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Original Research Article

Indoor metabolites and chemicals outperform microbiome in classifying childhood asthma and allergic rhinitis

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ABSTRACT

Indoor microorganisms impact asthma and allergic rhinitis (AR), but the associated microbial taxa often vary extensively due to climate and geographical variations. To provide more consistent environmental assessments, new perspectives on microbial exposure for asthma and AR are needed. Home dust from 97 cases (32 asthma alone, 37 AR alone, 28 comorbidity) and 52 age- and gender-matched controls in Shanghai, China, were analyzed using high-throughput shotgun metagenomic sequencing and liquid chromatography-mass spectrometry. Homes of healthy children were enriched with environmental microbes, including *Paracoccus*, *Pseudomonas*, and *Psychrobacter*, and metabolites like keto acids, indoles, pyridines, and flavonoids (astragalin, hesperidin) (False Discovery Rate < 0.05). A neural network co-occurrence probability analysis revealed that environmental microorganisms were involved in producing these keto acids, indoles, and pyridines. Conversely, homes of diseased children were enriched with mycotoxins and synthetic chemicals, including herbicides, insecticides, and food/cosmetic additives. Using a random forest model, characteristic metabolites and microorganisms in Shanghai homes were used to classify high and low prevalence of asthma/AR in an independent dataset in Malaysian schools ($N = 1290$). Indoor metabolites achieved an average accuracy of 74.9% and 77.1% in differentiating schools with high and low prevalence of asthma and AR, respectively, whereas indoor microorganisms only achieved 51.0% and 59.5%, respectively. These results suggest that indoor metabolites and chemicals rather than indoor microbiome are potentially superior environmental indicators for childhood asthma and AR. This study extends the traditional risk assessment focusing on allergens or air pollutants in childhood asthma and AR, thereby revealing potential novel intervention strategies for these diseases.

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1. Introduction

The prevalence of asthma and rhinitis has substantially increased since the 1940s, and they are the most prevalent chronic respiratory diseases today, affecting over one billion individuals worldwide [1]. Asthma and allergic rhinitis (AR) often occur as comorbid diseases, and patients with AR have a higher chance of developing asthma and vice versa [2].

Cortisol is the most widely used medication for asthma, but it can only alleviate symptoms without curing the disease. Consequently, preventing the onset and development of these diseases is of primary importance in asthma control. Various environmental factors have been suggested to be associated with asthma and AR, including air pollution, tobacco smoking, allergens, and indoor mold exposure [3]. However, they are still not enough to explain the increasing trend of the disease, such as air pollution and the decreased number of smokers globally [4].

Recent progress indicates that indoor microbial exposure is an important factor for disease onset and development [5]. Children living in the farm environment have a lower prevalence of asthma than children living in the urban environment [6], which has been connected with a high indoor microbiome diversity or specific composition [7–9]. Indoor microorganisms facilitate the maturation of the occupants' immune systems and regulate the homeostasis of leukocytes and cytokines, thus mitigating the risk of asthma and rhinitis [10].

Although the importance of indoor microorganisms has been acknowledged, it is still challenging to apply theoretical advances to practical applications, such as building an environmental microbiome index for allergic diseases. Major challenges include the extremely high diversity of indoor microorganisms, estimated to be approximately one trillion [11], and the geographic variation in indoor microbial composition [12,13]. Different regions have different health-related microorganisms [8,14]. Thus, it is almost impossible to build a universal microbial reference catalog for health assessment.

Alternative approaches, such as profiling microbial functional genes and metabolites, have shown promise. A study in Shanxi, China applied shotgun metagenomics to profile microbial functional genes in indoor dust [8]. The study found associations between the abundance of human disease pathways (immune systems, bacterial, and viral infectious diseases) and higher asthma/AR risks, and lower asthma/AR risks with metabolism pathways (amino acid metabolism, metabolism of co-factors, and vitamins). Microbial volatile organic compounds (MVOCs) have been characterized as risk factors for asthma [15]. Total indoor lipopolysaccharide exposure during childhood and adolescence was identified as a protective factor for asthma [16,17]. However, these studies used culture-dependent or low-throughput techniques to assess specific microbial metabolites. A recent study in Malaysia applied high throughput approaches and reported that indoor metabolites and chemicals could significantly affect asthma and wheezing outcomes in junior high school students [18].

In this study, we conducted a case–control study to compare home indoor microbes and metabolites in healthy children and diseased children with doctor-diagnosed asthma and/or AR. High-throughput shotgun metagenomic sequencing and liquid chromatography–mass spectrometry (LC–MS) were performed to characterize the home indoor microbial taxa and diversity, functional genes and pathways, and metabolites and chemicals. Our hypothesis is that indoor metabolites/chemicals may serve as better classifiers of asthma and AR than the indoor microbiome. Therefore, we aim to identify microbial features consistently associated with diseased status and develop models based on these features to differentiate cases and controls. We will validate the classification power of our models using an independent dataset of 1290 middle school students from three centers in Malaysia. Our findings have the potential to inform both environmental assessment and disease intervention strategies in the future.

2. Methods and materials

2.1. Study design and participants

Children (4–11 years old) with diagnosed asthma and/or AR (cases) and without any allergic diseases (controls) were enrolled simultaneously from November 2019 to March 2021 in Shanghai. Both cases and controls had lived in Shanghai for at least one year before recruitment.

Cases were recruited at Ruijin Hospital in Shanghai, Affiliated with the Shanghai Jiao Tong University Medical School. Ruijin Hospital is a public hospital open to all residents in Shanghai. The inclusion criteria of cases were diagnoses of asthma and/or AR with asthma attacks or AR symptoms in the last 12 months. The diagnosis followed the Guideline for the diagnosis and treatment of pediatric AR by the Chinese Medical Association [18,19], which was adopted from the Global Initiative for Asthma (GINA) guideline.

Controls were age- and gender-matched healthy children who had never had asthma or AR nor any related symptoms in the last 12 months, and did not have any other respiratory system diseases validated by clinical doctors. They were recruited from local communities in Shanghai. By comparison, the spatial distribution of children's home addresses was similar between cases and controls if grouped into 4 layers: "within the Inner Ring" "the Inner Ring to Middle Ring", "the Middle Ring to Outer Ring", and "outside the Outer Ring", according to the population distribution characteristics in Shanghai city (Table S1).

In total, 32 asthma, 37 AR, 28 comorbidity (with both asthma and AR), and 52 healthy children were included (Fig. 1). Personal information such as gender, age, birth weight, family income, number of siblings, and length of breastfeeding was obtained from a self-administered questionnaire answered by parents. The study design and protocols received approval from the ethical committee of the School of Public Health at Fudan University (ethics approval number IRB#2019-09-0778) and Ruijin Hospital (ethics approval number #2020-306). Informed consent was acquired from all individuals involved in the study.

2.2. Home dust sampling and indoor environment

Home dust samples were collected by customized vacuum cleaners. The vacuum process lasted 4 min per sample, with dust gathered in a sterile sampler with a 6 μm filter pore size. Dust samples consisted of dust deep in mattresses, pillows, and sofas, in particular the dust at the corner which was not easy to reach in regular cleaning, as well as the dust on the surface of curtains, floors, and tables in children's bedrooms [13,20]. To reduce the bias of room cleaning, we informed all parents not to clean children's bedrooms in the 3 days before we went to collect the dust. The vacuumed settled dust was passed through a metal mesh screen (pore size 0.3 mm) to obtain fine dust, which was then stored in a –80 °C freezer.

Indoor relative humidity and CO_2 levels were measured using Q-TRACK IAQ-validated air monitors from TSI (St. Paul, USA). Trained investigators documented indoor characteristics, including indoor signs of dampness/mold, presence of pets or plants, and children's exposure to passive smoking. According to the *Exposure Factors Handbook of Chinese Population: Children: 0–5 Years and 6–17 Years* [21,22], children aged 0–5 years and 6 years or older spend around 10–13 h and 10–14 h a day at home.

2.3. Shotgun metagenomic sequencing

Shotgun metagenomic sequencing was performed on the vacuum dust samples to analyze the indoor microbial composition. Total microbial DNA was extracted using a DNeasy PowerSoil Pro Kit from Qiagen (Hilden, Germany) and assessed by a NanoDrop™ 1000c Spectrophotometer purchased from Thermo Fisher Scientific (Waltham, USA). A TruSeq Nano DNA Preparation Kit from Illumina (San Diego, USA) was utilized to construct a shotgun metagenomics library with 2 × 150 bp paired-end

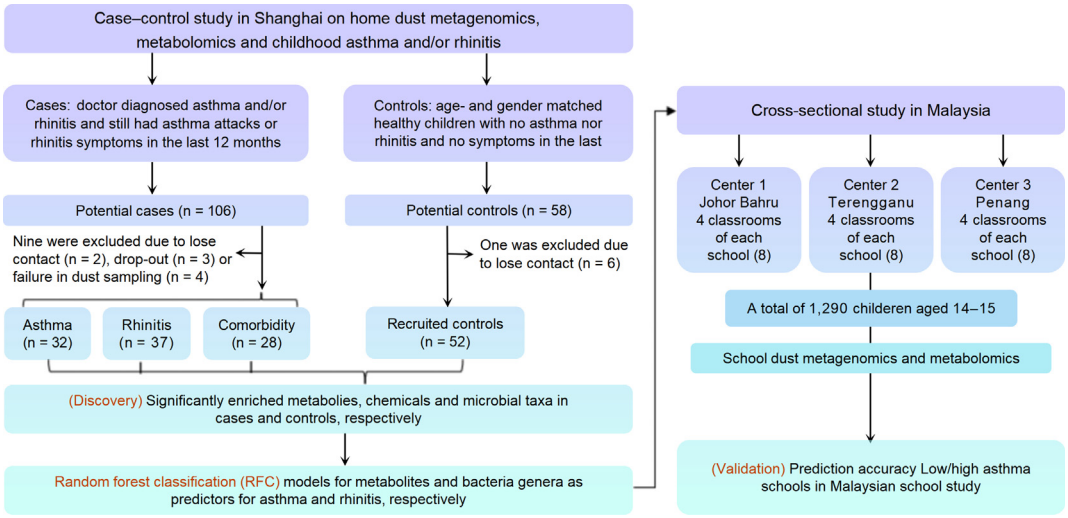

Fig. 1. Flowchart of study design on subject recruitment and data analysis.

reads and a 400 bp insert size. Dual-indexed barcodes were added to the primer for multiplexing. The prepared library was sequenced on the Illumina HiSeq X-ten platform, with raw sequences deposited in the Genome Sequence Archive (accession number PRJCA008482).

2.4. Untargeted metabolomic and chemical profiling

Untargeted LC-MS was performed on 10 mg vacuum dust samples to characterize indoor metabolomics and chemicals at BioNovoGene (Suzhou, China). Chromatographic separation was conducted using an Acquity HSS T3 Column from Waters Corporation (Milford, USA) at 40 °C with a 0.25 mL/min flow rate. LC-MS detection was performed using a Quadrupole Orbitrap Mass Spectrometer purchased from Thermo Fisher Scientific (Waltham, USA). Electrospray ionization (ESI-MSn) experiments involving multiple stages were conducted using spray voltages set at 3.5 kV for the positive mode and –2.5 kV for the negative mode. The mass spectrometer scanned a range from m/z 81 to 1,000 with a mass resolution of 60,000. Quality control (QC) and quality assurance (QA) are performed in order to obtain reliable and high-quality metabolomics data. Metabolites and chemicals were identified and annotated by consulting public databases, including METLIN (metlin.scripps.edu), mzCloud (www.mzcloud.org), Human Metabolome Database (www.hmdb.ca), MassBank (www.massbank.jp), and MoNA (mona. fiehnlab.ucdavis.edu).

2.5. Multi-omic data analysis

The primary tool for analyzing shotgun metagenomic data was QIIME2 [23]. Removal of adaptors, low-quality reads, and human reads was accomplished using Cutadapt (v3.1), BMTagger (v3.101), and KneadData (v0.10.0) [24]. Clean reads were assembled with MEGAHIT (v1.2.5) [24] and predicted using MetaGeneMark (v1.2) [24]. Gene abundance calculations were performed using SOAPdenovo2 (r240) [25]. Microbial taxonomies were annotated using BLASTN algorithms in the NCBI-NT database (e-value < 0.001) [26]. Functional genes were identified by searching the KEGG database with the DIAMOND protein aligner (v2.0.11) [27]. To characterize the distinguishing microbial taxonomic and functional genes/pathways, linear discriminant analysis effect size (LEfSe) analyses [28] were performed. Estimations of microbe-metabolite interaction co-occurrence probabilities were conducted using neural network algorithms with mmvec (v1.0.6) [29].

2.6. Using random forest analysis to classify asthma and AR in an independent Malaysian dataset

We conducted a random forest analysis to test whether the characteristic microorganisms and metabolites in Shanghai could be used to classify the probability of asthma and AR cases in an independent dataset. The independent dataset was obtained from a study conducted in various Malaysian schools (Fig. 1) [18]. This cross-sectional epidemiological survey aimed to analyze the occurrence of asthma among 1290 students across 24 middle schools. These schools were strategically chosen from three geographically distinct regions of Malaysia: Terengganu, Johor Bahru, and Penang. In each region, we randomly selected eight junior schools, choosing four classrooms from each school for dust sampling. A health questionnaire, presented in Malay, was administered to 15–20 students aged between 14 and 15 in each classroom. The questions on asthma symptoms were derived from a questionnaire in the International Study of Asthma and Allergies in Childhood (ISAAC), and contained the following questions: “In the last 12 months, have you had wheezing or whistling in the chest when you DID NOT have a cold or the flu?”, “In the past 12 months, did you have sneezing or a runny or a blocked nose when you DID NOT have a cold or the flu?”. Personal data, such as age and gender, was also collected. The dust microbial composition and metabolites present in each school were evaluated using the same methodologies as in the Shanghai case–control study, involving identical sequencing providers, protocols, and bioinformatic analysis pipelines. This methodological consistency ensured that the technical bias was substantially minimized.

Our random forest model was formulated through SciPy (v1.5.4), an open-source Python library designed for scientific computing. Initially, we identified the characteristic microorganisms and metabolites in the Shanghai dataset using LEfSe and mixed-effect regression models. We then selected those features that were shared with the Malaysian dataset to construct our classification model. The random forest classifier was employed with the number of estimators set to 1,000, and out-of-bag samples were utilized to estimate the generalization score. All other parameters remained at their default values. For the assessment of classification performance, the Malaysian dataset was partitioned into five equal segments, each maintaining the same phenotype distribution. This approach allowed for an unbiased 5-fold cross-validation.

3. Results

3.1. Personal and environmental characteristics

In total, 149 children participated in this study, comprising 97 cases and 52 healthy controls. Among the cases, 32, 37, and 28 children were diagnosed with asthma, AR, or comorbidity, respectively. Between each subgroup of cases and controls, no statistically significant differences (false discovery rate [FDR] $P > 0.05$; Table S2) were observed on gender, age, family income, birth mode and weight, the number of siblings, indoor and outdoor environmental characteristics (indoor relative humidity and CO_2 concentration, signs of home dampness/mold, environmental tobacco smoke, outdoor levels of fine particulates [$PM_{2.5}$] and NO_2 in the last 12 months), cleaning frequency in children's bedrooms and use of air purifiers. However, there was a higher proportion of parental history of asthma or AR and a lower percentage of longer exclusive breastfeeding (≥6 m) in cases than in controls. These results indicated that normal environmental characteristics could not differentiate between cases and controls.

3.2. Indoor microbiome diversity and composition

A total of 6,247 microbial species were characterized in children's homes, comprising 5,975 bacteria, 140 eukaryotes, 38 archaea, and 94 viruses. The most abundant indoor bacteria were human skin commensals (*Staphylococcus aureus*, *Cutibacterium acnes*, *Staphylococcus epidermidis*, *Streptococcus mitis*), environmental commensals (*Micrococcus luteus*, *Pseudomonas aeruginosa*, *Kocuria palustris*, *Paracoccus sphaerophysae*, which are mainly derived outdoor), and a few opportunistic pathogens (*Moraxella osloensis*, *Mycobacterium tuberculosis*; Table S3). The most abundant eukaryotes were fungal species, including common indoor molds (*Alternaria alternate*, *Aspergillus glaucus*, *Penicillium rubens*, *Chaetomium globosum*) and human skin fungi (*Malassezia restricta*, *Malassezia globosa*). Indoor archaeal and viral species were present in low abundance (<0.01%), mainly consisting of bacterial phage and human herpesvirus.

The indoor microbial α-diversity was compared. Healthy children had a higher indoor microbial Shannon index than asthma, AR, and comorbid children ($P < 0.05$) (Fig. S1A). However, the number of observed species did not differ among groups. Actinobacteria, Bacilli, Gammaproteobacteria, and Alphaproteobacteria were the most abundant bacteria class, accounting for >80% of the total microbial load (Fig. S1B).

We assessed indoor microbial β-diversity and found a small but significant variation between the homes of asthma, AR comorbid, and healthy children (PERMANOVA, $R^2 = 0.03$, $P = 0.005$; Fig. S1C). A higher number of outdoor species were enriched in the homes of healthy children compared with diseased children (Fig. S1D), including *Paracoccus yeei*, *Paracoccus* sp. 228, *Blautia obeum*, *Acinetobacter lwoffii*, *Pseudomonas* sp. 286, *Pseudomonas fluorescens*, *Pseudomonas fragi* and *Psychrobacter* sp. In contrast, only a few species, including *Mycolicibacterium mucogenicum*, *Comamonas aquatica*, *Jeotgalicoccus marimus*, and *Micrococcus* sp., were enriched in the homes of diseased children compared with healthy children. These species were first-time reported to be associated with asthma and AR. These results confirm that health-related indoor microorganisms exhibit high geographical variation.

3.3. Microbial functional genes and pathways

In this study, 2.76 million functional genes were annotated against the KEGG database. Approximately half of the genes were metabolic genes (Fig. S2A). No statistical differences were observed in the overall composition of functional genes in asthma, AR, comorbidity, and healthy children (PERMANOVA, $R^2 = 0.01$, $P = 0.70$, Fig. S2B). We further tested the abundance variation in each pathway. Compared to cases, three metabolic pathways were enriched in the homes of healthy children, including starch and sucrose metabolism, galactose metabolism, and secondary bile acid biosynthesis pathways (Fig. S2C). Also, the species diversity was correlated with functional pathway diversity ($R = 0.48$, $P < 0.0001$; Fig. S2D), indicating that diverse microbial exposure also led to diverse functional gene exposure. No functional pathway was enriched in the homes of asthma or AR children.

3.4. Metabolites/chemicals enriched in homes of healthy children

In total, 1,442 metabolites and chemicals were characterized in the home dust. The results of QA and QC are shown in Fig. S3. Four classes of metabolites were exclusively enriched in the homes of healthy children, not in diseased children (fold change > 1.5, $P < 0.01$, FDR < 0.05; Tables 1 and 2). These classes included derivatives of keto acids (acetoacetic acid, ketoleucine, 2-ketobutyric acid, alpha-ketoisovaleric acid, and pyruvic acid), indoles (5-hydroxy-L-tryptophan, indole-3-carboxylic acid), pyridines (1-methyl-6-phenyl-1H-imidazo[4,5-b]pyridin-2-amine, 6-hydroxynicotinic acid) (Table 1) and flavonoids (astragalin, 3-hydroxyflavone, hesperidin, naringin, tangeritin, apigenin, and baicalein) (Table 2). These enriched compounds were consistent in sub-pair comparisons between healthy children and either asthma, AR, or comorbid children.

Using neural network co-occurrence probability analysis, we found that the enriched keto acids, indoles, and pyridines in homes of healthy children were closely involved in the production of the potentially protective indoor microorganisms, while flavonoids were from a class of secondary metabolites derived from multiple plants (Fig. 2). Specifically, *Psychrobacter* species had high co-occurrence probabilities with acetoacetic acid, 2-ketobutyric acid, 3-methylindole, 2-hydroxypyridine and D-1-piperideine-2-carboxylic acid. Similarly, *Abyssicoccus albus*, *Facklamia hominis*, an uncharacterized Firmicutes, and *P. fragi* had high co-occurrence probabilities with multiple metabolites. Acetoacetic acid and 3-methylindole had high co-occurrence probabilities with many species.

The interconnections between metabolites within each class were explored by searching the literature (Fig. S4). Interconnections were observed within the class of keto acid and the class of indoles and their derivatives, while compounds in the class of flavonoids tended to be independent of each other, indicating their different sources.

3.5. Metabolites/chemicals enriched in homes of asthma, AR, and comorbid children

Two categories of metabolites/chemicals, mycotoxin and synthetic chemicals, were exclusively enriched in the homes of children with asthma, AR, or comorbid (Table 3).

Mycotoxins are toxic secondary metabolites produced by fungi. Nivalenol, deoxynivalenol (vomit toxin), and diacetoxyscirpenol were trichothecenes, a large mycotoxin family mainly produced by *Fusarium graminearum*, *Fusarium tricinctum*, and *Fusarium culmorum* [30]. Tentoxin is a natural cyclic tetrapeptide produced by *Alternaria alternata*. Analyzing the fungal abundance in metagenomic data, we found *F. graminearum* and *A. alternate* were present in significantly higher abundance in homes of children with asthma than in homes of healthy children ($P < 0.05$, Wilcoxon test), consistent with the enrichment of metabolites in children with asthma. These mycotoxins have been classified as hazardous chemicals with fatal or toxic effects in contact with skin or ingestion by the Globally Harmonized System (GHS). Inhaling or swallowing these mycotoxins leads to inflammation, vomiting, liver injury, and gastrointestinal diseases.

The synthetic chemicals mainly included herbicide (pelargonic acid), insecticide (benfuracarb, cyromazine), and food and cosmetic additives (2-methylpropanal, 4-Hydroxy-3-methoxy-benzaldehyde). These chemicals have also been classified as hazardous, causing skin and eye irritation and adverse symptoms including gastrointestinal diseases and inflammation.

We further performed a gender-specific subgroup analysis to compare the identified enriched metabolites/chemicals. Our analysis found that

Table 1
Potentially protective metabolites enriched in homes of healthy children compared with all diseased and each subgroup with asthma, AR, and comorbidity, respectively ($P < 0.01$, FDR < 0.05, fold change > 1.5 indicated by "√").

Metabolite Classes	Metabolites/compounds	Molecular formula	Healthy vs. Diseased	Healthy vs. Asthma	Healthy vs. AR	Healthy vs. Comorbid	Evidence of the mechanistic roles in the inflammation pathway
Keto acids and derivatives	Acetoacetic acid	$C_4H_6O_3$	√	√	√	√	
	Ketoleucine	$C_6H_{10}O_3$	√	√	√	√	
	2-Ketobutyric acid	$C_4H_6O_3$	√	√	√	√	
	alpha-Ketoisovaleric acid	$C_5H_8O_3$	√	√	√	√	
	Pyruvic acid	$C_3H_4O_3$	√	√	√	√	Attenuates inflammation by suppressing inflammatory cytokine HMGB1 [23]
	3-Sulfinylpyruvic acid	$C_3H_4O_5S$	√	√	√		
	2-Ketohexanoic acid	$C_6H_{10}O_3$	√		√		
Indoles and derivatives	5-Hydroxy-L-tryptophan	$C_{11}H_{12}N_2O_3$	√	√	√	√	
	Indole-3-carboxylic acid	$C_9H_7NO_2$	√	√	√	√	Attenuates oxidative stress by suppressing IL-6, IL-8, and NF-κB [24]
	Indolelactic acid	$C_{11}H_{11}NO_3$	√	√	√		
	Indole-3-acetate	$C_{10}H_8NO_2^-$			√	√	Attenuates liver inflammation [31]
	3-Methylindole	C_9H_9N		√			
	N-Acetylserotonin	$C_{12}H_{14}N_2O_2$			√		Attenuates inflammatory hyperalgesia by inhibiting NF-κB [25]
	Indoleacetaldehyde	$C_{10}H_9NO$	√		√		
Pyridines and derivatives	1-Methyl-6-phenyl-1H-imidazo[4,5-b]pyridin-2-amine	$C_{13}H_{13}ClN_4$	√	√	√	√	
	6-Hydroxynicotinic acid	$C_6H_5NO_3$	√	√	√	√	
	Chlorpheniramine	$C_{16}H_{19}ClN_2$		√			Attenuates IgE-mediated inflammation by suppressing histamine [26]
	Picolinic acid	$C_6H_5NO_2$				√	
	D-1-Piperideine-2-carboxylic acid	$C_6H_9NO_2$	√			√	
	2-Hydroxypyridine	C_5H_5NO	√			√	

The ticks "√" refer to the metabolites significantly enriched in the homes of healthy children with a fold of more than 1.5, compared with all diseased and each subgroup of diseases with asthma, AR, or comorbidity, respectively. The mechanistic roles of enriched metabolites were obtained by searching the metabolite names and the keyword "inflammation" in PubMed.

the patterns were largely consistent across males, females, and overall groups (Tables S4 and S5). Most metabolites enriched in the overall group remained so in both male and female subgroups. Notably, a few exceptions were observed. For instance, 3-Hydroxyflavone, Tangeritin, and 3-Methylindole were still enriched in the homes of healthy male children (FDR < 0.05) but not significantly detected in the female group (FDR > 0.05). Conversely, Epicatechin was enriched in homes of healthy female children (FDR < 0.05) but not significantly detected in male group (FDR > 0.05). However, we interpret that these differences are likely due to reduced statistical power resulting from data stratification and sample size limitations. Generally, our analysis did not reveal significant gender variations in the associations.

3.6. Random forest classification for asthma and AR in an independent Malaysian dataset

We employed characteristic metabolites and microorganisms in Shanghai to classify the probability of asthma and AR in Malaysian schools (Fig. 3, Table S6). For asthma, using 7 indole derivatives, 15 flavonoids, and 4 mycotoxins as marker metabolites could achieve an average accuracy of 74.9% in differentiating high asthma prevalence schools from those of low prevalence (Fig. 3A). Adding the model with keto acids, pyridines, and synthetic chemicals slightly decreased the classification accuracy for classification of asthma prevalence (Area Under Curve, AUC = 62.2%–71.4%; Fig. S5A–C). Conversely, using characteristic microbial genera from the Shanghai dataset resulted in a classification accuracy of only 51.0% in Malaysia (Fig. 3B). This finding implies that indoor microorganisms had a lower power in differentiating schools for children's prevalence of asthma.

Similar classification results were observed for AR. Using indole, flavonoids, and mycotoxins as marker metabolites achieved an average accuracy of 77.1% in differentiating high AR prevalence schools from those with low prevalence (Fig. 3C). When we added keto acids, pyridines, and synthetic chemicals to the model, the classification accuracy slightly decreased (area under curve, AUC = 70.7%; Fig. S5D). Using the characteristic microbial genera in the Shanghai study for classification only resulted in a classification accuracy of 59.5% in Malaysia (Fig. 3D), indicating that the indoor microorganisms have limited utility in distinguishing schools for children's prevalence of AR.

In conclusion, our findings suggested that indoor metabolites have promising potential in environmental assessment and health outcome classification for asthma and AR.

4. Discussion

This study is the first to utilize high-throughput metagenomics and untargeted metabolomics to profile both microbial and chemical exposure in home environments, providing a comprehensive assessment of microbial taxa and diversity, functional genes and pathways, metabolites, and chemicals in homes of children with asthma, AR, comorbid, and healthy children. More than 20 indoor species, three metabolic pathways, and metabolites including keto acids, indoles, pyridines, and flavonoids were enriched in the homes of healthy children, while mycotoxin and synthetic chemicals were exclusively enriched in homes of asthma, AR, or comorbid children.

The microbial species and functional gene pathways screened in this study were different from those observed in other studies, including studies in China, Malaysia, Europe, and the US [8,9,14,19], confirming

Table 2
Potentially protective flavonoids enriched in homes of healthy children compared with all diseased and each subgroup with asthma, AR, and comorbidity, respectively ($P < 0.01$, FDR < 0.05, fold change > 1.5 indicated by "√").

Subclass of Flavonoids	Metabolites/ compounds	Molecular formula	Healthy vs. Diseased	Healthy vs. Asthma	Healthy vs. AR	Healthy vs. Comorbid	Representative plants	Evidence on their potential roles in the respiratory inflammation pathway
Flavones and Flavonols	Astragalin	$C_{21}H_{20}O_{11}$	√	√	√	√	*Phytolacca americana*, *Phegopteris connectilis*	
	3-Hydroxyflavone	$C_{15}H_{10}O_3$	√	√	√	√	*Acacia doratoxylon*, *Acacia verniciflua*	
	5,7-Dihydroxyflavone	$C_{15}H_{10}O_4$	√		√	√	*Scutellaria adenostegia*, *Picea abies*	
	Tangeritin	$C_{20}H_{20}O_7$		√			*Citrus nippokoreana*, *Croton caudatus*	Attenuates inflammation by suppressing NF-κB and proinflammatory cytokines [32]
	Apigenin	$C_{15}H_{10}O_5$	√		√		*Asyneuma campanuloides*, *Dendrobium loddigesii*	Attenuates airway hyperresponsiveness and inflammation by suppressing NF-κB in murine model of asthma [33]
	Kaempferide	$C_{16}H_{12}O_6$			√		*Nothofagus nervosa*, *Deinandra increscen*	
	Eupatilin	$C_{18}H_{16}O_7$	√			√	*Artemisia*	Attenuates adhesion of monocytes and eosinophils to bronchial epithelial cells by suppressing Akt and NF-κB pathway [27]
	Luteolin	$C_{15}H_{10}O_6$	√			√	*Pteridophyta*, *Bryophyta*, *Magnoliophyta*, *Pinophyta*	Attenuates airway inflammation by inducing $CD4^+$ $CD25^-$ to $CD4^+$ $CD25^+$ regulatory T cells [34]
	Baicalein	$C_{15}H_{10}O_5$				√	*Chinese herbal medicine Scutellaria baicalensis*	Attenuates allergic airway inflammation by suppressing NF-κB signaling pathway [35]
Flavanones	Hesperidin	$C_{28}H_{34}O_{15}$	√	√	√	√	*Citrus excelsa*, *Cyclopia intermedia*	Attenuates airway inflammation and Th2 cytokines in a mouse allergic asthma model [36]
	(2S)-Liquiritigenin	$C_{15}H_{12}O_4$	√	√	√		*Physochlaina physaloides*, *Centrolobium robustum*	Attenuates oxidative stress and inflammation by suppressing NF-κB and NLRP3 inflammasome pathways [37]
	Naringin	$C_{27}H_{32}O_{14}$	√	√	√		*Citrus maxima*, *Citrus medica*	
	Hesperetin	$C_{16}H_{14}O_6$	√	√			*Cyclopia intermedia*, *Brassica*	Ameliorates acute lung injury in mice through regulating TLR4-MyD88-NF-κB signaling pathway [38]
Proanthocyanidins	Procyanidin B2	$C_{30}H_{26}O_{12}$	√		√		*Rubus idaeus*, *Davallia divaricata*	Attenuates inflammation and apoptosis by suppressing NF-κB in human epithelial cells and lung fibroblasts [39]
Flavans, Flavanols, and Leucoanthocyanidins	Epicatechin	$C_{15}H_{14}O_6$	√		√		*Davallia divaricata*, *Cinnamomum tenuifolium*	Attenuates lung inflammation by suppressing ROS/ NLRP3 inflammasome pathway in rats with COPD [40]

Flavonoids were classified into different subclasses based on their structure, including the attachment position of C ring on B ring and the degree of unsaturation and oxidation of the C ring. Evidence on their potential roles in respiratory inflammation was obtained by searching the metabolite names and keyword "respiratory inflammation" in PubMed.

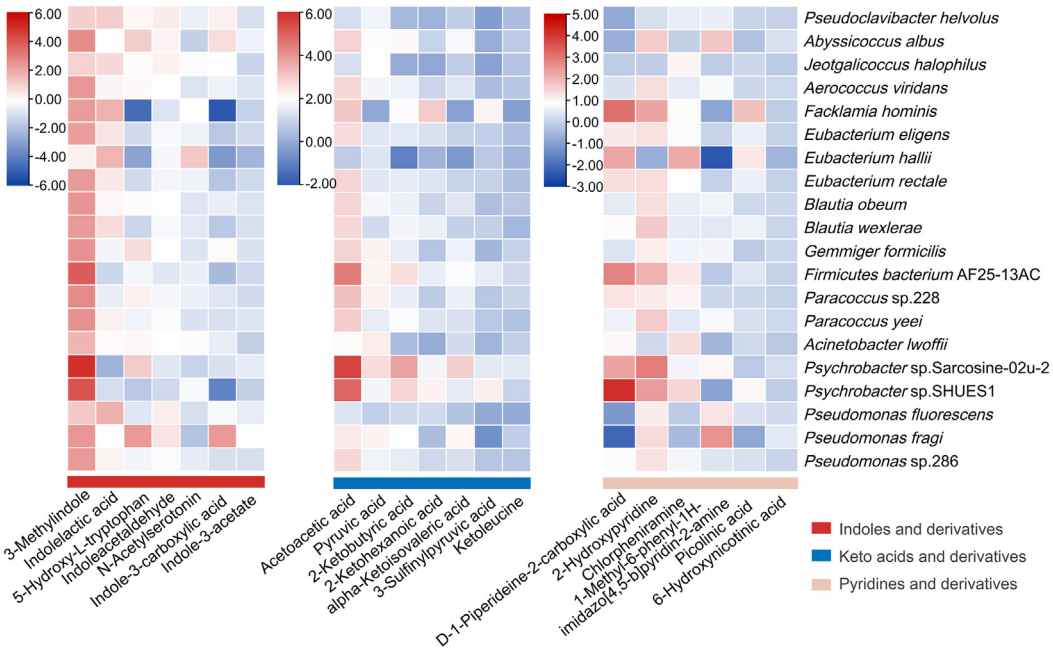

Fig. 2. Co-occurrence probability of potentially protective environmental microbial species and metabolites. The microbial species were presented at the y-axis of the heat plot. Indoor microbial species that are potentially derived from humans were not presented in the figure. The human-derived microorganisms are defined as abundance >0.01% in the human gut, skin and oral cavity in the Human Microbiome Project [41]. The potential protective metabolite in keto acids and derivatives, indoles and derivatives, and pyridines and derivatives were presented at the x-axis. The potential protective taxa were defined as microbial species enriched in the homes of healthy children (LDA > 2). The potential protective metabolites in the three classes were defined as metabolites enriched in homes of healthy children compared with homes of diseased children ($P < 0.01$, FDR < 0.05, fold change > 1.5).

the previously reported significant geographic variations. Similarly, health-associated functional pathways were also inconsistent among studies. For example, a shotgun metagenomic microbial survey in Shanxi, China, reported that the abundance of pathways related to immune systems, bacterial/viral infectious diseases, and cancer was associated with higher asthma and rhinitis risks [8], whereas in our study, no such pathway was associated with higher asthma and rhinitis risks. Additionally, the potential protective pathways in the Shanxi study, including amino acid metabolism and metabolism of co-factors and vitamins, were different from the protective functional pathways in our study, like starch and sucrose metabolism, galactose metabolism, and secondary bile acid biosynthesis pathways.

Compared to microbial taxa and functional genes, indoor metabolites/chemicals have shown more consistent associations with asthma and AR. A recent cross-sectional epidemiological survey in Malaysia [18] observed similar characteristic metabolites as in the Shanghai home study, including indole-derivatives (indole-3-carboxylic acid, 5-Hydroxy-L-tryptophan) and flavonoids (astragalin, tangeritin), but the health-related indoor microorganisms (*Actinomycetospora*, *Fischerella* and *Truepera*) differed. Using a random forest classification model, we found that the enriched metabolites (74.9% and 77.1%) in Shanghai provided better classification accuracy than enriched microorganisms (51.0% and 59.5%) on asthma/AR prevalence in Malaysia, respectively, indicating that the larger potential of indoor metabolomics rather than microbiome in classifying health outcomes. This result aligns with findings from other fields of study. For instance, a multi-omics analysis of cervicovaginal specimens found that the metabolome was a stronger predictor of genital inflammation than the microbiome [29]. Different microorganisms can participate in similar metabolic pathways, which are closely related to disease mechanisms [30]. Therefore, the metabolome might serve as a more universal and reliable classifier and environmental risk assessment index for asthma and AR in the future. And, we hope to enlarge the sample size and conduct research in different regions to retest the current findings.

Many microbial metabolites can influence the occurrence of allergic diseases. It has been reported that microbial cell wall compounds and metabolites, including endotoxin and microbial volatile organic compounds (MVOCs), are related to the development of asthma and AR. For example, early-life exposure to endotoxins can potentially reduce airway sensitivity and reactivity in later stages, a principle known as endotoxin or lipopolysaccharide tolerance [35]. In this study, we reported that keto acids, indoles, pyridines, and their derivatives could potentially provide protection against asthma, and these metabolites might be produced by microorganisms in the indoor environment. Previous studies have reported that environmental microorganisms, such as *Pseudomonas* and *Psychrobacter*, could produce 2-keto acid, indole-3-carbaldehyde, and pyridine-2,6-dithiocarboxylic acid [20,23,24]. These findings are consistent with our neural network analysis. In addition, several laboratory studies have reported the health effects of the metabolites characterized in this study. For instance, pyruvic acid can ameliorate chronic gut inflammation in multiple animal models by inhibiting inflammatory cytokine HMGB1 [25]. The anti-inflammatory effects of indole derivatives are primarily mediated by NF-κB (nuclear factor κB). Indole-3-carboxylic acid and N-acetylserotonin can attenuate oxidative stress and inflammatory hyperalgesia by inhibiting the expression of NF-κB [26,32]. Chlorpheniramine, a derivative of pyridine, can suppress the level of histamine to reduce IgE-mediated inflammation [33]. However, the health benefits and molecular mechanisms of the remaining metabolites are still unclear and require further clarification through in vitro or in vivo experiments, which could facilitate the translational application of these metabolites in asthma and rhinitis treatment.

Fifteen flavonoids were characterized as potential protective metabolites for asthma and AR. These flavonoids had anti-inflammatory effects on the respiratory tract by suppressing the expression of NF-κB and inflammatory cytokines, as supported by in vitro and in vivo evidence. For example, tangeritin and apigenin can down-regulate NF-κB in a murine model of asthma, attenuating lung injury and airway hyperresponsiveness [27,28]. Similarly, other flavonoids, such as eupatilin,

Table 3
Enriched risk environmental metabolites and chemicals in homes of diseased children and in each subgroup with asthma, AR, and comorbidity, compared with healthy controls, respectively ($P < 0.01$, FDR < 0.05, fold change > 1.5 indicated by "√").

	Metabolites/Compounds	Molecular formula	Diseased vs. Healthy	Asthma vs. Healthy	AR vs. Healthy	Comorbid vs. Healthy	Key features	Potential fungal producer	GHS hazard statements	Symptoms, disorders, and diseases
Natural metabolites	Nivalenol	$C_{15}H_{20}O_7$	√	√	√	√	Mycotoxin	*Fusarium graminearum*	H310 (100%): Fatal in contact with skin H330 (100%): Fatal if inhaled	Inflammation, vomiting
	Deoxynivalenol	$C_{15}H_{20}O_6$	√	√	√		Mycotoxin	*Fusarium graminearum*	H300 (100%): Fatal if swallowed	Inflammation, vomiting, dermatitis, gastrointestinal diseases
	Diacetoxyscirpenol	$C_{19}H_{26}O_7$	√		√		Mycotoxin	*Fusarium graminearum*	H300 (100%): Fatal if swallowed H310 (100%): Fatal in contact with skin H319 (100%): Causes serious eye irritation H330 (100%): Fatal if inhaled	Liver injury
	Tentoxin	$C_{22}H_{30}N_4O_4$	√		√	√	Mycotoxin	*Alternaria alternata*		Gastrointestinal diseases
Synthetic chemicals	Benfuracarb	$C_{20}H_{30}N_2O_5S$		√		√	Insecticide agrochemical		H331: Toxic if inhaled	Poisoning
	2-Methylpropanal	C_4H_8O	√	√		√	Food additive		H319 (98.24%): Causes serious eye irritation	Eye Injuries, Crohn's disease, ulcerative colitis
	Pelargonic acid	$C_9H_{18}O_2$	√	√	√	√	Herbicide		H315: Causes skin irritation H319: Causes serious eye irritation	Poisoning
	Cyromazine	$C_6H_{10}N_6$		√			Insecticide		H315 (21.57%): Causes skin irritation H319 (21.08%): Causes serious eye irritation H335 (20.59%): Causes respiratory irritation	Low birth weight
	4-Hydroxy-3-methoxy-benzaldehyde	$C_8H_8O_3$	√		√		Food and cosmetic additive		H317 (14.8%): Causes an allergic skin reaction H319 (77.46%): Causes serious eye irritation	

Chemical characteristics were obtained from the PubChem database maintained by NCBI. Chemicals identified as drugs or common metabolites in humans, animals, or plants were excluded from the table. The Globally Harmonized System (GHS) encompasses criteria for classifying health, physical, and environmental hazards. It also outlines the information that should be featured on labels of hazardous chemicals and safety data sheets. The table presented GHS classification information, such as GHS hazard codes, notified classification ratio, and hazard statements. Hazard codes ranging from H300 to H336 were included in the table, as these statements primarily pertain to allergic irritations.

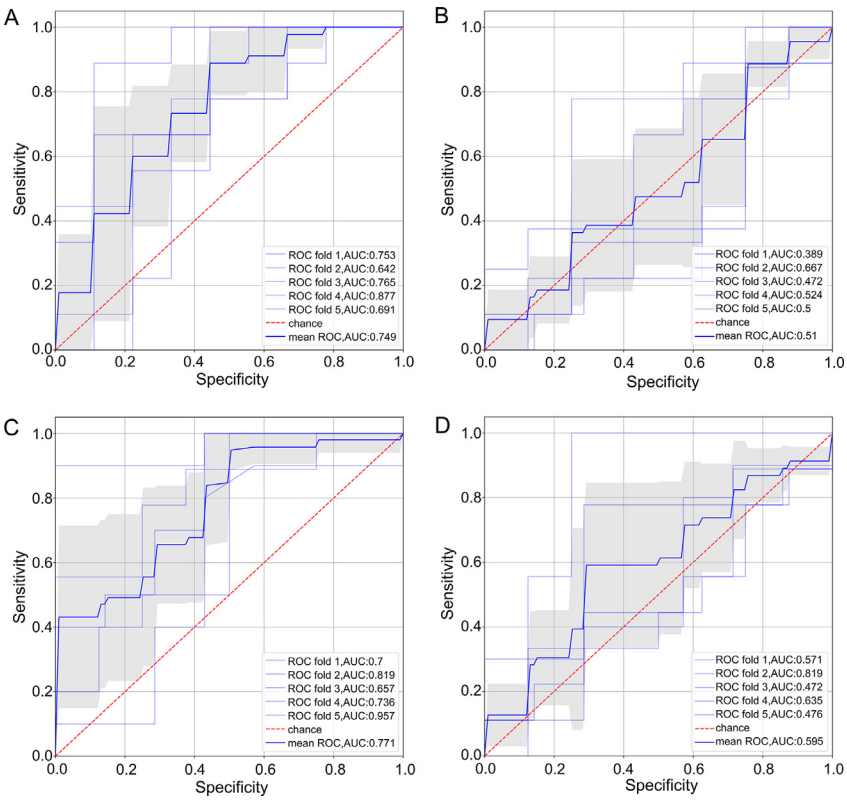

Fig. 3. Microbial markers identified in Shanghai home study as classifiers of asthma and AR in an independent Malaysian study. Metabolites (A) and microbial taxa (B) from the Shanghai home study served as classifiers of asthma in the Malaysian study. Metabolites (C) and microbial taxa (D) in the Shanghai home study served as classifiers of AR in the Malaysian study. The metabolite markers included indole derivatives (5-Hydroxy-L-tryptophan, Indole-3-carboxylic acid, Indolelactic acid, Indole-3-acetate, 3-Methylindole, N-Acetylserotonin, Indoleacetaldehyde), flavonoids (Astragalin, 3-Hydroxyflavone, 5,7-Dihydroxyflavone, Tangeritin, Apigenin, Kaempferide, Eupatilin, Luteolin, Baicalein, Hesperidin, (2S)-Liquiritigenin, Naringin, Hesperetin, Procyanidin B2, Epicatechin), and mycotoxins (Nivalenol, Deoxynivalenol, Diacetoxyscirpenol, Tentoxin) characterized in Tables 1–3. The microbiome markers included characteristic genera in the LEfSe analysis in the Shanghai study (*Staphylococcus*, *Dolosigranulum*, *Bifidobacterium*, *Prevotella*, *Faecalibacterium*, *Blautia*, *Porphyromonas*, *Anaerostipes*, *Akkermansia*, *Ruminococcus*, *Abyssicoccus*, *Eubacterium*, *Roseburia*, *Subdoligranulum*, *Dorea*, *Gemmiger*, *Aerococcus*, *Coprococcus*, *Facklamia*, *Alistipes*). The classification model was constructed by five-fold cross-validation on a random forest model. The classification accuracy was expressed as an Area Under the Curve (AUC) value. An AUC of 1 indicates 100% accuracy, whereas an AUC of 0.5 indicates the model fails to differentiate schools with varying occurrences of asthma and AR.

baicalein, liquiritigenin, hesperetin, and procyanidin [42–46], have been reported to exhibit similar anti-inflammatory effects by targeting NF-κB.

In contrast, four mycotoxins, namely nivalenol, deoxynivalenol, diacetoxyscirpenol, and tentoxin, were characterized as potential risk factors for asthma and AR. Deoxynivalenol is the most widely spread mycotoxin in human food and has been detected in more than 80% of food commodities globally [38]. Numerous studies have reported the adverse health effects of these mycotoxins in the human digestive tract, activating the expression of NF-κB and causing inflammation in the gastrointestinal tract [45–47]. However, only a few studies have reported their health effects on the respiratory tract. An experimental study showed that nivalenol and deoxynivalenol administration induced the expression of inflammation cytokines, including IL-6 and IL-8, in multiple airway epithelial cells [48]. Epidemiological surveys reported that the mycotoxin concentration in classrooms was low in Europe and Malaysia [49,50]. Nevertheless, these studies did not survey the mycotoxins characterized in this study, including deoxynivalenol and nivalenol, highlighting the need for further research to clarify their role in respiratory health.

Synthetic chemicals were also characterized as enriched compounds in the home dust of asthma and rhinitis children. Two studies reported that 2-methylpropanal and pelargonic acid could induce inflammation in respiratory epithelial cells and vascular endothelial cells [51,52]. Interestingly, these chemicals, along with diacetoxyscirpenol and pelargonic acid, can activate the expression of NF-κB, which leads to subsequent inflammatory symptoms [44,50]. Overall, many indoor metabolites/chemicals characterized in this study can either inhibit or induce the expression of NF-κB and subsequent inflammation, indicating the central role of this marker in diseases. Developing antagonists to inhibit NF-κB could be potential therapies for asthma and rhinitis treatment.

This study contributed to the identification of metabolite characteristics to differentiate the home environment of asthma/AR/comorbidity and healthy children. Methodologically, the study combined clinical diagnosis of health status (rather than parental reports in a questionnaire) and shotgun metagenomics analysis (rather than 16S rRNA sequencing) of dust samples. We proposed that indoor metabolites and chemicals have the potential to be used as an environmental assessment indicator for asthma and AR. The information can be used to distinguish potential health outcomes by indicating whether a room is “metabolite healthy” or not, and providing a tool for asthma and AR prevention. In addition, current environmental risk assessment approaches for childhood asthma and AR typically focus on allergens (such as dust mites, pet dander, cockroaches, and molds), environmental tobacco smoke, air pollution (indoor and outdoor), and certain chemicals. Our study extends the scope of the indoor environment examination to include both microbial and chemical characterizations, providing a more comprehensive and integrated assessment of exposure risk. The study opens up new possibilities for intervention strategies, such as modifying the indoor microbiome or reducing certain chemical exposures, to prevent or manage asthma and AR.

There are several limitations in this study. Firstly, our study was a case–control design that restricted the ability of causal inference. Another concern of this study design was whether the enriched metabolites reflected the use of drugs or medications, especially in the diseased children since they had already been diagnosed with asthma/AR. In our analysis, we have excluded chemicals identified as drugs in the case subjects (Legends in Table 3) and they were not applied in the classification model. Further studies are needed to explore the sources or factors determining or influencing the metabolite enrichment characteristics. Secondly, our sample size was relatively small, which may limit the power of our study to detect small effects and may introduce variability. This limitation is particularly pertinent in the context of asthma and allergic rhinitis, which are influenced by a myriad of confounding factors leading to substantial variability in exposure features during the development of these conditions. Thus, the classification model developed in this study should be interpreted with caution and may benefit from further validation in larger cohorts. Thirdly, the fact that our study only used a random forest model presents a limitation. While this model is known for its robustness and capacity to handle high-dimensional data, the performance may differ if other models were used. It’s important to

clarify that our findings were obtained using this approach and other models might yield different results. Fourthly, we did not collect dust samples from moist areas such as in the restroom, which might differ from the dust in the bedroom. However, there was no statistical difference in the signs of home dampness/mold by questionnaire between case and control groups. This suggests that the variation in health outcomes is unlikely to be attributed to differences in indoor moisture levels or the presence of dampness and mold. Furthermore, the history of parental asthma or AR might influence their behavior in room cleaning, such as increasing the cleaning frequency. However, we did not observe such differences between cases and controls. Also, our dust sampling methods tended to represent a mixed exposure to both long-lasting and current environmental exposure. We do not believe the dust samples were largely biased by room cleaning or sampling. Finally, LC-MS was used to quantify the relative concentration of metabolites/chemicals based on intense peaks of mass spectra. However, the approach is affected by ion suppression of the co-eluting compounds, and the peak areas may not accurately represent the absolute metabolite concentration [52]. Therefore, we could not assess whether these chemicals exceeded the health regulation levels (GHS classification). Future studies using stable isotope-labeled MS could provide a more accurate assessment of the absolute quantification of indoor metabolites/chemicals [53]. Additionally, the association between indoor metabolites and microorganisms was calculated by a bioinformatic approach, and future studies are needed to verify the results in laboratory conditions.

5. Conclusions

This study contributed in connecting the asthma/AR/comorbidity and healthy children with the dust metabolite characteristics in the home environment, where children spend the largest part of their time in their early life. The results showed that indoor metabolites and chemicals had the potential to be used as an environmental classifier for asthma and AR, and testing whether the room is “metabolite healthy” could be applied in asthma and AR prevention in the future. This study also paves the way for innovative intervention strategies, including the alteration of the indoor microbiome or curbing specific chemical exposures, as potential methods to prevent or manage asthma and AR.

Data availability statement

Sequencing data were publicly available at the Genome Sequence Archive with accession number PRJCA008482. Individual participant data can be shared after applying to the authors.

Author contributions

Data curation: Z.H.Z, H.T., S.D., J.S.,Y.C., Z.Y.Q., M.Z., Z.R.C., Z.W.T., D.J.Z., T.Y.C., J.H.H. and Z.H. Investigation, project administration, software, validation and writing–original draft: Y.S, H.T, S.D., J.S., X.F. and Z.H.Z. Supervision, writing–review and editing: Y.S., H.T., S.D., Y.Y.X., J.F.L., D.N., J.S., X.F. and Z.H.Z. All authors read and approved the final manuscript.

Declaration of competing interest

The authors have declared no conflicts of interest.

Ethical statement

Patient consent for publication

All participants signed informed consent and consent for publication.

Ethics approval

The study design and protocol were approved by the ethical committee of School of Public Health, Fudan University (IRB#2019-09-0778) and Ruijin Hospital (the ethical approval number #2020-316).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eehl.2023.08.001.

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