

Metagenomic characterization of indoor dust fungal associated with allergy and lung inflammation among school children

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ABSTRACT

The exposure of school children to indoor air pollutants has increased allergy and respiratory diseases. The objective of this study were to determine the toxicodynamic interaction of indoor pollutants exposure, biological and chemical with expression of adhesion molecules on eosinophil and neutrophil. A self-administered questionnaire, allergy skin test, and fractional exhaled nitric oxide (FeNO) analyser were used to collect information on health status, sensitization to allergens and respiratory inflammation, respectively among school children at age of 14 years. The sputum induced were analysed to determine the expression of CD11b, CD35, CD63 and CD66b on eosinophil and neutrophil by using flow cytometry technique. The particulate matter (PM_{2.5} and PM₁₀), NO₂, CO₂, and formaldehyde, temperature, and relative humidity were measured inside the classrooms. The fungal DNA were extracted from settled dust collected from classrooms and evaluated using metagenomic techniques. We applied chemometric and regression in statistical analysis. A total of 1869 unique of operational taxonomic units (OTUs) of fungi were identified with dominated at genus level by *Aspergillus* (15.8%), *Verrucaria* (5.5%), and *Ganoderma* (4.6%). Chemometric and regression results revealed that relative abundance of *T. asahii* were associated with down regulation of CD66b expressed on eosinophil, and elevation of FeNO levels in predicting asthmatic children with model accuracy of 63.6%. Meanwhile, upregulation of CD11b expressed on eosinophil were associated with relative abundance of *A. clavatus* and regulated by PM_{2.5}. There were significant association of *P. bandonii* with upregulation of CD63 expressed on neutrophil and exposure to NO₂. Our findings indicate that exposure to PM_{2.5}, NO₂, *T. asahii*, *P. bandonii* and *A. clavatus* are likely interrelated with upregulation of activation and degranulation markers on both eosinophil and neutrophil.

1. Introduction

Many recent reviews and studies have showed that interaction exposure to indoor pollutants and spectrum of allergens could contribute to allergic responses, increase the risk of developing asthma and exacerbating existing asthma (Caillaud et al., 2018; Eguiluz-Gracia et al., 2020; Esty et al., 2019; Vincent et al., 2018). Children are more

vulnerable than adults to these conditions as they breathe more air and immaturity of respiratory system, especially during the window of susceptibility (Munoz-Pizza et al., 2020). Moreover, the heterogenous, dynamic combination of dysregulated immune response, tissue remodelling and hyperresponsiveness in affected tissues explained the complexity of asthma and others allergic diseases (Agache and Akdis, 2019). Thus, biomarkers represent a measurable indicators that linking

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endotype with phenotype (Carr and Kraft, 2018). Presently, biomarkers such as fractional exhaled nitric oxide (FeNO), cytokines, chemokines, lipid mediators, enzymes, adhesion molecules, and other growth factors, have been considered as the indicators of allergic airway inflammation (Tiotiu, 2018). However, there has been limited studies that explore the multi-dimensional characters of adhesion molecules (CD11b, CD35, CD63, and CD66b) on eosinophil and neutrophil. Activation of these markers in response to inflammatory signals (Vijverberg et al., 2013) stimulate degranulation and release of cytotoxic mediators including cysteinyl leukotrienes, eosinophil peroxidase, matrix metalloproteinases and neutrophil endopeptidase, which significantly contribute to airway inflammation (Figueiredo and Neves, 2018; Pelaia et al., 2015; Xu and Hakansson, 2002).

Recently, researches about the potential health impact on taxonomic diversity of atmospheric fungi have attracted a lot of attention. Metagenomic technologies have provided more comprehensive assessment of fungi by amplifying prime targets rDNA, such as internal transcribed spacer (ITS) 1, ITS2 and 5.8 S, followed by next-generation sequencing (NGS) and characterizing thousands of species, referred to as OTUs (operational taxonomic units) (Baeza et al., 2017; Donovan et al., 2018). The selection of prime targets are still controversial, but it is proposed that ITS1 as an universal fungal barcode is able to capture taxonomic profiles similar to shotgun metagenomic technique (Mbareche et al., 2020; Yang et al., 2018). Yet, relatively sparse metagenomic studies have been applied to characterized the fungi in the dust collected from school area. Fungi diversity in the settled dust can be used as surrogate for allergy reactions through their effects from inhalation and potential ingestion exposure. Also, there is a complex interplay between environmental factors, including PM₁₀, PM_{2.5}, NO₂, CO₂ and climate changes with the fungal richness, growth, dynamics and release into the biosphere (Roy and Bhattacharya, 2020). Therefore, the interaction of these environmental factors and allergenic fungi with the associated inflammatory mediators are deserves deep investigation.

The purposes of this study was to use amplicon sequencing approach to characterize and identify the fungal in the dust samples collected inside the classrooms that may be associated with the exacerbation of asthma among school children. Moreover, it also aims to predict the toxicodynamic effects of fungal richness and indoor pollutants measured in the classrooms towards marker expression on the eosinophils and neutrophils in sputum samples.

2. Methodology

2.1. Study population

The study was carried out in Hulu Langat District, Selangor Malaysia, which experiencing urban sprawl from rapid urbanization of Kuala Lumpur Metropolitan and Putrajaya. In this district, there are massive construction development projects for the industrial and property estates (Bakeri et al., 2020). Eight randomly selected secondary schools in this district were included and the school areas were classified as urban and suburban based on locale classification of ecological measures. The school children from the selected schools (N = 470) aged 14 years old with guardian's consent and started attending the same school since January 2017 (or more than 18 months) were randomly selected from four classrooms of each school. Among them, only 50 (10.6%) children were clinically diagnosed with asthma based on questionnaire administered. Non asthmatic school children who produced adequate sputum leukocytes cells count (< 80% of squamous epithelial cells) from the same class and school were randomly selected as a control group. Out of the control group, a total of 46 children were able to produce adequate leukocytes cells count. In total, 96 school children were included in the final study group. School children were excluded from the study if they had received antibiotic treatment in the previous four weeks and history of smoking in the last 12 months. The data collection from the questionnaire, clinical assessments and indoor air monitoring were carried

out at the same time from August until November 2018 and in February 2019.

The study was approved by the Ethics Committee for Research Involving Human Subjects Universiti Putra Malaysia (JKEUPM) (JKEUPM-2018-189). Written informed consent were obtained from the guardians before enrollment in this study, with the addition of the children's own assent.

2.2. Assessment of health data

The validated European Community Respiratory Health Survey (ECRHS) and the International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire were self-administered by school children to obtain information on demographic characteristics, asthma and allergic condition and symptoms for the last 6 months (Norback et al., 2017a, 2017b). These information was verified during face-to-face interviews and telephone calls with the children's respective guardians.

2.3. Assessment of fractional exhaled nitric oxide (FeNO) and allergy skin test

The FeNO levels were measured with chemiluminescence analyser (NIOX VERO, Circassia, Sweden) with a single breath maneuver for about six to ten seconds at a constant flow rate (50 mL s⁻¹) as recommended by the American Thoracic Society/ European Respiratory Society (ATS/ERS) (Dweik et al., 2011). This procedure was repeated two times and the average value of reproducible FeNO measurements was used as the main outcome variable. The detection limits and accuracy for this FeNO analyser are 5–300 ppb and ± 5 ppb, respectively.

Atopy status was determined using allergy skin test by means of prick-puncture method on the volar side of the participants' forearm. The tested allergens included *Dermatophagoides pteronyssinus* (Derp1) (house dust mite), *Dermatophagoides farina* (Derf1) (house dust mite), *Cladosporium herbarium* (fungi), *Alternaria alternata* (fungi), and *Felis domesticus* (cat), also Histamine (10 mg mL⁻¹) and normal saline were employed as a positive and negative control, respectively (Prick-Test Diagnostic, ALK-Abelló, Madrid, Spain). Positive sensitization was considered by a wheal diameter at least 3 mm and atopy was defined as a positive sensitization to at least one of the applied allergens (ASCLA Australasian Society of Clinical Immunology and Allergy, 2016).

2.4. Sputum induction and processing

School children who fulfilled the criteria were requested to inhale nebulised sterile mixture of 4.5% sodium chloride through an ultrasonic nebuliser (Model CUN60 Citizen System Japan Co. Ltd., Tokyo, Japan) and salbutamol 200 µg (by metered dose-inhaler) to achieve successful sampling. Then, they were encouraged to cough and expectorate the airway secretions into a plastic container. The collected sputum samples were kept in icebox and further processed within two hours and analysed by using flow cytometry technique. Details of the sputum processing and flow cytometry analysis are to determine the expression of activation and degranulation of CD11b, CD35, CD63 and CD66b on eosinophil and neutrophil have been reported earlier (Mohd Isa et al., 2020). The gating strategy of all the markers is highlighted in the Fig. S1, while Fig. S2 depicts the markers analysis in sputum sample as an example.

2.5. Assessment of school environment

In each school, four classrooms were randomly selected to monitor the indoor parameters, which include particles with aerodynamic size of 10 µm (PM₁₀) and 2.5 µm (PM_{2.5}) by using Dust-Trak monitor (Model 8532 TSI Incorporated, Minnesota, USA) set to a sampling rate of 1.7 L min⁻¹ with a detection limit of 0.001–150 mg m⁻³. Temperature (°C), relative humidity (RH) (%) and carbon dioxide (ppm) were monitored

by using Q-Trak™ IAQ monitor (Model 7565 TSI Incorporated, Minnesota, USA) with the average logging values of over one minute. The accuracy of this device on temperature, relative humidity, and CO₂ are ± 0.6 °C, $\pm 3\%$, and ± 50 ppm, respectively. The concentration of formaldehyde (mg m⁻³) was measured by using PPM Formaldemeter™ htV-m (PPM Technology Ltd, Wales, UK) with accuracy of 10.0% at 2 ppm. Monitoring devices were placed one meter from the ground in the center of the classroom and all devices used were periodically calibrated. In each school, a total of four hours measurements were taken from four randomly selected classrooms for period of an hour's time each during learning session and has been previously described (Kamaruddin et al., 2015; Norback et al., 2014; Suhaimi et al., 2017). The NO₂ ($\mu\text{g m}^{-3}$) concentration was measured by means of passive monitoring with diffusion sampler (IVL, Goteborg, Sweden) for one week period with the limit of detection (LOD) of $0.5 \mu\text{g m}^{-3}$ and 10.0% of measurement uncertainty (Foldvary et al., 2017). NO₂ analysis was performed with spectrophotometer technique by the IVL Swedish Environmental Research Institute Laboratory (Goteborg, Sweden), an accredited laboratory.

Settled dust in the classrooms were collected by a 1800 W vacuum cleaner (Model PVC-31A, Pensonic, Penang, Malaysia) equipped with a special dust Millipore Filter with a pore size of 6 μm (ALK Abello, Horsholm, Denmark). The dust Millipore Filter was attached inside the filter holder (ALK Abello, Horsholm, Denmark) and fitted at the end of vacuum's tube. Dust vacuuming procedures were performed for a period of four minutes for each sample; two minutes on floor and another two minutes on other surfaces like desk, chair, bookshelf and softboard. The classroom was divided into the window (part 1) and the corridor (part 2), which were sampled separately. The collected dust samples were kept in an individual sterile zip lock plastic bag, transported to laboratory and stored in a low temperature freezer at -80 °C until extraction procedures. The dust sample from part 1 and part 2 were combined and sieved through a 0.3 mm mesh screen prior extraction procedures (Norback et al., 2014).

2.6. DNA extraction and amplicon sequencing and analysis

Fungal genomic DNA was extracted from 32 samples with a commercial EZ-10 Column Soil DNA Mini Prep Kit (BioBasic, Markham, Canada) according to the manufacturer's instruction with additional glass beads. Briefly, about 1.0 g of sieved dust sample was mixed well with 1.0 g of sterile 0.6 mm diameter glass beads (Sigma, St. Louis, MO), previously acid-washed (Rodrigues et al., 2018) and 0.5 mL of SCL solution. Then, it was incubated at 65 °C for 5 min. Afterward, the mixtures were centrifuged at 13,000 g at room temperature for 3 min. Supernatants were mixed well with equal amount of SP solution and the microtubes were cooled on ice for 10 min. The mixtures were centrifuged at 13,000 g for 3 min at room temperature. The supernatants were mixed with 0.2 mL chloroform and 1.5 vol of SB solution followed by centrifuged for 3 min at 13,000 g. Final mixtures were transferred into EZ-10 spin column and washed twice with 1.0 mL wash solution by centrifuging each time at 12,000 g for 30 s to pellet cells debris. The spin column was then transferred into a new microtube and the DNA was eluted from the column with 50 μL of elution buffer. After incubation of two minutes at room temperature, the samples were then centrifuged for one minute at 12,000 g. The evaluation of DNA purity and concentration was done using NanoPhotometer P-Class (P360) (IMPLEN, Schatzbogen, Germany) by determining the spectrophotometer ratios A_{260}/A_{280} . The DNA purity and concentration of all samples were in the range between 1.8 and 2.0 and 25–816 ng/ μL , respectively. The DNA products were stored at -20 °C before further analysed using amplicon sequencing technique.

Amplification of the fungal internal transcribed spacer (ITS) 1 region used the primer pair ITS1–30 F/ITS1–217R (Usyk et al., 2017) with partial Illumina Nextera adapter incorporated their 5' end. The PCR reaction was performed using YourTaq™ Direct-Load PCR Mix

(Biotechrabbit, Hennigsdorf, Germany) supplemented with 2.5% DMSO, with PCR conditions of 95 °C for 180 s, followed by 30 cycles of 95 °C for 15 s, 60 °C for 20 s and 72 °C for 30 s. The indexed PCR products were pooled, bead-purified and quantified using Denovix high-sensitivity fluorescence quantification kit (Denovix, Delaware, USA). The library was sequenced on an ISeq100 (Illumina, San Diego, CA) using the run configuration of 1×300 bp to maximize information on the Read 1. Sequencing was performed at certified laboratory GeneSEQ, Rawang, Selangor Malaysia.

Single-end fastq demultiplexed fastq files generated were first trimmed with Cutadapt v1.18 (Martin, 2011) to remove the non-biological forward and reverse primer sequences located on the 5' and 3' ends of each read, respectively. The trimmed reads were used as the input for amplicon sequence variant (ASV) generation and abundance table construction using DADA2 (Callahan et al., 2016) which is part of the QIIME2 pipeline (Bolyen et al., 2019). Taxonomic assignment of the ASVs used the QIIME2 scikit-learn naive Bayes machine-learning classifier (Bokulich et al., 2018) that was trained on the QIIME2 Classifier with UNITE database (version 04.02.2020) (Nilsson et al., 2019). Finally, MicrobiomeAnalyst, web-based platform was used in the generation of taxa abundance plots, rarefaction curves and alpha diversity from QIIME2. For the estimation of core microbiota, we defined ASVs that are present in more than 75% of the samples as core ASVs.

2.7. Statistical analysis

Descriptive test analysis was performed by the Statistical Package for Social Science (SPSS) 25.0. The Chi-square and Mann-Whitney tests were used to evaluate the association and differences between two groups. Exploration of data was performed using Principle Component Analysis (PCA) and Agglomerative Hierarchical Clustering (AHC) to reduce the variables dimensionality and clustering the the biomarkers with relative abundance of taxa. Apart from that, correlation of taxa with indoor parameters were estimated with Spearman correlation test. The final prediction models of asthma development were generated by logistic regression analysis with take in the potential predictors atopy, gender, parental asthma/allergy and family member smoking status. The models performance were based on the coefficient of determinant, overall accuracy, sensitivity and specificity. We used log-transformed data of indoor parameters and biomarkers to improve normality in the chemometrics and regression analysis. The multivariate analysis was carried out by using the Statistical Package XLSTAT Evaluation 2019.2.3 (Addinsoft, New York, US).

3. Results

The characteristics of 96 school children are described in Table 1. Atopy (65.0%) and parental asthma/allergy (65.9%) were more prevalent in the groups of clinically-diagnosed asthma ($p < 0.05$). There were no significant difference of asthma prevalence between gender, school areas and family member smoking status ($p > 0.05$). The majority of asthmatic children were tested positive for both house dust mites (Derp1 and Derf1) (60.4%), followed by cat dander (22.9%) (Table S1). The FeNO levels was statistically higher among doctor diagnosed asthmatic than healthy children ($p < 0.05$). In addition, the expression of CD11b on eosinophil and neutrophil in sputum samples of doctor diagnosed asthma children were significantly higher compared to healthy children ($p < 0.05$). Similarly, the expression of CD63 on the neutrophil was higher in samples of doctor diagnosed asthma children ($p < 0.001$).

3.1. Levels of indoor pollutants

A comparable analysis of indoor pollutants between suburban and urban school area are shown in Table S2. The concentration of NO₂, PM_{2.5}, PM₁₀ and formaldehyde were significantly higher in classrooms at urban area than suburban ($p < 0.001$). All the classrooms for both

Table 1

School children's characteristic and inflammation status for doctor diagnosed asthma and control group (N = 97).

Characteristics	Doctor diagnosed asthma (n = 50)	Healthy (n = 46)	p-value
Female (%)	44.2	55.8	0.094
Male (%)	61.4	38.6	
Atopic (%)	65.0	35.0	< 0.001 **
Non atopic (%)	30.6	69.4	
Parental asthma/ allergy-Yes (%)	65.9	34.1	0.020*
Parental asthma/ allergy-No (%)	41.8	58.2	
Family member smoking-Yes (%)	56.9	43.1	0.169
Family member smoking-No (%)	41.9	58.1	
School area- urban (%)	60.4	39.6	0.102
School area- suburban (%)	43.8	56.3	
Clinical characteristics			
FeNO levels (ppb)	56 (66.5)	23 (32.3)	0.002*
Eosinophil count (%)	11.6 (8.7)	10.2 (9.2)	0.259
Neutrophil count (%)	11.6 (5.1)	13.4 (14.0)	0.130
CD11b (Eos) ^a	13.0 (6.1)	7.1 (6.7)	< 0.001 **
CD35 (Eos) ^a	16.6 (6.6)	20.1 (19.1)	0.242
CD63 (Eos)	25.1 (15.5)	16.3 (39.1)	0.346
CD66b (Eos) ^a	1.2 (1.3)	0.6 (2.6)	0.788
CD11b (Neu) ^b	27.1 (6.8)	23.6 (10.8)	0.022*
CD35 (Neu)	0.9 (0.8)	1.5 (2.2)	0.221
CD63 (Neu)	31.3 (26.7)	22.4 (14.2)	< 0.001 **
CD66b (Neu)	0.6 (1.4)	0.4 (1.1)	0.647

Values are median (IQR) for clinical characteristics. IQR= Interquartile range.

Eos= eosinophil, Neu= neutrophil.

* p < 0.05;

** p < 0.001

^a Activation marker for eosinophil,

^b Activation marker for neutrophil.

areas were designed with natural ventilation and equipped with windowpanes on both sides of the wall. There were an average of three ceiling fans in each classroom and the floor surface was furnished with concrete. The classroom were equipped with bookshelves, whiteboard, and softboards.

3.2. Diversity of fungal

A total of 32 settled dust samples were collected inside the classrooms from both areas, urban and suburban. The diversity of fungal was successfully characterized by application of DNA extraction and amplicon gene sequencing. Based on 97% similarity, the clustering sequences recovered 1869 unique of OTUs from the dust samples. Overall, four fungal phyla were identified as shown in Fig. 1. The observed OTUs richness for Ascomycota, Basidiomycota, Glomeromycota and Chytridiomycota were 60.7%, 36.4%, 2.9% and 0.04%, respectively. Ascomycota and Basidiomycota phyla were relatively more abundant in dust samples collected from urban schools (33.2%) and suburban schools (20.7%), respectively. Taxonomic identification at family levels revealed that the Aspergillaceae (20.0%), Teratosphaeriaceae (11.8%) and Didymosphaeriaceae (5.5%) were the top three highest abundant families. Only 154 (33.9%) taxa were identified at genus levels and 109 (25.6%) at species levels. At the genus level, the top eight highest abundant genera in all dust samples were *Aspergillus* (15.8%), *Verrucoconiothyrium* (5.5%), *Ganoderma* (4.6%), *Hyphoderma* (4.2%), *Heterochaete* (3.8%), *Cladosporium* (3.4%), *Wallemia* (3.3%), *Cystobasidium* (1.3%) and *Duportella* (1.1%) (Fig. S3). The remaining genera represented less than 1.0% of the overall abundance and 50.7% of the sequences were not classified. At the species levels, *Aspergillus clavatus* was the most abundant in urban samples followed by *Hyphoderma multicystidium*, *Verrucoconiothyrium prosopidis*, *Ganoderma tuberculosum* and *Heterochaete shearii* with the total percentages of 37.8%, 13.6%, 11.2%, 9.2% and 5.9%, respectively. Similarly, for suburban samples, *A. clavatus* was the most abundant followed by *Cladosporium halotolerans*, *H. multicystidium*, *G. tuberculosum*, *Wallemia tropicalis* and *H. shearii* with the total percentages of 15.2%, 10.8%, 10.6%, 9.2%, 9.1% and 8.5%, respectively (Fig. 2). The relative abundance at class and family levels were presented in Fig. S4 and Fig. S5, respectively. Over 109 species were identified in all dust samples, 25 major species with

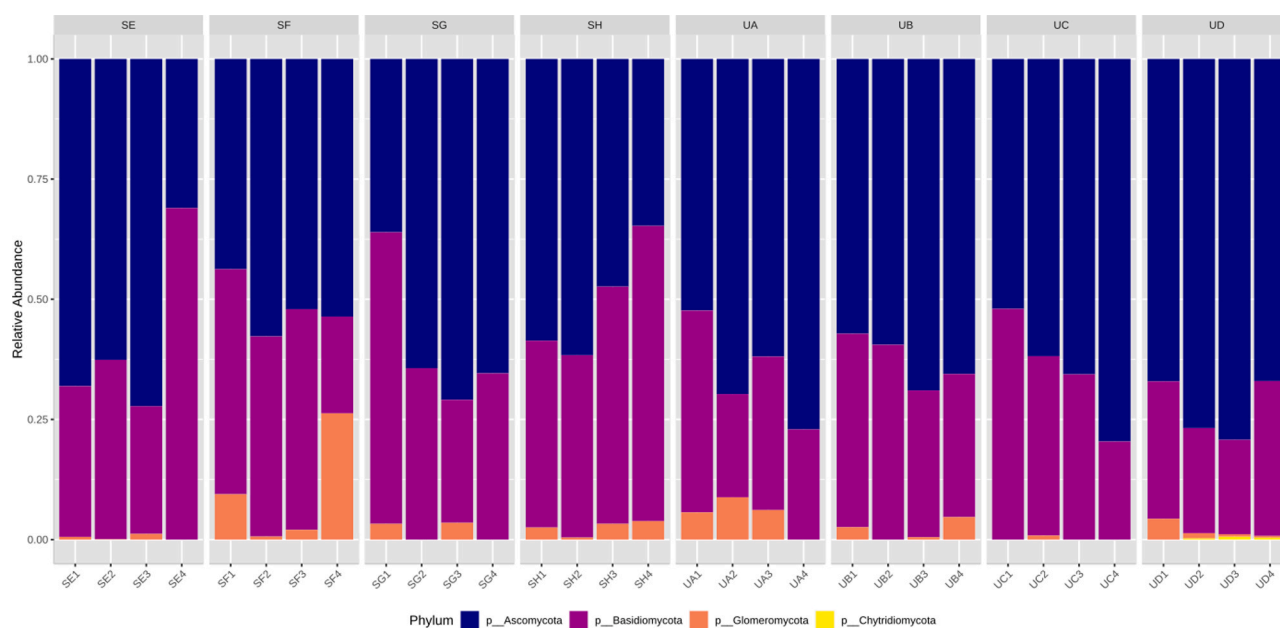


Fig. 1. Relative abundance of fungal identified at the phylum level in settled dust collected in 32 classrooms. Each sample was labelled with area, school name and class number. Samples for suburban schools (S) were labelled SE, SF, SG, and SH, while for urban schools (U) were labelled UA, UB, UC and UD.

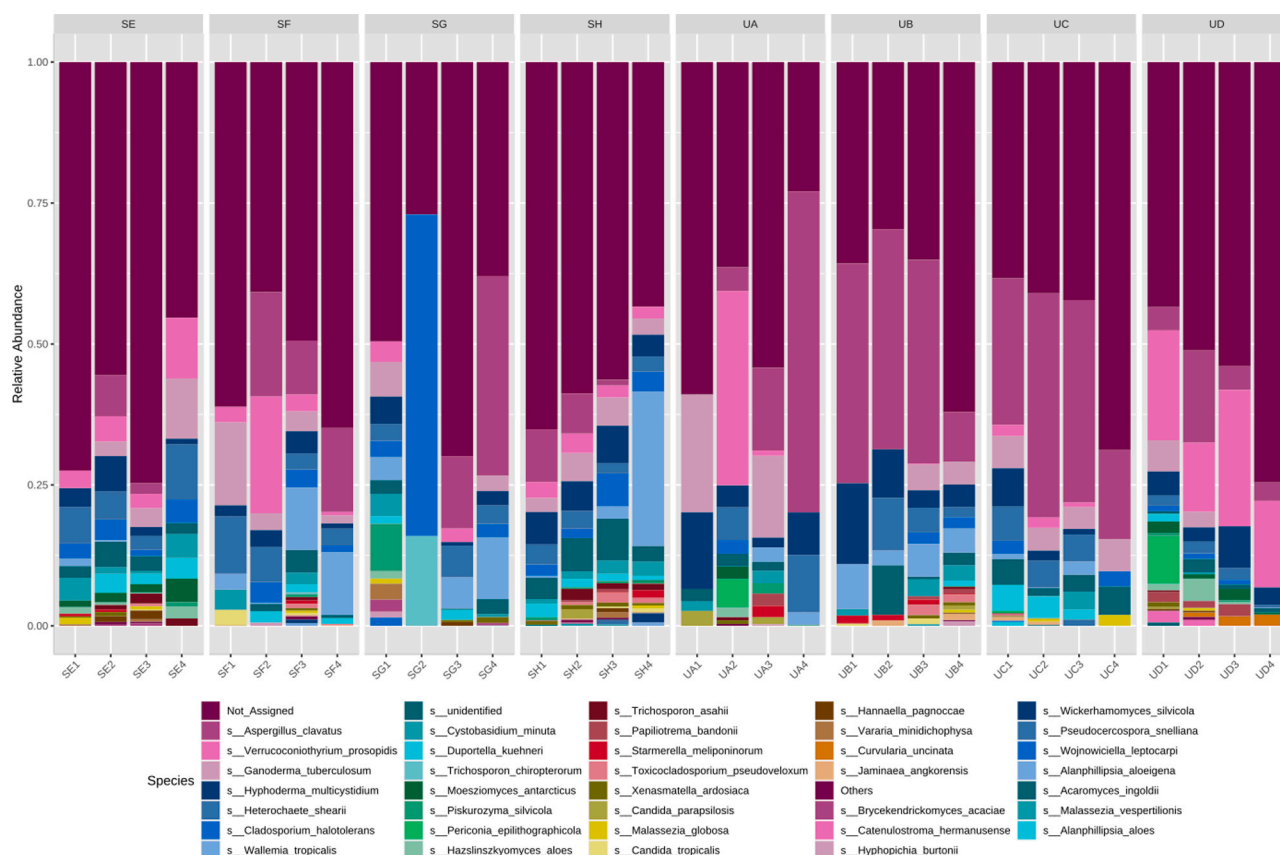


Fig. 2. Relative abundance of fungal identified at the species level in settled dust collected in classrooms.

percentage higher than 1% in one sample were chosen for the analysis in revealing the association with indoor pollutants and biomarkers towards development of asthma among school children (Table S3). We performed principle component analysis (PCA) to reduce the dimensionality of the OTUs by considering the smaller percentage of important variables before proceeding with Agglomerative Hierarchical Clustering

(AHC). Only taxa with loading factors > 0.5 in Factor 1 to Factor 3 with Eigenvalue > 1 and showed interaction with indoor pollutants were further analysed in the regression models (Table S4).

The alpha diversity in each sample was evaluated using OTU richness and Shannon index with rarefaction curves based on 97% similarity. The continually rarefaction curves against the sequencing depth suggest

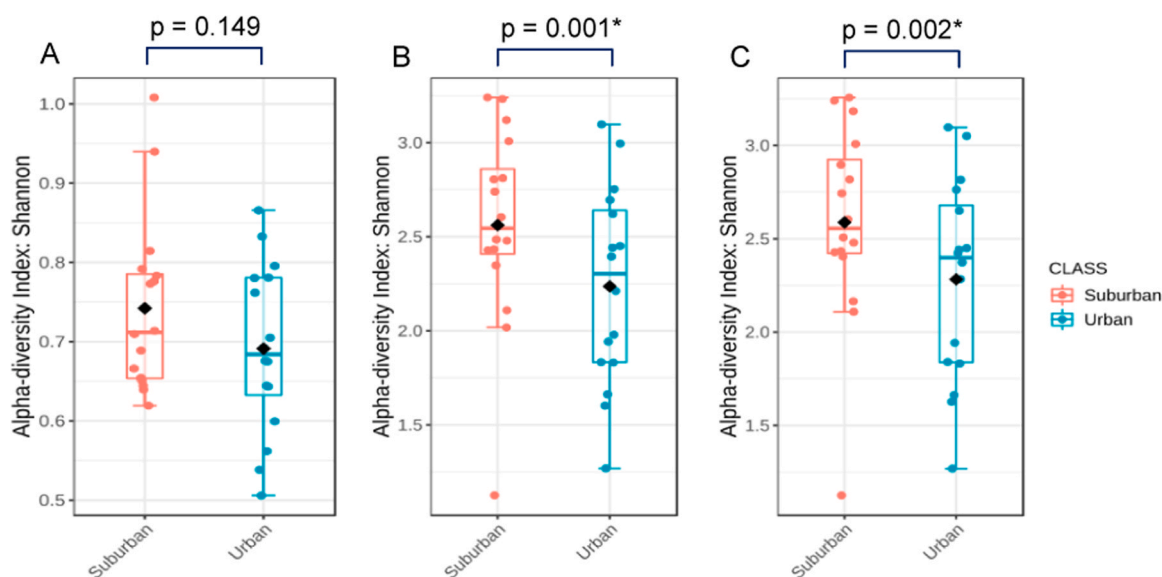


Fig. 3. Comparison diversity of fungal at phylum (A), genus (B) and species (C) levels between settled dust collected from suburban (red) and urban (blue) school areas using Shannon diversity index. The significance of difference between urban and suburban school areas was tested using Mann-Whitney test. The line inside the box represents the median value and whiskers represent the lowest and highest values within 1.5 interquartile range. The outliers values were shown as small circles. * $p < 0.05$.

that the sampling sequencing process was sufficient to capture the overall fungal diversities in the samples and reflect the fungal richness and evenness (Fig. S6). Furthermore, analysis of OTUs in eight schools samples indicated that samples from SH, followed by SE and UB had more fungal species than other schools. This was confirmed by the Shannon index, which fungal diversity of SH (Shannon = 6.15), UB (Shannon = 5.64) SG (Shannon = 5.42) and UD (Shannon = 5.41) was higher than SE (Shannon = 5.20), UC (Shannon = 4.80), UA (Shannon = 4.57) and SF (Shannon = 4.10) (Table S5). Analysis between subcategory of suburban and urban area of school location showed that samples from suburban had more fungal diversity than urban area at phylum, genus and species levels. However, there were significant difference in fungal diversity at genus and species level between these two school locations ($p < 0.05$) (Fig. 3).

3.3. Contribution of environmental parameters to fungal diversity

We identified several significant correlation between relative abundance at species levels and environmental parameters (Fig. 4). The relative abundance of *C. halotolerans* ($r = 0.359$, $p = 0.044$) was positively associated with relative humidity, while, *H. shearii* ($r = -0.361$, $p = 0.043$), *Papiliotrema bandonii* ($r = 0.560$, $p < 0.001$), and *Trichosporon asahii* ($r = -0.391$, $p = 0.028$) were correlated with temperature. *Hazslinszkyomyces aloes* ($r = -0.473$, $p = 0.007$), *Jamniaea angkorensis* ($r = 0.427$, $p = 0.016$) and *Moesziomyces antarcticus* ($r = -0.407$, $p = 0.021$) showed correlation with the concentration of CO_2 . Whereas, *Starmerella meliponinorum* ($r = -0.475$, $p = 0.006$) and *T. asahii* ($r = -0.359$, $p = 0.044$) were negatively correlated with the concentration of NO_2 . Only *C. halotolerans* ($r = -0.369$, $p = 0.039$) and

Xylodictyochaeta lusitanica ($r = -0.364$, $p = 0.041$) showed negative association with the concentration of PM_{10} and $\text{PM}_{2.5}$. The relative abundance of *C. halotolerans* ($r = -0.481$, $p = 0.006$), *Toxicocladosporium pseudoveloxum* ($r = -0.365$, $p = 0.041$), *S. meliponinorum* ($r = -0.401$, $p = 0.024$), and *T. asahii* ($r = -0.372$, $p = 0.037$) were negatively correlated with the concentration of formaldehyde. Overall, relative abundance of most fungal have relatively lower dependencies with indoor parameters tested in this correlation ($p > 0.05$).

3.4. Chemometrics analysis of environmental parameters and biomarkers

We further characterized the indoor pollutants, relative abundance of fungal at species levels with biomarkers expression on eosinophil and neutrophil based on their homogeneity levels using Agglomerative Hierarchical Clustering (AHC) (Fig. 5). There were three cluster generated for eosinophils. Cluster 1 with 59.4% of variance within-class consisted of relative humidity, concentration of CO_2 , relative abundance of *C. halotolerans*, *T. pseudoveloxum*, *Brycekendrickomyces acaciae*, *Candida tropicalis*, *S. meliponinorum*, *X. lusitanica*, *Xenasmattella ardosiaca*, *J. angkorensis*, *Hannaella pagnoccae*, and *T. asahii*, FeNO levels, percentage of eosinophil and CD66b. Cluster 2 showed 37.1% of variation within-class for concentration of PM_{10} , and $\text{PM}_{2.5}$, and formaldehyde, temperature, relative abundance of *A. clavatus* and *H. shearii*, *Hyphoderma multicystidium*, *W. tropicalis* as well as CD11b. The concentration of NO_2 , relative abundance of *H. aloes*, *V. prosopidis*, *Candida parapsilosis*, *Malassezia globosa*, *P. bandonii*, and *M. antarcticus* were grouped in Cluster 3 with 96.7% of variation within-class. Likewise, three clusters were generated for neutrophil with 90.4% of variation within-class for Cluster 1 which consisted of concentration of NO_2 , FeNO levels, CD11b,

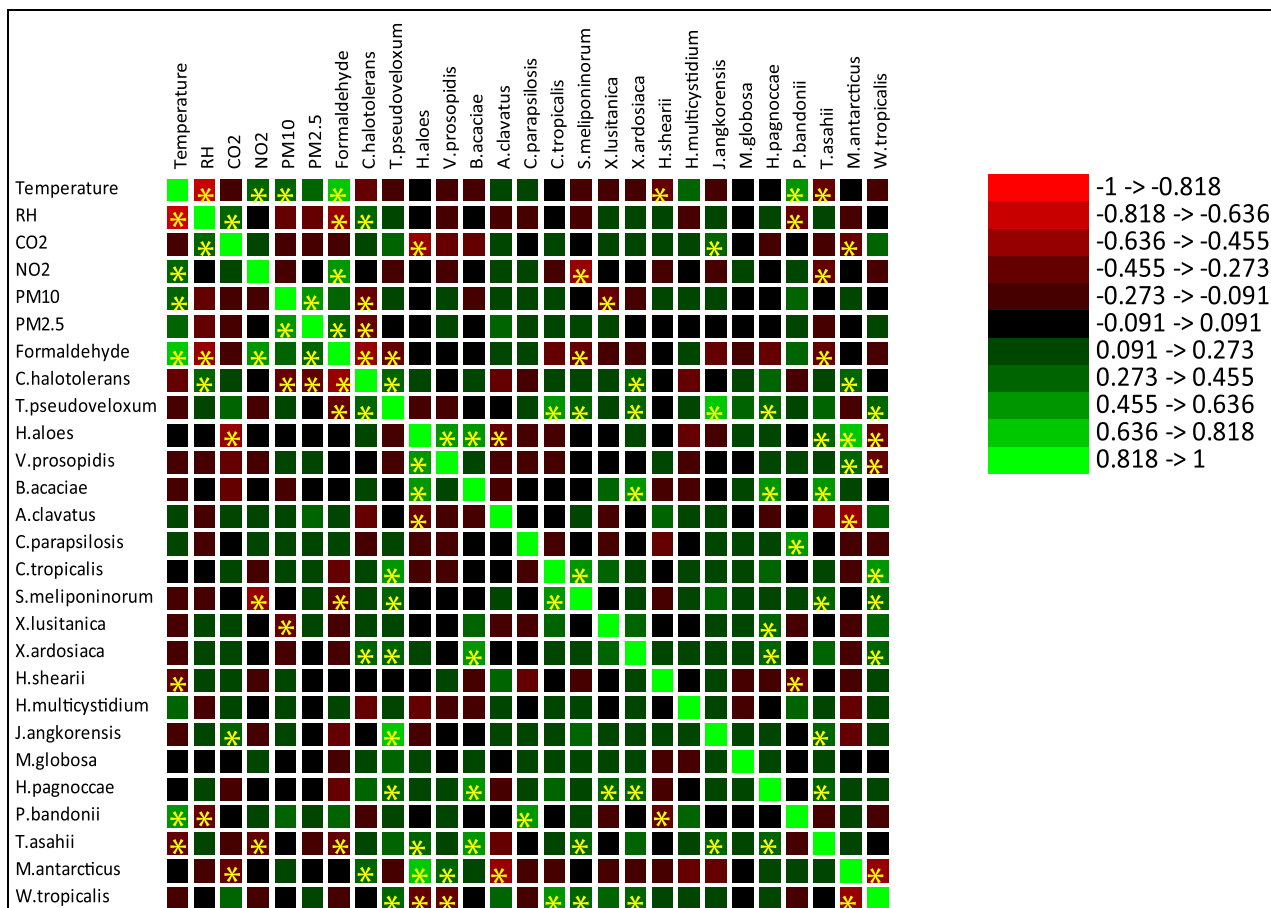


Fig. 4. Association analysis of environmental parameters with relative abundance of fungi at the species levels. The heat maps shows the correlation coefficient (r) values, which are indicative of the association. Significant association at $p < 0.05$ was marked with asterisk symbol (*).

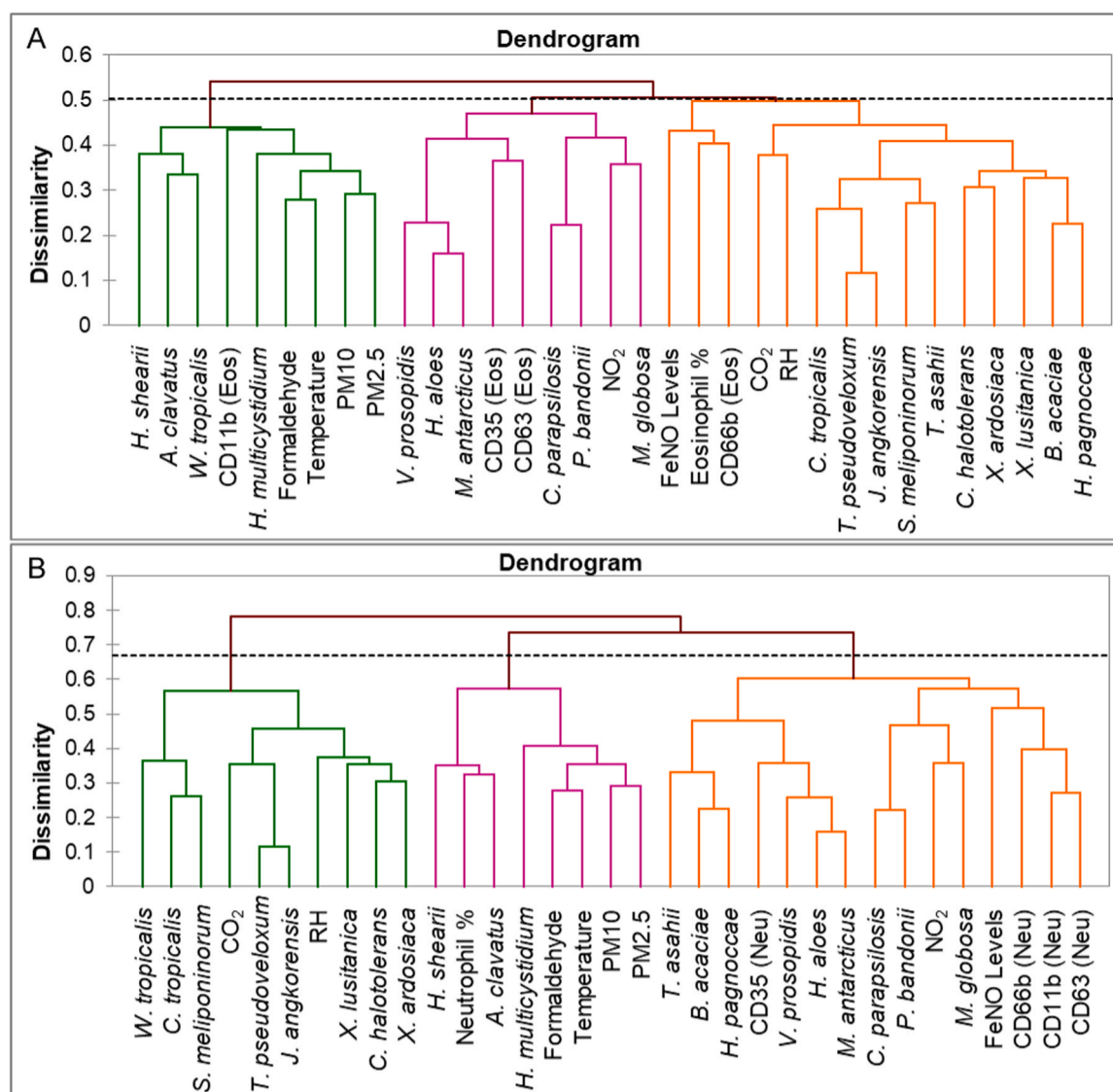


Fig. 5. Agglomerative Hierarchical Clustering (AHC) analysis using Ward linkage method and exploiting Euclidean distance to generate the clustering of environmental pollutants, relative abundance of fungal at species level and biomarkers for (A) eosinophil and (B) neutrophil.

CD35, CD63, CD66b, relative abundance of *H. aloes*, *V. prosopidis*, *B. acaciae*, *C. parapsilosis*, *M. globosa*, *H. pagnoccae*, *P. bandonii*, *T. asahii*, and *M. antarcticus*. Cluster 2 presented temperature, concentration of PM₁₀, PM_{2.5}, and formaldehyde, relative abundance of *A. clavatus*, *H. shearii* and *H. multicystidium*, and percentage of neutrophil with 56.0% of variation within-class. The concentration of CO₂, relative humidity, relative abundance of *C. halotolerans*, *T. pseudoveloxyum*, *Candida tropicalis*, *S. meliponinorum*, *X. lusitanica*, *X. ardosiacae*, *J. angkorensis* and *W. tropicalis* were grouped in Cluster 3 with 31.2% of variation within-class.

3.5. Binary Logistic Regression analysis

We further built models to predict the toxicodynamic effects of indoor air pollutants and relative abundance of fungal at species level towards the marker expression on the eosinophil and neutrophil in the sputum samples among doctor diagnosed asthma and healthy children. To gain insight into these objectives, we applied the binary logistic regression with the potential confounders of atopy, parental asthma/allergy status, gender, family member smoking status and area of schools. For Cluster 1, we observed that relative humidity, and relative

abundance of *T. asahii* were associated with down regulation of CD66b expressed on eosinophil, and elevation of FeNO levels in predicting asthmatic children with accuracy of 63.6% and 88.0% sensitivity. The likely risk were observed among school children in urban area and parental with asthma/allergy condition. Cluster 2 showed an overall accuracy of 58.1% and 82.0% sensitivity in predicting asthmatic children with positive significant association of relative abundance of *A. clavatus* with exposure to concentration of PM_{2.5} with upregulation of CD11b expressed on eosinophil. Cluster 3 demonstrated that concentration of NO₂ were positively associated with relative abundance of *P. bandonii* and upregulation of CD63 expressed on eosinophil in predicting asthmatic children with accuracy of 58.6% and 82.0% sensitivity (Table 2).

In the model generated for neutrophil, Cluster 1 presented an overall accuracy of 59.1% and sensitivity of 86.0% in predicting asthmatic. Exposure to the concentration of NO₂ and *P. bandonii* were associated with upregulation of CD63. Children from urban school area with the condition of atopy, parental with asthma/allergy status, and family member smokers were more likely to develop the risk. There were significant association of *A. clavatus*, *Hy. multicystidium* and *H. shearii* with the concentration of formaldehyde with neutrophil percentage in

Table 2

Logistic regression analysis based on the clusters generated from AHC analysis.

Predictors	β	SE	OR (95% CI)	p-value
Eosinophil				
Cluster 1				
Intercept	-89.025	36.552		0.015*
FeNO Levels	5.122	2.062	1.677 (2.945–9.550)	0.013*
CD66b	-2.788	1.361	0.062 (0.004–0.887)	0.041*
Relative humidity	40.122	17.372	2.661 (4.343–16.332)	0.021*
<i>T. asahii</i>	-9.829	3.129	0.120 (0.002–0.025)	0.002*
Urban	6.023	2.297	4.126 (4.577–37.204)	0.009*
Parental Asthma/ Allergy ^a	2.952	1.2	19.153 (1.823–20.125)	0.014*
R ²	0.847			
Accuracy/Sensitivity/ Specificity	63.6%/	88.0%/	55.6%	
Cluster 2				
Intercept	-33.329	12.822		0.009*
CD11b	3.534	1.394	3.426 (2.231–5.261)	0.011*
PM _{2.5}	19.634	9.005	3.371 (7.279–15.611)	0.029*
<i>A. clavatus</i>	0.316	0.081	1.371 (1.170–1.608)	< 0.001**
<i>H. shearii</i>	-0.902	0.335	0.406 (0.210–0.782)	0.007*
Atopy	2.403	0.862	11.06 (2.043–59.873)	0.005*
R ²	0.732			
Accuracy/ Sensitivity/ Specificity	58.1%/	82.0%/	50.3%	
Cluster 3				
Intercept	-24.233	8.388		0.004*
CD63	2.508	1.219	12.275 (1.126–13.376)	0.040*
NO ₂	16.481	4.953	1.437 (8.735–23.611)	0.001*
<i>P. bandonii</i>	10.386	3.501	3.239 (3.391–30.938)	0.003*
Suburban	3.165	1.24	23.683 (2.084–26.914)	0.011*
Atopy	1.822	0.814	6.185 (1.254–3.489)	0.025*
Female	1.991	0.934	7.322 (1.173–4.571)	0.033*
Parental Asthma/ Allergy ^a	2.463	0.978	11.735 (1.724–7.986)	0.012*
Family Member Smoking ^b	2.853	1.059	17.344 (2.176–13.822)	0.007*
R ²	0.742			
Accuracy/ Sensitivity/ Specificity	58.6%/	82.0%/	51.0%	
Neutrophil				
Cluster 1				
Intercept	-45.47	19.252		0.018*
CD63	15.733	5.83	6.804 (7.417–62.411)	0.007*
NO ₂	21.634	8.91	2.490 (6.472–9.551)	0.015*
<i>P. bandonii</i>	17.488	6.406	3.935 (1.387–11.213)	0.006*
<i>T. asahii</i>	-5.371	2.289	0.005 (0.002–0.413)	0.019*
R ²	0.675			
Accuracy/Sensitivity/ Specificity	59.1%/	86.0%/	50.3%	
Cluster 2				
Intercept	-5.655	2.407		0.019*
Neutrophil %	-3.724	1.941	0.024 (0.002–0.840)	0.045*
Formaldehyde	3.953	1.376	52.081 (3.514–7.718)	0.004*

Table 2 (continued)

Predictors	β	SE	OR (95% CI)	p-value
<i>A. clavatus</i>	0.385	0.093	1.469 (1.224–1.763)	< 0.001**
<i>H. shearii</i>	-1.566	0.463	0.209 (0.084–0.518)	0.001*
<i>H. multicystidium</i>	0.501	0.205	1.651 (1.103–2.469)	0.015*
Atopy	2.318	1.057	10.153 (1.279–80.573)	0.028*
Parental Asthma/ Allergy ^a	1.819	0.865	6.163 (1.131–33.572)	0.036*
Family Member Smoking ^b	1.721	0.873	5.593 (1.011–30.952)	0.048*
R ²	0.767			
Accuracy/Sensitivity/ Specificity	58.6%/	84.0%/	50.3%	
Cluster 3				
Intercept	-89.892	33.333		0.007*
CO ₂	33.248	12.337	2.749 (8.663–87.285)	0.007*
<i>T. pseudoveloxtum</i>	-2.010	0.692	0.134 (0.034–0.520)	0.004*
<i>S. meliponinorum</i>	-1.174	0.550	0.309 (0.105–0.909)	0.033*
<i>X. ardosiacca</i>	1.756	0.681	5.787 (1.524–21.982)	0.010*
<i>J. angkorensis</i>	2.310	1.028	10.075 (1.342–75.622)	0.025*
Urban	1.078	0.446	2.940 (1.227–7.045)	0.016*
Atopy	1.534	0.404	4.635 (2.100–10.234)	< 0.001**
Parental Asthma/ Allergy ^a	1.308	0.398	3.699 (1.694–8.076)	< 0.001**
Family Member Smoking ^b	1.040	0.414	2.829 (1.258–6.364)	0.012*
R ²	0.441			
Accuracy/ Sensitivity/ Specificity	55.2%/	86.0%/	45.1%	

OR = Odd ratio, CI = Confidence interval, R² = Coefficient of determination, SE = Standard error

^a Parental with asthma/allergy status;

^b Family member who are smoking

* p < 0.05,

** p < 0.001

Cluster 2. As for Cluster 3, the concentration of CO₂ and relative humidity were not significantly associated with *C. halotolerans*, *T. pseudoveloxtum*, *C. tropicalis*, *X. ardosiacca* and *W. tropicalis*.

4. Discussion

The prevalence of doctor's diagnosed asthma among school children in Hulu Langat, Selangor (10.6%) was marginally similar to study conducted in Penang, Malaysia (10.3%) (Norback et al., 2017a, 2017b), nevertheless higher than reported among children from Bangkok, Thailand (8.8%) (Chinratanaipisit et al., 2019) and Middle East (7.57%) (Mirzaei et al., 2017). Moreover, allergy to house dust mites were common among asthmatic children (Derp1 = 70.0%, Derf1 = 68.0%, mix = 55.7%) and this prevalence was higher than previous study reported (Fu et al., 2020a). According to Nadzrah et al. (2015), 82.4% of the Asian population had a positive response toward house dust mites compared to Europeans (41.2%). Literature had also demonstrated that the prevalence of allergy among children was increasing substantially in many countries (Downs et al., 2001). Similar pattern of association was observed between atopy and doctor diagnosed asthma children with other studies conducted previously (Ferrante and Grutta, 2018; Rhee et al., 2018; Simpson et al., 2010).

The fact that FeNO levels and integrin are strongly associated with lung inflammation ascends its roles as useful biomarkers in evaluating the aetiology, phenotyping, and treatment of respiratory illness (Kim

et al., 2016). In this study, the FeNO levels were significantly higher among asthmatic school children with the average value above the threshold of 50 ppb, which could reflect a high degree of airway inflammation. This similar pattern was observed in previous studies conducted in Peninsular Malaysia (Ma'pol et al., 2019; Norback et al., 2017a, 2017b) and China (Xu et al., 2011). The finding of this study revealed that upregulation of tertiary and azurophilic/crystalloid granules markers, CD11b and CD63 on both eosinophil and neutrophil cells as reported by Tak et al. (2015) among asthmatic patients. The upregulation of these classical activation markers is associated with the circulating cytokines that bind with plasma membrane or stress signal which in response to the stimulus (Kim et al., 2017). The mitogen-activated protein kinase (MAPK) pathway is believed to be central to the degranulation process of these markers (Carr et al., 2016).

The concentration of PM₁₀ and PM_{2.5} measured inside the classroom were below the 24 h mean of the World Health Organisation (WHO) guideline, the National Ambient Air Quality Standard by USEPA, and new Malaysian Ambient Air Quality Standard 2018 Interim Target-2. Similarly, the median levels of NO₂ and CO₂ for urban and suburban schools were below the WHO guideline of 40 µg/m³ (annual mean) for NO₂ and American Society of Heating, Refrigerating and Air-Conditioning Engineers (ASHRAE) recommended limit of 1000 ppm for CO₂ (ASHRAE, 2001; WHO, 2005). Overall, the levels of indoor air pollutants measured in schools were below the guideline limits. This was due to inflow of outdoor air by natural ventilation and adequately ceiling fans could have a large impact on indoor air parameters. The classroom designs with a wider windows on both side of the classroom suit the temperature of the equatorial region and is able to reduce the NO₂, CO₂ and particles concentrations (Schibuola et al., 2016).

Commonly, fungal association studies with asthma have focused on detecting several fungal genera such as Aspergillus, Penicillium, Alternaria and Cladosporium (Shin et al., 2015). However, amplicon sequencing analysis have recently merged to identify the potential allergenic fungi which may be influenced by the indoor air pollutants and affects human health. In this study, it has shown that Ascomycota and Basidiomycota were most abundant phylum in both areas, urban and suburban. This is consistent with the predominance of both the phyla in the biosphere, where Ascomycota have single-celled or filamentous vegetative growth forms and easily aerosolized compared to Basidiomycota (Guo et al., 2020; Yan et al., 2016). In this study, there is more similarity in fungal diversity at genus level compared to previous study conducted at Johor Bahru, Malaysia using the same sampling technique, where Aspergillus (16.6%) followed by Penicillium (10.2%) and Cladosporium (7.8%) (Fu et al., 2020b) as the three most dominant genera. Interestingly, *A. clavatus* was detected in almost all samples and one previous study reported that phylogenetic analysis suggested that strains of *A. clavatus*, *A. hiratsukae*, *A. lentulus* and *A. fumigatus* were in the same monophyletic clade (Visagie et al., 2014). They also reported that *A. clavatus* was only isolated from dust collected in Thailand and it is possible that this strain is geographically restricted in tropical country.

The variation of fungal richness can be contributed by the environmental characteristics. Some crucial factors influencing the fungal growth and release into the atmosphere include NO₂, PM₁₀, SO₂, CO₂, temperature, relative humidity, urbanization, rainfall, and vegetation have been identified (Xu et al., 2017; Yan et al., 2016). Notably, *C. halotolerans* was abundantly identified in samples from suburban than urban schools and this is in accordance with the pure culture experiment conducted by Segers (2017), where the species grew optimally in temperature ranges of 5–30 °C and relative humidity of 75–84%. Results also showed that *C. halotolerans* and *X. lucitanica* were negatively correlated with concentration of PM₁₀ and PM_{2.5} and this could be due to other meteorological factors that affecting the release of different sizes of fungi debris in the atmosphere then transported by the particles and lead to uncertainty of fungal richness (Du et al., 2018). Moreover, the ventilation, air circulation and activities inside the classrooms, such as cleaning could also have impact on the richness of fungi (Cox et al.,

2017). The relationship of indoor pollutants and fungal diversity variation should be investigated in more detail.

To the researchers' knowledge, this study is the first study that explores the interrelation of different indoor air pollutants and richness of fungal with CD11b, CD63, CD35, and CD66b marker expression on eosinophils and neutrophils. This study revealed that *P. bandonii* and NO₂ were clustered together in the AHC analysis and regression results has shown they were positively associated with CD63 expressed on both eosinophil and neutrophil. *P. bandonii* as first described by Sampaio et al. (2002) with basidiospores allantoides size of 2–3 × 8–12 µm and considered to be phylogenetically close to *Cryptococcus allantoinivorans*, *Cryptococcus nemorosus*, *Cryptococcus perniciosus*, *Cryptococcus aureus*, *Cryptococcus flavescens* (*P. flavescens*) and *Papiliotrema laurentii* (Golubev et al., 2003; Middelhoven, 2004; Sampaio, 2004). Inhalation of the propagules from these taxa were associated with skin, lung and neurologic infection (Cogliati et al., 2017). Recently, study reported the ability of *P. laurentii* to produce allergenic extracellular enzymes including, amylase, lipase, pectinase, protease and xylanase (Kumla et al., 2020). This could explained the activation of CD63 expression on eosinophil and eosinophil in the sputum samples and attributed to inflammation reactions, resulting in a Th2-like cytokine response and the destruction of mucosal barrier functions (Denning et al., 2014; Inoue et al., 2020; Knutsen et al., 2012). Little information is available on the *P. bandonii* and the associated health effects, potentially because of its rare occurrence. Previous studies had demonstrated that cryptococcus phagosome development in the phagocytosis involves recruitment of major histocompatibility complex (MHC II), CD63 and lysosomal associated membrane protein 1 (LAMP1) to complete the degradation process (Johnston and May, 2013; Smith et al., 2015). Thus, *P. bandonii* can be an opportunistic pathogen and demanding further studies in the future. Furthermore, *P. bandonii* is generally more abundant and frequently found in soil and bird excreta. Similar finding was observed on the elevation of NO₂ exposure effects (18 h) on the circulating eosinophil and neutrophil counts in previous study conducted by Steenhof et al. (2014). They suggested that these results could have been due to the replenishment of granulocytes occur after more than 24 h exposed to the allergen or stimuli.

This study also showed that *A. clavatus* was associated with CD11b expression on eosinophil and elevation of neutrophil percentage. Aspergillus spp. are the most commonly encountered fungi associated with allergy and pulmonary respiratory diseases, which including *A. fumigatus*, *A. niger*, *A. oryzae*, *A. flavus*, and *A. terreus*. We found studies reported that *A. clavatus* is an opportunistic human pathogen which is also able to cause invasive aspergillosis, extrinsic alveolitis, endocarditis, mycotoxicoses-related diseases and neurologic disorders (Aquino et al., 2018; Richardson et al., 2019; Swilaiman et al., 2017). However, no previous reports were found regarding interaction between *A. clavatus* and leukocytes either in field nor in vitro. Therefore we alluded to the similar interaction possibility to *A. fumigatus* as it is phylogenetically close to the *A. clavatus* (Krimitzas et al., 2013; Samson et al., 2014). Moreover, this result is consistent with the results in the previous in vivo experiment conducted by Teschner et al. (2019) using CD11^{−/−} mice infected with *A. fumigatus*. They found that polymorphonuclear neutrophil (PMN) were highly infiltrated, which contribute to fungal killing and attenuated of CD11b in the bronchoalveolar lavage (BAL) after 24 h infection. Attenuation of CD11b activation could be due to early innate responses toward *A. fumigatus* infection, which CD11b⁺ had a significant roles in mediating the Th2 cell and eosinophil recruitment in the airways via STAT6-dependent chemokine production (Medoff et al., 2009). In addition, Figueiredo and Neves (2018) provided an evidence to reinforce our finding and stated that human eosinophils cytolytically release the extracellular DNA traps (EETs) through CD11b activation and the Syk kinase pathway in response to *A. fumigatus* to immobilize, trap, and potentially kill them. Similarly, Tak et al. (2015) reported that of the classical activation markers, CD11 and CD63, in both eosinophil and neutrophil cells were

increased in sputum samples of asthmatic children which associated with the circulating cytokines in response to the stimulus. Besides, a number of previous studies have reported that *aspergillus* spp. were positively associated with the concentration of PM_{2.5} in the air and soil samples (Cao et al., 2014; Degobbi et al., 2011; Wei et al., 2019). Interestingly, we found one study reported that *Aspergillus* spp. were tolerant to the formaldehyde and able to grow in the presence of formaldehyde up to 4000 mg/L (Yu et al., 2014).

Recently, systematic review yield a total of 140 cases of *T. asahii* infection for year 1996–2019 throughout Asia, Europe, North and South America and Africa, which only 10.7% were related to lung infection (Li et al., 2020). In this recent study, *T. asahii* was negatively associated with FeNO levels, CD63 expressed on neutrophil and down regulation of CD66b expressed on eosinophil in predicting asthma among school children. The discrepancy between the above mentioned studies can be justified by the different in samples used, which *T. asahii* was isolated from clinical samples. In fact, *T. asahii* is an opportunistic fungus that colonize the human skin, respiratory and gastrointestinal tracts (Montoya et al., 2018), which regulate the immune response with production of the capsular polysaccharide GXM, melanin, extracellular enzymes (hemolysins, proteases, lipases) and contains galactosaminogalactan (GAG) in the cell wall (Duarte-oliveira et al., 2017). However, few investigations have demonstrated that similar polysaccharides present in fungi possess antioxidant, anti-tumor, and anti-inflammatory characteristics that underlying the protective response (Liu et al., 2017; Ullah et al., 2019). Therefore, further investigation is warranted to justify the interaction between *T. asahii* isolated from environmental samples with asthma phenotyping and their potential protective characteristics. Similarly, so far no information is available on the medical importance of *H. shearii* and further studies are required on its interaction and habitual diversity as it was isolated from 27 dust samples (84.4%) in this current study.

Many epidemiology studies have reported that CO₂ was positively associated with the asthma prevalence (Aquino et al., 2018; Sims et al., 2020). Elevation of CO₂ concentrations appear to stimulate the sporulation of allergenic fungi and has implication for increasing the prevalence of allergy and asthma severity (Gabriel et al., 2016; Liu et al., 2019; Pringle, 2013). Similarly, in the last regression results for Cluster 3, the CO₂ was positively associated with relative abundance of *X. ardosiaca* and *J. angkorensis* in the development of asthma. School children from urban area with parental asthma/allergy and family member smoking status pose a significant risks, especially with atopy. It is possible that these taxa could trigger asthma because of their allergenic protein structure and small in size, which allows them to lodge deep in the airway system, and lead to allergic response and inflammation (Sipiczki and Kajdacs, 2009; Tham et al., 2017). CO₂ were emitted into the air through fossil fuels combustion and mobile exhaust which closely related to the anthropogenic activities in urban area (Gurney et al., 2019). It is unclear why the relative abundance of *T. pseudoveloxyum* and *S. meliponinorum* were negatively associated with asthma. Nevertheless, a further study is more necessary to explore the medical importance and environmental pollutants effects of rare and unculturable fungi including *H. multicystidium*, *H. shearii*, *T. pseudoveloxyum*, *X. ardosiaca* and *J. angkorensis*, which distributed in different ecosystems. Many fungi taxa that are potentially allergenic still undocumented and leave a barrier to establishing a clear link between respiratory problems and fungi exposure (Mbareche et al., 2020). Moreover, rare fungi have newly been implicated in human infection due to continues expansion of the investigative technology, human behaviors and environmental factors (Liu et al., 2018).

Several limitations of this study should be recognized. First, selection of the control group is biased by its inclusion and exclusion criteria, but we have been as inclusive as possible. Furthermore, the leukocyte cells are almost absent in samples from healthy individuals. Second, the physiologic parameters were not included in the analysis, which considered clinically relevant to verify the interrelationship between

asthma severity, airway inflammation, and environmental exposure. Moreover, the outdoor air quality was not monitored to represent overall exposure. Nevertheless, the indoor and outdoor ratios for all pollutants are expected to be constant throughout the year. This is supported by several studies conducted throughout Peninsular Malaysia (Abidin et al., 2014; Norback et al., 2017). Finally, it would be ideal to evaluate home environmental factors which potentially attribute to the respiratory outcomes.

5. Conclusion

In summary, the profile of fungi taxa in settled dust samples identified through metagenomic in this study provide a new perspective of intricate connections between indoor pollutants and asthma phenotype. Moreover, application of chemometrics technique for drawing the interactions have a greater accuracy and flexibility to be applied in environmental health study. In this context, findings from this study have revealed that rare and unculturable fungi taxa does potentially attributable to the risk of asthma development among school children, especially *P. bandonii*, *Aspergillus clavatus*, *T. asahii* and *H. multicystidium*. This study reinforced that activation and degranulation of eosinophil and neutrophil may play an important role in airway inflammation and asthma development through upregulation of CD11b and CD63, which also regulated with increase concentration of NO₂ and PM_{2.5}. Future endeavors to determine the temporal patterns of inflammatory biomarkers throughout air pollution exposure are anticipated to investigate this possibility.

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CRediT authorship contribution statement

Khairul Nizam Mohd Isa: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Data curation, Project administration. **Juliana Jalaludin:** Conceptualization, Methodology, Validation, Writing - review & editing, Supervision, Funding acquisition. **Saliza Mohd Elias:** Investigation. **Leslie Thian Lung Than:** Conceptualization, Methodology, Writing - review & editing. **Mohammed Abdulrazzaq Jabbar:** Investigation. **Ahmad Shakir Mohd Saudi:** Software, Funding acquisition. **Dan Norbäck:** Methodology, Resources. **Jamal Hisham Hashim:** Conceptualization, Methodology. **Zailina Hashim:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing - review & editing, Supervision, Project Administration, Funding acquisition.

Declaration of Competing Interest

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

Availability of Data

The datasets generated and/or analyzed in this current study are available in the NCBI database: <https://www.ncbi.nlm.nih.gov/sra/PRJNA670294>.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2021.112430](https://doi.org/10.1016/j.ecoenv.2021.112430).

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