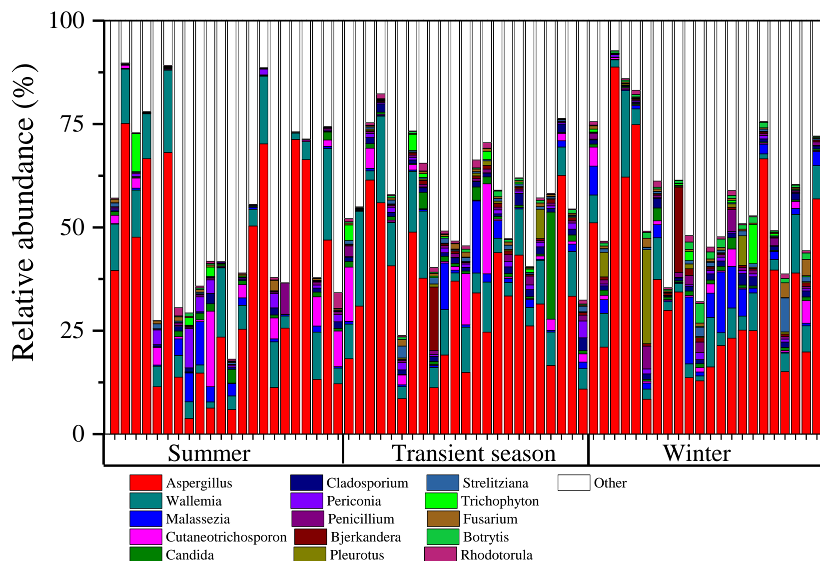


Figure 5 OUT level bar plot in different homes and seasons.

3.3.1 Fungal community and composition

Figure 6 shows the relative abundance of the selected top 15 fungi at genus and species respectively. At genus level in Figure 6(a), the *Aspergillus* was the predominant and the relative abundance accounted for the highest proportion in majority of the dust samples. The *Wallemia* was subsequently higher. In contrast, the values varied significantly by different homes, rather than by seasons, and no remarkable trends were found among different seasons. For the varied species analysis in Figure 6(b), the *Aspergillus_penicillioides* was the most common fungal taxa, which was followed by *Fungi_sp*, *Wallemia_sebi*. The relative abundance values of these three fungal taxa had totally accounted for more than 50% of the top 15 representative species. Similar to Figure 6(a), the differences among seasons were not significant, compared to home variations. A heat map for the top 15 fungal genera OTUs is provided in Figure S1 of the appendix. The clustering results were grouped under different seasons. The red side reflected a higher relative abundance, while the blue side indicated a lower relative abundance. Based on the results, the relative abundance of the fungal genus was comparatively high in winter.



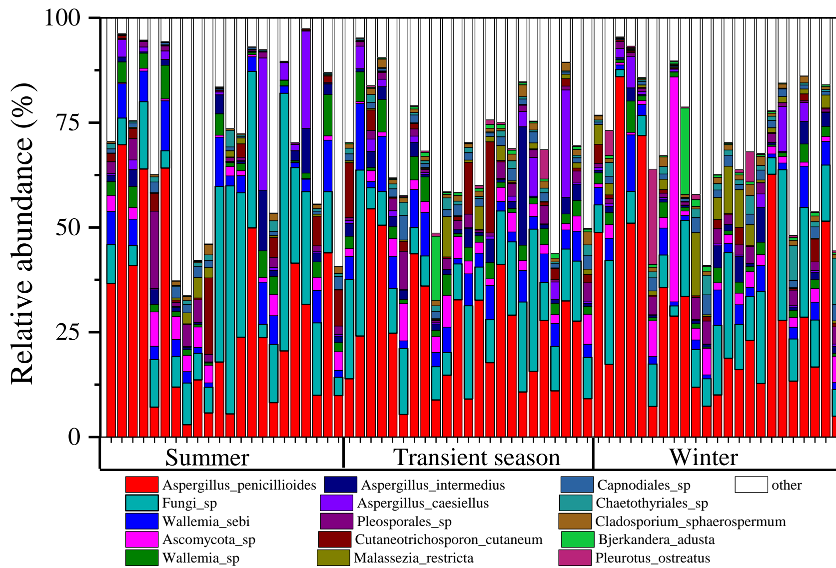


Figure 6 Community structure of the top 15 fungi: (a) genus taxa (b) species taxa.

3.3.2 Analysis of α diversity

Figure 7 shows the results of the rarefaction measure of Chao 1. With increasing the extraction sequences for per sample, the curves in Figure 7 (a) tend to be flat, indicating the current sequences were reasonable and a further sequencing just had a small amount of OTU numbers. Taking the season as the baseline for grouping, Figure 7(b) gives the distribution of exponential values of α diversity analysis, and the significant differences of the values were found among different seasons.

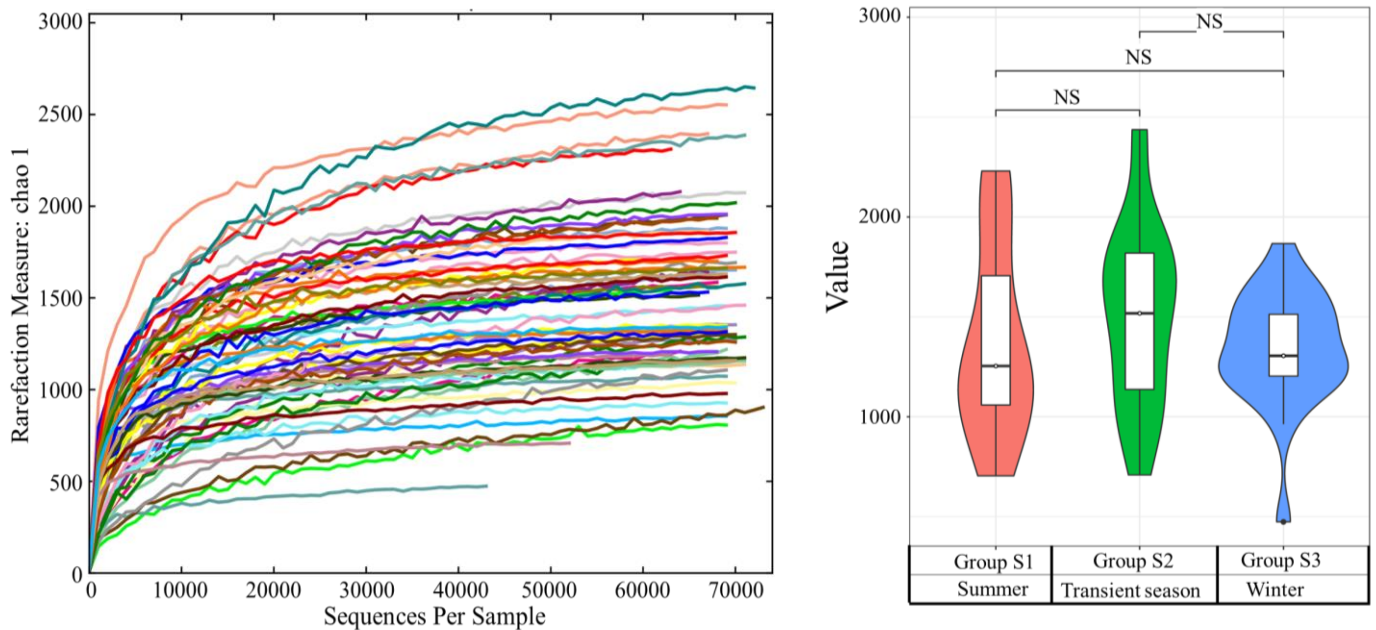


Figure 7 The α diversity of the 68 samples: (a) rarefaction curves (b) distribution exponentials

3.3.3 Analysis of β diversity

The result of the 2D plot for PCoA analysis is showed in Figure 8. The closer the samples are in the same group and the further among different groups, the better the grouping results. Based on the grouping, the differences for the dust samples among summer, transient seasons and winter were not remarkable. However, the Adonis analysis shows statistical significances for the different groups ($p<0.001$).

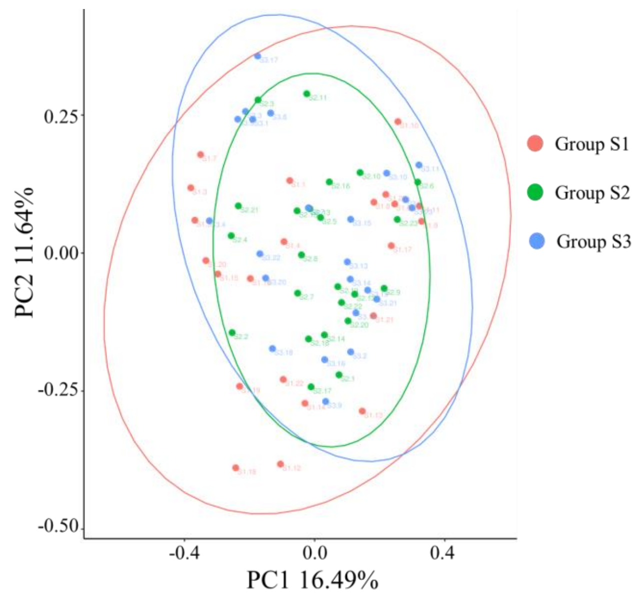


Figure 8 Principal coordinate analysis (PCoA) of community structure based on bray_curtis method.

Taking the genus distribution as an example, we chose the top 10 genus and plotted the box for relative abundance (exponential distribution) in Figure 9. The results are differentiated by different seasons (Group S1: summer, Group S2: transient season, Group S3: winter). From Figure 9, there were significant differences in different seasons. Compared to transient season and winter, the values were relatively lower for the top genus in summer, except for *Peniophora*, *Schizophyllum* and *Trametes*, which was responded to the statistical tests in Figures 7 and 8.

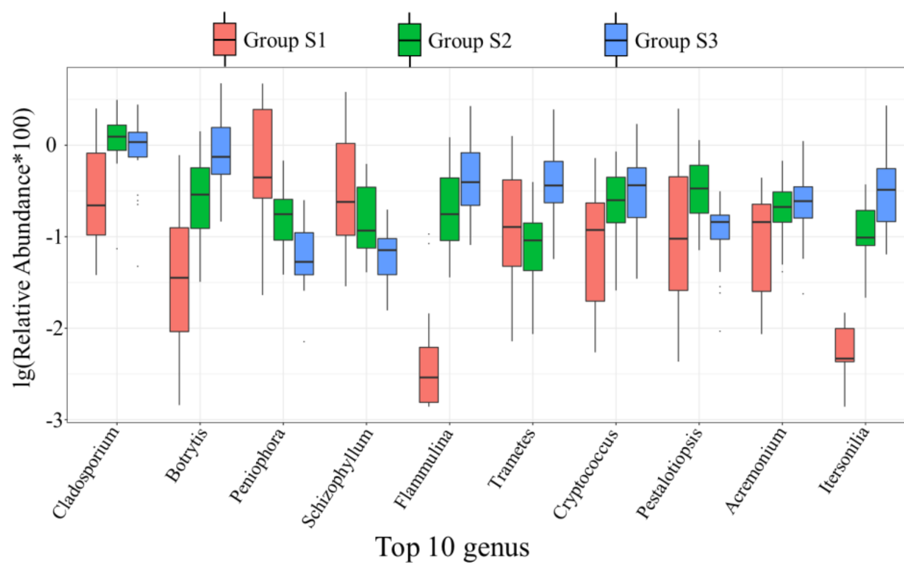


Figure 9 Box-plot for relative abundance of the top 10 genus.

3.4 Relations of fungal exposures with environmental parameters and children' targeted diseases

3.4.1 Correlations of environmental parameters and airborne fungal concentrations

Considering indoor fungal concentrations in air would be affected by indoor temperature, humidity, as well as the outdoors, the Pearson' correlation was conducted for bedroom and living room respectively, stratified by seasons. The results are shown in Table 1. From Table 1, the indoor fungal concentrations were positively related to the indoor relative humidity in each room and outdoor airborne fungal concentrations at the measured time. Particularly, the correlations were

statistically significant for outdoor and indoor fungal concentrations in summer and winter in both bedroom and living room, e.g., the coefficient of r was about 0.885 for bedroom indoor and outdoor concentrations in winter ($p<0.05$). In contrast, it showed inconsistent correlations between airborne fungal concentrations and indoor temperature and CO_2 concentration. For example, a negative correlation was found between indoor temperature and airborne fungal concentration in summer, suggesting the airborne fungal concentrations would decrease when the temperature increased in summer. Coupled with the indoor CO_2 concentration, it was inferred by the occupants' behaviors for heating and cooling in summer and winter time investigated during measurements. However, this should be further explored.

Table 1 Pearson' correlation between indoor airborne fungal concentration and environmental parameters

Seasons	Room type	Pearson coefficient, ***r*** (p value)			
		Outdoor fungal concentration (CFU/m^3)	Indoor temperature (^{o}C)	Indoor relative humidity (%)	Indoor CO_2 concentration (ppm)
Summer	Bedroom	**0.73***	**-0.568***	0.273	-0.117
	Living room	**0.771**	-0.289	0.147	0.13
Transient Season	Bedroom	0.346	0.245	0.291	0.004
	Living room	0.324	0.336	0.346	0.121
Winter	Bedroom	**0.885***	-0.242	0.167	**-0.457***
	Living room	**0.65***	-0.327	0.334	-0.146

*two-tailed test, $p<0.05$. The bold values are significant based on statistical test.

To further explore the relation between the environmental temperature and humidity and the airborne fungal concentrations in different seasons in Table 1, we first grouped the measured indoor and outdoor data in three seasons and calculated the THI values according to Equation (3). Then the measured airborne fungal concentrations were averaged via a THI interval of 0.5 °C. The results are demonstrated in Figure 10, which shows the changes of airborne fungal concentrations with the THI index. From Figure 10, the airborne fungal concentrations were decreased with the increased THI values in winter and summer and it was more remarkable in summer. In contrast, the airborne fungal concentrations were increased significantly in transient season, with the comprehensive THI values from about 12°C to 20°C. Overall, the changes trends in Figure 10 was in line with the correlation tests in Table 1. It was inferred that in transient season, the mild thermal environments contributed to the mold growth while the higher temperatures might inhibit the growth of mold, resulting in a negative relation between both. This is in line with the current findings that the mold growth is favored by appropriate temperature and relative humidity[59-61]. However, from the whole, the values for airborne fungal concentrations were still higher in summer, and lower in winter, which was in consistent with that in Figure 3.

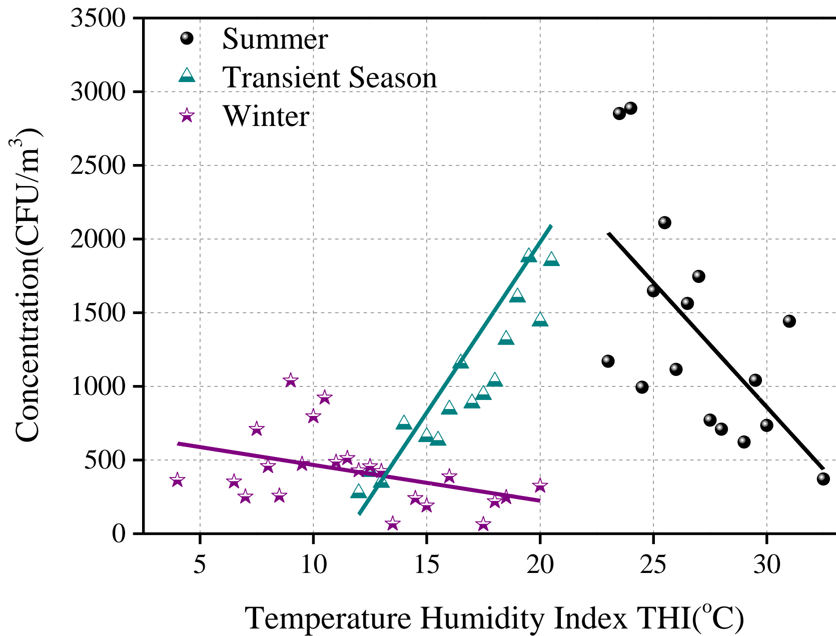


Figure 10 Relation of airborne fungal concentration and THI in different seasons.

3.4.2 Association of indoor fungal concentration and prevalence of children' diseases

During the home visits, we asked the parents to complete the same questionnaires again. To explore the relations between the studied diseases of children and the exposures to airborne fungi, responding to Figure 2, we counted the prevalence of the target diseases (i.e., asthma, wheeze, allergic rhinitis, eczema) of children for lifetime ever and during the past 12 month. For indoor fungal concentration, we took the median value as baseline and divided the data into binary variables (low concentration exposure, 0 for $<$median value; high concentration exposure, 1 for $\geq$median value). The logical regression was conducted and the adjusted aOR values with 95%CI are plotted, as illustrated in Figure 11. Since the data from a small sample from the investigated 23 homes, no asthma diseases or symptoms were reported and only wheeze, allergic rhinitis and eczema were analyzed. From Figure 11, the associations between measured airborne fungal concentrations and childhood diseases were not significant in summer and winter, with the aOR values lower than, or approximately to 1. In contrast, the exposure to airborne fungi was risk factor in transient seasons, where the aOR values were higher than 1. For example, for lifetime ever eczema and eczema during the past 12 months, the aOR values were 4.33 (95%CI:0.76,9.47) and 3.58(95%CI:0.12,5.7) respectively. This was slightly different from the significant associations between childhood allergic symptoms or diseases and residents' reported indoor dampness/mold indicators based on cross-sectional survey (Figure 2), which might be possibly explained by the complex and confounding factors in different research stages.

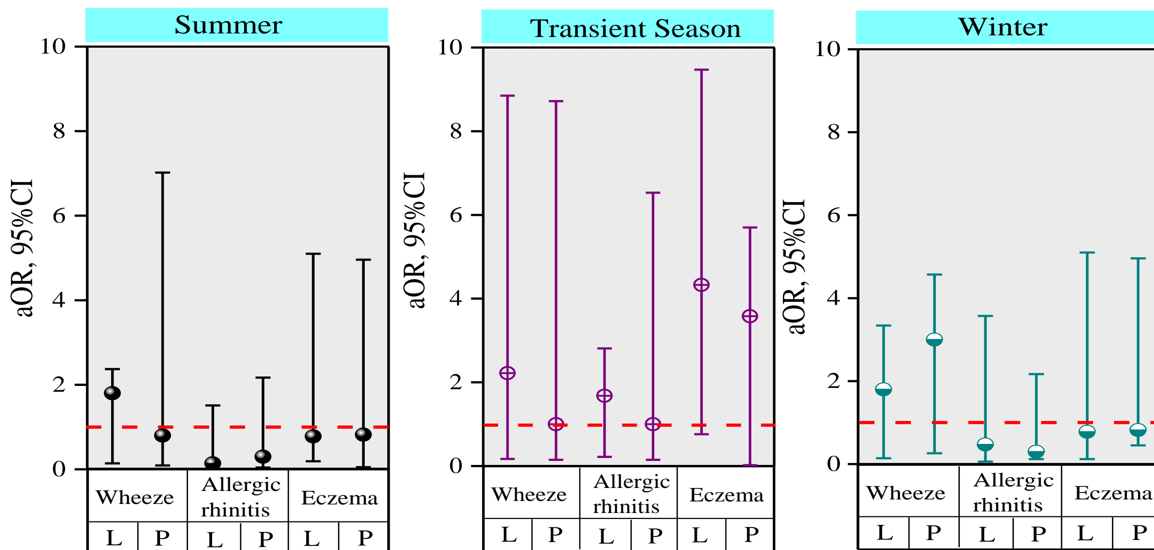


Figure 11 Associations of indoor airborne fungal concentrations and children' diseases (note: L lifetime ever, P the past 12 months)

4 DISCUSSION

4.1 Combinations of cross-sectional surveys and onsite measurements

An increasing body of epidemiological studies have shown clear and consistent associations between indoor dampness/mold occurrence and increased probability of adverse health effects of children, such as development and exacerbation of asthma, allergic rhinitis, and significant respiratory infections[12-15,18,43,44,62]. These findings are consistent with our results, where the logistics regression showed increased aORs of the children studied diseases with indoor dampness/mold indicators in residences (Figure 2).

However, due to the limitation of questionnaire-based survey, the data collection did not include any inspection or measurement of damp/mold exposure situations. Therefore, to better understand the direct relations of household damp/mold exposures and respiratory health outcomes, we conducted further comprehensive walk-through home inspection based on the first-stage questionnaire survey and a case-control design was performed. The onsite measurements revealed that most studied homes have exposure risks to higher airborne fungal concentrations and medium pollution level (Figure 3). Moreover, the results in Figure 11 further verified the close relations between airborne fungal concentration and children' specific diseases, which provides further understanding of what the household dampness/mold situations are and how it is related to children' adverse health outcomes.

Although the air sampling/dust collection during home inspection and the culturable method have been conducted by several studies[26,63-66], to evaluate the indoor airborne fungal concentrations by quantification, the one-time air sampling is only helpful to determine current exposure at the sampling location at that time. The results do not facilitate evaluation of long-term exposure of fungi in air and their dynamic variation temporally and spatially. This study filled in this gap by repeated and tracked measurements for the same residences in different seasons. Figure 3 reveals the seasonal variations of airborne fungal concentrations in living rooms and children' bedrooms. The concentrations were the highest in summer and lowest in winter, regardless of the home differences. Such findings are consistent with previous studies where the fungal concentrations are peaking from spring to summer and declining throughout fall to winter[67]. Table 1 and Figure 10 further explored the influencing factors between environmental variables and measured fungal concentrations via bivariate correlations. The results showed that there were significant relations between indoor and outdoor fungal concentrations and a temperate and moisture climate may provide favorable conditions for growth and propagation of airborne fungi[68]. Interestingly, Figure

10 display an inconsistent changes of fungal concentrations with the air temperature and humidity, where the concentrations were positively increased with THI values in transient seasons, while the change trends were converse in summer and winter. However, such relations are probably influenced by complex surrounding factors, like the climatic conditions for mold growth, the home characteristics (location, year of construction, airtightness) and the residents' behaviors (heating and cooling, ventilation, window opening), which are warranted for further study.

4.2 Combination of culture-based and DNA sequencing methods to characterize fungal populations

The ubiquitous distribution of fungal spores leads to their inevitable exposure by breathing inhalation or skin contact. Especially for children, due to the not fully developed respiratory system and the higher breathing rates[69], they are considered as more susceptible and vulnerable to airborne fungi pollution. As the particle with 2.5-10 μm in diameter are respirable allergenic molds, this study analyzed the size distributions of the culturable airborne fungi. Results from Figure 4 showed that the particle diameters in the range of 2.1-4.7 μm (III and IV) totally accounted for more than 50%, which were similar to the results observed in residences and other buildings[31,32,70]. This fills in the gaps that most of the current studies only quantitatively analyze the number of fungal colonies but are limited to provide a view of fungal distribution. The findings indicated that most fungal spores in homes were inhalable for children and the much smaller fine particles posed much higher risks for health exposure, which should be critically managed in children residences.

Considering the cultivation and counting method did not enable the evaluation of the composition of fungi, the adoption of the DNA sequencing method made up for the deficiency. The 16S ITS sequencing enables a comprehensive understanding of fungal populations, including diversity, composition, taxa analysis, etc. Since the composition and concentration of airborne fungi might be influenced by seasons, regions, weather, residents' activities and habits, etc., we collected dust samples from the floor and bedding in children' bedroom as an alternative (Figures 5-6). The species richness for each home (α diversity) and compared the similarities of fungal communities across each season (β diversity) were analyzed (Figures 7-8).

From Figure 6, the *Aspergillus* was the predominant taxa at genus level, and the *Aspergillus_penicillioides* was the most common at species level. Moreover, the species composition and community structure of the settled dusts were different among homes, and the season differences were found among summer, transient season and winter (Figure 9). According to numerous culture and nonculture based studies[71,72], the *Penicillium*, *Aspergillus*, *Cladosporium*, *Alternaria* and other fungal genera are the most commonly found in dust in residential environments, which is in line with our results. However, due to different dust type, collecting method, type of room, testing time and seasons, etc., the identified dominant genera generally varied between studies[10,73,74]. For example, Fan et al [65] found that the indoor fungal communities had strong geographic pattern and were affected by the season and outdoor sources. From Figure 9, the significant differences among the top 10 genus were found among three seasons. It is explained by the different release mechanisms of fungi [75,76] that different types of fungal spores may have different released fragments and external environments. However, this is beyond our research and does not discuss further.

4.3 Significant associations between indoor fungal exposures and prevalence of children' specific diseases

It has been well documented and widely accepted that mold inhalation and sensitivity is a risk factor to the cause of respiratory problems; different fungal species may have different mechanisms, releasing the taxonomic composition or only causing allergy [75]. For instance, it was verified that *Penicillium* and *Cladosporium* species are significantly associated with asthma morbidity and its severity[77,78]. Exposure to the higher concentrations of *Alternaria*, *Aspergillus*, *Cladosporium* and *Penicillium* species increased number of current asthma symptoms by 36% to

48% compared to those exposed to lower levels [8]. Despite this, as the prevalence of human health effects is not always related to a specific cause or factor, but rather depends on various parameters and their coupled effects, the study is currently unable to provide strong evidences for the direct causal relation between children' disease and indoor airborne fungal exposure. A study from Mendell et al. [79] did not find the strong associations of the quantitative microbiological measurements and the studied health effects; it was inferred by inaccurate microbial factors, or the influence of location and time. In this study, though the measured mean airborne fungal concentrations were higher in summer (Figure 3), the concentrations were decreased with increased THI values (Figure 10), which may also affect the associations between fungal exposures in air and children' investigated diseases. Moreover, the environmental temperature and humidity are possible factors influencing the prevalence of children' allergic diseases/symptoms. While this study assumed the thermal environments directly affected the indoor mold growth and spore spread in air and indirectly affected children health. Considering the focus of this study was fungi exposure in air, the logistic regression analysis in Figure 11 confirmed the associations of indoor airborne fungal concentrations and the probability of the studied diseases of children, and the multiple variables and the coupled effects are expected in further study.

In addition, the adverse outcomes caused by mold exposure require a sufficient concentration for sufficient amount of time [3]. For majority populations, we may be sensitized to mold spores but do not reflect symptoms. For susceptible individuals, one possible mechanism is the activation of immune responses and sensitization to mold. This may cause sensitization via non Immunoglobulin E (IgE) mediated pathways so that people with allergic asthma are often poly-sensitized, thus producing IgE antibodies [80]. In addition, some molds can even form toxins and cause intoxication, e.g., mycotoxins, which are produced by *Aspergillus spp.*, *Fusarium sp.*, and *Penicillium spp.*[81,82]. Such toxicity may cause the body to expand its immune responses and lead to epithelial cell damage, inflammatory cell infiltration (e.g., eosinophils), and inflammatory factor release, thus inducing bronchospasm and asthma attacks. However, the mechanisms behind mold inhalation exposure and asthma, allergies, and other related symptoms are still not fully unveiled and possible toxicology research at molecular level is regarded as a good way to reveal the mechanism and determine the safety thresholds.

4.4 Limitations and future work

The purpose of this study is to in-depth verify the association of indoor dampness and mold contamination, and children health effects, based on the first-stage epidemiological survey and the second-stage home visiting and measurement. The findings ultimately provide references for understanding indoor mold exposure characteristics in homes and reducing the presence of mold contamination in indoor environments. However, any measurements have demonstrable merits but also have limitations. First, this study was conducted with a small number of longitudinal cases. Coupled with the COVID-19 pandemic, the homes visits were performed from June to February next year and the spring measurements were not covered. As the climate characteristics may have differences in spring and autumn, as well as the airborne fungi, further interpretation of the results may be limited by the small sample size. Secondly, the investigated childhood allergic diseases and symptoms were only based on the self-reported symptoms and lack of availability of diagnose by doctors or clinical tests. The accurate detection of sensitization to certain fungal allergens were unable to provide. Therefore, it is currently insufficient to build the solid casualty between measured mold species or components and the occurrence of the studied diseases. Third, this study, as well as previous studies, collected the dust samples (floor or bedding settled dust). Some findings [15,65,83] identified the airborne fungal concentrations were poorly related to the concentrations in home dusts, due to the optimal propagation conditions and survival potential differences among fungal species. For example, the dust sampling is significantly affected by the frequency of cleaning, the users of the bedding, etc. We asked residents to not clean the room on the measuring

day and they had the common daily activities as usual. Regardless of this, the results from dust analysis may not adequately represent the inhalation exposures of fungal concentrations in air.

In addition, the current codes/standards/guidelines provide several practical strategies for estimating dampness-related risks, but these assessments and exposure thresholds still need further refinement. As the exposure-dose-response related health effects are much complicated, the detailed knowledge about the human regulatory response under mold exposure has not been well established yet. Both epidemiological and toxicological approaches are needed to further elucidate the role of the microbiome in human health. Therefore, a combination with clinical observation is encouraged to the development of new microbiota-targeting therapies and diagnostics, which is instructive for setting health-protective thresholds.

As for the mold treatments, the designers' choice of building envelope materials, construction details and systems of heating and ventilating are the sources to minimize the mold growth risk. A good prediction of mold growth in buildings is necessary during design, in order to minimize the potential risks. In addition, reasonable conditions of use for occupants liking heating and sufficient ventilations are key to improve indoor hygrothermal performance and avoid surface condensation in buildings. The auxiliary purification facilities like air cleaners and filters are encouraged in some contaminable situations in buildings but the maintenance should be noticed. Moreover, with the growing focus on building energy efficiency and carbon emission reduction, a trade-off between building energy-efficient designs and indoor air quality guarantees should be balanced, to ensure comfortable and healthy indoor environments and human well-beings.

5 CONCLUSION

The present study fills in the gap to reveal the dynamics variations of dampness/mold in residential environments, through systematically combing cross-sectional survey, case-control measurements and DNA sequencing.

The questionnaire survey verified the positively significant associations of indoor dampness/mold indicators and the prevalence of children' asthma, wheeze, allergic rhinitis and eczema (aORs>1), regardless of lifetime ever or in the past 12-month exposure.

Based on a case-control design and repeated home visit measurements in 23 homes, the culturable airborne fungal concentrations were the highest in summer and lowest in winter, e.g. 1515 CFU/m^3 and 462 CFU/m^3 in living room respectively. The proportions for fungal particle size distributing in the range of 2.1–3.3 μm (IV) were the highest in all seasons, indicating a higher risk of fine particles penetrating and depositing in the lower airways of human. The DNA sequencing from settled dust samples identified the predominant genus of the *Aspergillus* and *Wallemia*, while the community structure and composition varied significantly by homes, and seasonal differences.

The indoor airborne fungal concentrations are correlated to indoor air temperature, humidity, CO_2 as well as the outdoor fungal concentrations. The concentrations were increased with increasing THI in transient season while the change trends were converse in summer and winter. Further, the logistic regressions showed positive associations between indoor fungal concentration and the prevalence of children' specific diseases like wheeze, allergic rhinitis and eczema in transient seasons, with aOR values higher than 1.

The outcomes help underlying the complex issues of indoor fungal contaminations in homes and its' relations to building environments and adverse health effects for children, benefiting to public health protection, microorganism management and indoor air quality improvement in future.

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measurement and data collection in the two periods.

CONFLICT OF INTEREST STATEMENT

No conflict of interest declared in this study.

AUTHOR CONTRIBUTIONS

All the authors approve the final version and agree to submit to journal, and be accountable for all aspects of the work related to the accuracy or integrity of any part of the work

Chenqiu Du: Conceptualization; Data curation; Formal analysis; Funding acquisition; Methodology; Project administration; Writing - original draft
Baizhan Li: Conceptualization; Methodology; Project administration; Supervision; Writing - review & editing
Wei Yu: Conceptualization; Methodology; Visualization; Writing - review & editing
Runming Yao: Methodology; Project administration; Resources; Supervision; review & editing
Jiao Cai: Data curation; Investigation; Resource; Formal analysis
Bicheng Li: Data curation; Investigation; Resources
Yinghui Yao: Data curation; Investigation; Resources
Yujue Wang: Data curation; Investigation; Resources
Min Chen: Data curation; Formal analysis
Emmanuel Essah: Conceptualization; Writing-review & editing

DATA AVAILABILITY STATEMENT

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

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