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Associations between indoor microbiome exposure and allergic and non-allergic rhinitis for junior high school students in Terengganu, Malaysia

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Abstract

Rhinitis is one of the most prevalent diseases in the world. Indoor microbiome is confirmed to associate with respiratory diseases such as asthma and infections, but no study reported the association between indoor microbiome and the occurrence of rhinitis. In this study, 370 students were randomly selected from 8 junior schools in Terengganu, Malaysia, and self-administered questionnaire and skin prick tests were conducted to define the allergic and non-allergic rhinitis among students. Vacuum dust was collected from the floor and chair/desk surfaces in the classrooms, and culture-independent high-resolution amplicon sequencing and quantitative PCR were conducted to characterize the absolute concentration of bacterial and fungal species. Hierarchical logistic regression was applied in the association analyses. We found similar microbial associations for the students with allergic and non-allergic rhinitis. The microbial richness in Gammaproteobacteria was protectively associated with allergic and non-allergic rhinitis ($p = 0.02$ and 0.04), and total fungal richness was positively associated with allergic and non-allergic rhinitis ($p = 0.01$ and 0.03). The absolute concentration of two bacterial species, Aeromonas enteropelogenes and Brasilonema bromeliae, were associated with both types of rhinitis, and six bacterial and one fungal species was associated with either allergic and non-allergic rhinitis ($p < 0.005$). Four species previously reported as facultative pathogens, including A. enteropelogenes, Escherichia fergusonii, Enterobacter xiangfangensis and Streptococcus salivarius, were protectively (negatively) associated with rhinitis. A higher concentration of two radiation-resistant species, including Deinococcus gobiensis and Deinococcus grandis, were associated with an increased odds of rhinitis.

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Abstract

Rhinitis is one of the most prevalent diseases in the world. Indoor microbiome is confirmed to associate with respiratory diseases such as asthma and infections, but no study reported the association between indoor microbiome and the occurrence of rhinitis. In this study, 370 students were randomly selected from 8 junior schools in Terengganu, Malaysia, and self-administered questionnaire and skin prick tests were conducted to define the allergic and non-allergic rhinitis among students. Vacuum dust was collected from the floor and chair/desk surfaces in the classrooms, and culture-independent high-resolution amplicon sequencing and quantitative PCR were conducted to characterize the absolute concentration of bacterial and fungal species. Hierarchical logistic regression was applied in the association analyses. We found similar microbial associations for the students with allergic and non-allergic rhinitis. The microbial richness in Gammaproteobacteria was protectively associated with allergic and non-allergic rhinitis ($p = 0.02$ and 0.04), and total fungal richness was positively associated with allergic and non-allergic rhinitis ($p = 0.01$ and 0.03). The absolute concentration of two bacterial species, *Aeromonas enteropelogenes*and *Brasilonema bromeliae* , were associated with both types of rhinitis, and six bacterial and one fungal species was associated with either allergic and non-allergic rhinitis ($p < 0.005$). Four species previously reported as facultative pathogens, including*A . enteropelogenes* , *Escherichia fergusonii* ,*Enterobacter xiangfangensis* and *Streptococcus salivarius* , were protectively (negatively) associated with rhinitis. A higher concentration of two radiation-resistant species, including*Deinococcus gobiensis* and *Deinococcus grandis* , were associated with an increased odds of rhinitis. This is the first health association study conducted between the indoor microbiome and the occurrence of rhinitis, revealing that microbial exposure at school can affect both allergic and non-allergic students.

Keywords

Allergic rhinitis, non-allergic rhinitis, skin prick test, indoor microbiome, high-throughput sequencing

Introduction

Rhinitis is one of the most prevalent diseases in the world, affecting up to 30% of the total population[1-3]. The prevalence of rhinitis is still increasing in most of the countries in the world, especially in many developing countries[2]. The disease leads to substantial economic and medical loss as well as many indirect costs for patients, such as absence from work and school and reduced work productivity. The symptoms of rhinitis include rhinorrhea/runny nose, nasal blockage/stuffy nose, itching nose and sneezing. Allergic rhinitis is defined as rhinitis accompanied with allergen-specific IgE production. It is frequently associated with asthma and ocular symptoms, such as allergic rhinoconjunctivitis, and it has been estimated that 10-40% of the

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patients with rhinitis also have asthma[2, 4]. Non-allergic rhinitis includes a heterogeneous subgroup of rhinitis without any clinical signs of allergic inflammation, such as rhinitis of the elderly, occupational rhinitis, gustatory rhinitis, hormonal rhinitis and drug-induced rhinitis [1, 5, 6]. The mechanisms for non-allergic rhinitis are still mostly unclear. The diagnosis of allergic and non-allergic rhinitis is mainly based on allergen-specific IgE test. The test can be performed either on the skin surface by skin prick tests or in blood serum by enzyme immunoassay or radioimmunoassay [2].

The occurrence of rhinitis is suggested to be associated with genetic and environmental factors. Many environmental factors, such as pollen and fungal allergen, air and traffic pollution, climate change, ozone level, tobacco smoking, fragrance products, industrial chemicals and social class, are also suggested to be associated with the occurrence of rhinitis [7, 8]. In recent years, the development of culture-independent high-throughput sequencing technology enables researchers to survey microbiome composition in various human body sites, such as human gut, skin and respiratory tract, as well as indoor environments, such as homes, schools, hospitals, hotels and public transport systems [9-15]. These profiling studies showed that microbiome diversity and compositional variation are associated with many metabolic and immune diseases, such as asthma[16]. Studies identified many potential protective and risk microorganisms for asthma in the home and school environments[17-22]. Compared with extensive studies in asthma, no studies reported the relationships between indoor microbiome and occurrence of rhinitis, and thus the association pattern between the indoor microbiome and the development of rhinitis is unclear.

In this study, we investigated associations between indoor microbiome exposure and the occurrence of allergic and non-allergic rhinitis. The study was conducted among randomly selected junior high school students in Terengganu, Malaysia, and a self-administered questionnaire and skin prick test were conducted to assess the prevalence of allergic and non-allergic rhinitis. Species-level high-throughput amplicon sequencing was conducted to characterize microbiome composition in the classrooms. A regression model was applied to evaluate the associations between indoor microbiome and disease. The study aims to screen a list of potential protective and risk microorganisms for allergic and non-allergic rhinitis and evaluate the health effects of indoor microbiome exposure.

Materials and methods

Study design and ethical permission

In this study, 8 junior high schools were randomly selected from Terengganu, Malaysia. In each school, 4 classes were randomly selected to collect vacuum dust samples. Dust samples from 2 classrooms were used up in previous chemical analyses, and thus in total 30 samples were sequenced to character indoor microbiome composition. Students were randomly selected to conduct a skin prick test and report their health condition and personal information through the self-administered questionnaires. Vacuum dust, self-administered questionnaires and skin prick test were collected from November 2012 to February 2013. The study was approved by the Medical Research and Ethics Committee at the National University of Malaysia, Malaysia Ministry of Education, Education Department of Terengganu State and principals and teachers of each school. All participants gave their formal written consent, and the records were kept in the National University of Malaysia.

Health data and skin prick test

A self-administered questionnaire in the Malay language was distributed to students to survey the personal information, including age, gender, current smoking and parental asthma, and the occurrence of rhinitis. The questionnaire was also used in previous rhinitis studies in Sweden, China and Malaysia [23, 24]. One question asked whether the students had a stuffy nose or runny nose in the last 3 months, and 4 alternatives were available to choose, including (A) “Yes, everyday”, (B) “Yes, 1-4 times/week”, (C) “Yes, 1-3 times/month” and “No, never”. The students chose (A), (B) or (C) were defined as having rhinitis. One question asked whether the students had respiratory infections in the last 3 months, and 4 alternatives were available to choose, including (A) “Yes, everyday”, (B) “Yes, 1-4 times/week”, (C) “Yes, 1-3 times/month” and “No, never”. The students chose (A), (B) or (C) were defined as having recent respiratory infections, and these students

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were excluded in the further rhinitis analyses. The questionnaire was answered at home with the help of the students' parents. Medical staff from our group went through the questionnaire and had a face-to-face interview with students to clarify the uncertainties about the questions. When they answer the questionnaire, the students had no information regarding the data collected in the classrooms.

To define allergic and non-allergic rhinitis, we conducted skin prick tests for the selected students[25]. The tests were performed by experienced hospital nurses, and the participants were asked about their recent medication intake before the tests. Allergen extract was purchased from ALK-Abello, Horsholm, Denmark, including pollen (Birch mix, *Phleum pretense* and *Artemisia vulgaris*), animal epidermal (*Equus caballus* and *Felis domesticus*), house dust mite (*Dermatophagoides farinae* and*Dermatophagoides pteronyssinus*), molds (*Cladosporium cladosporioides* and *Alternaria alternate* /*Alternaria tenuis*). Each bottle contained allergen extract in 10 HEP (histamine equivalent). A standard saline solution and histamine solution (10 mg/mL) were used as negative and positive controls. Skin prick tests were performed on the forearm with a disposable ALK skin lancet. The skin surface was observed after 15 minutes, and a positive reaction was defined as wheal $>$ 3mm in diameter.

Vacuum dust sampling, DNA extraction and sequencing

A vacuum cleaner (400W) was used to collect dust in classrooms. A dust sampler (ALK Abello, Copenhagen, Denmark) with a Millipore filter was equipped in the vacuum cleaner. The Millipore filter was made of cellulose acetate. The pore size of the filter was 6 μm, which retains 74% particles of 0.3-0.5 μm, 81% particles of 0.5-1.0 μm, 95% particles of 1-10 μm and 100% particles larger than 10 μm according to manufacturer's instruction. Each selected classroom was divided into two parts with approximately the same size; one near the corridor and one near the window. Dust sample was collected from each part by 4 minutes vacuum sampling with 2 minutes on the floor surface and 2 minutes on the upper surfaces of desks and chairs. Two dust samples in each classroom were combined and sieved through a 0.3-mm mesh screen to obtain the fine dust, which was further stored in a -80 @C freezer until DNA extraction and sequencing.

DNA extraction and multiplex amplicon sequencing were performed by Personal Biotechnology Co., Ltd (Shanghai, China). First, the total bacterial and fungal DNA was extracted from vacuum dust by DNA kit D5625-01 (Omega Bio-Tek, Inc., Norcross, GA, USA) and Fast DNA SPIN kit (MP Biomedicals, Santa Ana, CA, USA). The quality and quantity of the extracted DNA were assessed by agarose gel electrophoresis and NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The full-length of bacterial 16S rRNA gene was amplified by the forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and the reverse primer 1492R (5'-ACCTTGTTACGACTT-3'), and the fungal ITS region was amplified by the forward primer ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and the reverse primer LR3 (5'-CCGTGTTTCAAGACGGG-3'). Then sample-specific tag sequences (16 bp) were incorporated into the PCR amplicons by the Barcoded Univ F/R Primers Plate-96v2 kit (Pacific Biosciences, Menlo Park, CA, USA). The PCR amplicons were purified by Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN) and further quantified by PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). The amplicons were sequenced by the Single-Molecule Real-Time (SMRT) technology combined with circular consensus sequencing (CCS) technique at the PacBio Sequel platforms [26, 27].

The total bacterial and fungal concentration in dust was quantified by quantitative PCR. First, the bacterial and fungal DNA was extracted from 10 mg dust, respectively. Universal primers targeted bacterial 16S rRNA gene (forward: 5'-GCAGGCCTAACACATGCAAGTC-3' and reverse: 5'-CTGCTGCCTCCCGTAGGAGT-3') and fungal ITS 1 regions (forward primer 5.8F1: 5'-AACTTTCAACAACGGATCTCTTGG-3', the reverse primer is 5.8R1: 5'-GCGTTCAAAGACTCGATGATTCAC-3') were used for the quantitative PCR[23, 28].

Bioinformatics and association analyses

The raw sequence data were deposited in the Genome Sequence Archive[29] with accession numbers CRA002825 and CRA002876. The sequenced circular consensus sequences were first processed by the PacBio SMRT Link portal (version 5.0.1.9585) with minfullpass as 3 and minPredictedAccuracy as 99. Sequences

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with predicted accurate < 99% were removed from further analyses. The Quantitative Insights Into Microbial Ecology (QIIME, v1.8.0) pipeline was used to process the filtered data[30]. Sequences were first assigned to the corresponding samples based on the sample-specific tag information. In each sample, sequences were clustered into operational taxonomic units (OTUs) with 97% of sequence similarity by UCLUST (v5.2.236) [31], and a representative sequence from each OUT was picked and annotated by searching against the Silva (release 115) and UNITE database (release 5) for bacteria and fungi[32, 33]. For each sample, an OTU table was generated to record the abundance of all characterized OTUs. OTUs counted for < 0.001% of total sequences were removed from further analyses. All samples were rarefied at even depth for the comparative analyses. Microbial compositional variation was visualized by NMDS (non-metric multidimensional scaling) analysis[34] based on Bray-Curtis distance metrics.

We performed three-level (class and school as second and third levels) hierarchical logistic regression between microbial richness/absolute concentration and occurrence of allergic/non-allergic rhinitis by StataSE 15.0 (StataCorp LLC). Gender, current smoking and parental asthma were included as adjustments in the regression analyses. The microbial richness was represented as the number of OTUs in bacteria and fungi. The absolute concentration of microbial species was calculated as multiplying the relative abundance of species with the total bacterial/fungal concentration from the qPCR[35]. The absolute concentration was first transformed in the logarithmic scale ($\log_{10}$ (absolute concentration + 1)) and then analyzed in the logistic regression model. The association between the absolute microbial concentration and allergic/non-allergic rhinitis were conducted for species presented in > 10 classrooms with average relative abundance > 0.1%. Only associations with p-value < 0.005 were considered as significant throughout the study. The false discovery rate (FDR) was calculated by the p.adjust function with the Benjamini-Hochberg procedure in R.

Results

Demographics and prevalence of rhinitis

In this study, we collected vacuum dust from 30 classrooms of 8 junior high schools in Terengganu, Malaysia. Four schools were from the urban area and four schools from the rural area. Self-administered questionnaires and skin prick test results were obtained from 370 students in the selected classes. Among them, 23 students reported recent respiratory infections in the last 3 months and thus were removed from the analyses. In total, 347 students were included in the final dataset, included 129 males and 218 females. Based on health information from questionnaire and skin prick tests, we defined 107 students as healthy subjects, 110 students with allergic rhinitis and 130 students with non-allergic rhinitis. The prevalence of house dust mite and cat allergy was high among students (38.3% and 11.0%; Table S1). There was no difference between male and female students in the prevalence of allergic and non-allergic rhinitis (p = 0.98; Table 1). Ten students reported current smoking, and there was no difference between smoking and non-smoking students in the prevalence of allergic and non-allergic rhinitis (p = 0.38).

Species-level microbiome composition and variation

The abundance of microbial taxa at the taxonomical class and species level were analyzed first. Alphaproteobacteria (17.2%), Gammaproteobacteria (15.9%), Cyanobacteria (14.3%), Actinobacteria (12.9%), Bacilli (10.5%), Deinococci (7.2%) and Betaproteobacteria (7.2%) were the high abundant bacterial classes in the classrooms.*Acinetobacter johnsonii* (4.0%), *Aliterella Antarctica*(2.7%), *Klebsiella pneumonia* (2.0%), *Methylobacterium radiotolerans* (1.9%) and *Saccharopolyspora halotolerans* (1.9%) were the high abundant bacterial species (Figure 1A; Table S2). For fungi, Dothideomycetes (30.7%), Eurotiomycetes (20.6%), Wallemiomycetes (12.2%), Agaricomycetes (11.9%), Sordariomycetes (7.8%) and Agaricostibomycetes (5.8%) were the high abundant fungal classes in the classrooms. *Wallemia sebi* (10.9%), an unidentified Pleosporales (7.1%), *Sterigmatomyces halophilus*(5.8%), *Aspergillus penicillioides* (5.7%), *Eupenidiella venezuelensis* (5.3%) and *Bambusaria bambusae* (5.2%) were the high abundant fungal species in the junior high school of Terengganu (Figure 1B; Table S3).

We further characterize and visualized the overall microbial compositional variation among classrooms by non-metric multidimensional scaling (NMDS) analysis. The bacterial and fungal composition in urban

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schools did not differ from rural schools (Figure 2A and 2B). Samples collected in the same school were compositionally more similarity compared with samples collected in different schools (Mann-Whitney U test on Bray-Curtis distance matrix, bacteria $p = 0.003$, fungi $p < 0.001$). However, there were also exceptions that samples from the same school had a low compositional similarity. For example, the bacterial composition in T7.3 (school 7 class 3) differed drastically from the bacterial composition in T7.1, T7.2, and T7.4, due to high abundance of *K. pneumoniae* in T7.3 (46.2%; Figure 1A and 2A).

Associations between indoor microbial richness/concentration and allergic/non-allergic rhinitis

In total, 4,977 bacterial OTUs (operational taxonomic units) were identified in this study. The overall bacterial richness (represented as the number of OTUs) in each classroom was not associated with the occurrence of rhinitis in the classroom ($p > 0.05$; Table 2). The same test was also conducted for the major bacterial classes (> 200 OTUs). The richness in Gammaproteobacteria was protectively/negatively associated with allergic and non-allergic rhinitis ($p = 0.02$ and 0.04). Similarly, 2,632 fungal OTUs were identified in this study, and the overall fungal richness was positively associated with allergic and non-allergic rhinitis ($p = 0.01$ and 0.03). We did not find significant associations between specific fungal class and the occurrence of rhinitis ($p > 0.05$). The richness in Agaricomycetes had a small trend to be associated with allergic rhinitis ($p = 0.06$) and non-allergic rhinitis ($p = 0.10$).

We further analyzed the associations between the absolute concentration of indoor bacterial and fungal species and the occurrence of allergic and non-allergic rhinitis. To control the number of tests, only species presented in at least 10 classrooms and mean abundance > 0.1% were included in the regression analyses. *Brasilonema bromeliae* from the phylum of Cyanobacteria and *Aeromonas enteropelogenes* and *Escherichia fergusonii* from the class of Gammaproteobacteria were protectively associated with allergic rhinitis ($p < 0.005$, FDR = 0.17; Table 3). These species were presented in 21, 10 and 23 classrooms out of the total 30 classrooms, and the geometric mean absolute concentration was $4.1*10^5$, $1.2*10^5$ and $2.6*10^5$ copies per gram dust. *Deinococcus grandis* from the class of Deinococci and*Chaetomium grande* from the fungal class Sordariomycetes were positively associated with allergic rhinitis ($p < 0.005$, FDR = 0.09 and 0.18). *D . grandis* was presented in 15 classrooms with a geometric mean concentration as $1.2*10^5$ copies per gram dust. *C . grande* was presented in 11 classrooms with a geometric mean of concentration as 399 copy per gram dust. The results indicate that the concentration of health-associated bacterial species is three orders of magnitude higher than the fungal species in the classrooms of Terengganu.

Interestingly, we also found *B. bromeliae* and *A. enteropelogenes* were protectively associated with non-allergic rhinitis ($p = 0.002$ and 0.003, FDR = 0.07; Table 4). *Enterobacter xiangfangensis* from the class of Gammaproteobacteria,*Streptococcus salivarius* from the class of Bacilli and*Patulibacter minatonensis* from the class of Thermoleophilia were also protectively associated with non-allergic rhinitis ($p < 0.05$, FDR < 0.1). *Deinococcus gobiensis* was the only bacterial species that were positively associated with non-allergic rhinitis. These species were presented in 10 to 23 classrooms in Terengganu with a geometric mean concentration from $1.2*10^5$ to $6.0*10^5$ copy per gram dust. No fungal species was associated with the occurrence of non-allergic rhinitis.

No association between environmental characteristics and rhinitis-related species

We further tested the associations between environmental characteristics and the identified rhinitis-related species. Environmental characteristics were tested separately with the rhinitis-related species, including location of schools (urban/rural), the concentration of house dust mite (Der p 1 and Der f 1) and cockroach allergen (Bla g 1 and Per a 1), indoor CO_2, NO_2 levels and relative humidity and indoor visible dampness and mold. We did not find any significant associations between the environmental characteristics and the rhinitis-related species ($p > 0.05$).

Discussion

Strengths and limitations of the study

Here, we report the first association study between indoor microbiome exposure and the occurrence of rhini-

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tis. With this approach, we identified a list of potential health-related species and found similar association patterns between allergic rhinitis and non-allergic rhinitis, which can be further tested in future studies. Another strength is that we applied high-resolution amplicon sequencing (PacBio) to characterize the bacterial and fungal composition and diversity. The species-level taxonomic resolution facilitates accurate microbial diversity estimation compared to standard second-generation sequencing, which can only resolve taxa at the genus level. Also, we applied absolute concentration rather than the relative abundance of microbial species in the association analysis. Previous studies confirmed that absolute concentration is a superior approach compared with the relative abundance in terms of finding the correct microorganism-phenotype associations [35].

There are also limitations in this study. The cross-sectional study design limits the causal inference in the relationship between indoor microbial exposure and rhinitis. The cross-sectional study design is widely used in epidemiological studies, as it is an economical approach to obtain health associations among populations. However, this study design cannot draw the causal relationships, and thus future longitudinal studies are needed to verify the patterns we observed in this study. Also, we applied amplicon sequencing to characterize microbiome composition in this study. Since only one marker gene was sequenced, this approach can provide microbial taxonomic information rather than accurate functional inference, which can be achieved by the shotgun metagenomics sequencing. We conducted the false discovery rate correction in this study, which is one of the widely used approaches to adjust the type I error in null hypothesis testing when conducting multiple comparisons[36]. The associations were not significant after adjustment with the Benjamini-Hochberg (BH) procedure ($0.06 < \text{FDR} < 0.19$). However, the BH procedure was confirmed to be over-conservative in microbiome analysis with much-reduced power in detecting the accurate significant findings. To solve the problem, a new approach DS-FDR has been proposed to improve the sensitivity of detection[37]. However, the new approach can only be applied in the microbiome dataset with relative abundance, and thus it is not applicable in this study. Future approaches dealing with microbiome dataset with transformed absolute concentration are needed to solve the issue.

Prevalence of allergic/non-allergic rhinitis and environmental stimuli

Rhinitis is one of the most prevalent diseases in the world. It is estimated that allergic rhinitis affects approximately 10%-40% of the population in different countries[3]. The international collaborated ISAAC and ECRHS studies reported a list of countries with high prevalence in allergic rhinitis, including Nigeria (>35%), Paraguay (30-35%), Sweden (31%), Hong Kong (25-30%) Australia (15-20%), New Zealand (15-20%), the United Kingdom (15-20%%) [2, 38, 39]. In this study, we reported that the prevalence of allergic rhinitis was 31.7% among junior high schools students in Terengganu, Malaysia, which is comparable with the countries with high prevalence. The prevalence of non-allergic rhinitis is less well defined, and no international collaboration has been conducted to survey the prevalence among different countries systematically. One study estimated that in the United States, 17 million people had non-allergic rhinitis and 22 million people had mixed rhinitis, a combination of allergic and non-allergic rhinitis[40]. Another study reported that the prevalence of non-allergic rhinitis was 9.6% among high school students in Belgium[41]. The prevalence of non-allergic rhinitis is higher in our study (37.5%) compared with previous studies. In this study, we applied skin prick tests to define allergic and non-allergic rhinitis, by using nine allergen extracts from plants, animals, mites and molds. The allergic reaction for students in Terengganu was mainly from the sensitization of house dust mites and cat allergen, which is consistent with a previous study in Malaysia that approximately 50% and 25% office workers had mites and cat allergy[42].

The mechanisms for allergic and non-allergic rhinitis are suggested to be different. Allergic rhinitis is related to the nasal inflammation caused by IgE-mediated allergy. Exposure to the indoor allergen and environmental stimuli lead to overproduction of IgE (immunoglobulin E), which can further activate the receptors on the surface of cells and lead to the allergic and inflammatory symptoms[43]. Compared with allergic rhinitis, the mechanisms for non-allergic rhinitis are less clear. Classic T-cell and cytokine inflammation are suggested to be the major mechanisms for non-allergic rhinitis[1]. Neurogenic dysregulation is another proposed pathway that leads to nasal obstruction and rhinorrhea[1, 44]. Rhinitis of the elderly is suggested to be mainly associated

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with the neural dysregulation.

Although the mechanisms for allergic and non-allergic rhinitis are quite different, the environmental factors associate with the development of rhinitis could be similar. For example, environmental and traffic pollution, including NOx, SOx and ozone, environmental tobacco smoking, mold growth and chemical exposures, including volatile organic compounds (VOCs) are related to both allergic and non-allergic rhinitis[7, 8, 45, 46]. In this study, we found that the association pattern for indoor microbiome was similar between the two types of rhinitis. The phenomenon was supported by analysis in microbial richness and absolute concentration. For example, the richness (number of observed OTUs) in Gammaproteobacteria and in fungi was associated with both allergic and non-allergic rhinitis. The absolute concentration of *B. bromeliae* and *A. enteropelogenes* and two species from the genus of *Deinococcus* were also associated with both allergic and non-allergic rhinitis. The detailed mechanisms for how microorganisms affect nasal inflammation still need to be explored. It is possible that certain microorganisms can stimulate the inflammatory symptoms by multiple pathways, either with allergen sensitization or disturb the epithelium barrier. Future studies can explore the mechanisms in depth by using the microorganisms identified in this study. Epidemiological surveys should also consider to routinely survey the indoor microbiome exposure, together with other environmental factors, to add a new dimension to the exposure science.

Health effects of the microbial species

As this is the first association study between indoor microbiome exposure and rhinitis, we cannot directly compare our results with studies with the same experimental design, but there are some studies available with similar framework. One study in junior high school of Johor Bahru, Malaysia reported that the levels of total endotoxin (lipopolysaccharide, LPS) was negatively associated with rhinitis[47]. LPS is a bacterial cell-wall component and is widely used as a chemical marker for Gram-negative bacteria. In this study, we found that the diversity of Gammaproteobacteria, one of the most diverse classes of Gram-negative bacteria, was protectively associated with rhinitis. Another study reported that the total fungal DNA, assessed by quantitative PCR, was a potential risk factor for rhinitis in the indoor environment[23]. This is also consistent with our finding in this study that total fungal diversity was positively associated with the occurrence of rhinitis.

As this is the first association study for indoor microbiome and rhinitis, all these species were first time reported to be associated rhinitis. Several of the identified species were from the outdoor environment. Two of the environmental species, including *B. bromeliae* , *P. minatonensis* , were protectively (negatively) associated with rhinitis, and two of the environmental species, including *D. gobiensis* , *D. grandis* , were positively associated with rhinitis. Interestingly, the two potential risk species,*D. gobiensis* and *D. grandis* were both extremely radiation-resistant bacteria[48, 49]. These species are physically protected from the UV damages due to their efficient DNA repair system[50]. No study reported their health effects for humans, and it is unclear whether the radiation-resistant feature of these bacteria is related to the potential adverse health effect. Four rhinitis-related bacteria species, including *E. fergusonii* , *A. enteropelogenes* , *E. xiangfangensis* and *S. salivarius* , were protectively associated the rhinitis, and interestingly, these bacteria were all reported to be facultative pathogens that can cause various diseases in human and animals, such as respiratory infections, diarrhea and neutropenia[51-54]. Although*S. salivarius* is a facultative pathogen that can infect immunocompromised patients, the species can also be used as probiotic that could reduced 80% of nasal streptococcus infections in adults[55]. The results suggest complex health effects of the species. The "hygiene hypothesis" proposes that having respiratory infections could reduce the occurrence of allergic diseases for children[56]. The role of infectious pathogens and the development of asthma in children have also been fiercely debated over the past years[57, 58]. Some studies suggest that rhinovirus infections and respiratory syncytial virus (RSV) infection were associated with increased odds of wheeze and asthma[57, 59], but other population-based studies reported that exposure to infections diseases could lead to reduced atopic disorders [60]. The potential protective effects of childhood infections are suggested to relate to the training and maturation of the immune system from the exposure [58]. A recent study in university dormitories reported that exposed to facultative pathogens, including *Haemophilus* , *Klebsiella* ,*Buttiauxella* , and *Raoultella* , increased the odds

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of having respiratory infections[61]. Overall, more studies are needed to verify the pattern we observed in this study and further disentangle the health effects of the indoor microbiome on different respiratory diseases.

Acknowledgements

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Figure 1. Relative abundance of top (A) bacteria and (B) fungal species level in the classrooms.

Table 1. Prevalence of allergic and non-allergic rhinitis among junior high school students (N=347) in Terengganu, Malaysia. P-values were calculated by Chi-square test.

	Number	Prevalence (%)	Male (%)	Female (%)	p-value	Smoking (%)	Non-smoking	p-val
Healthy	107	30.8	31.0	30.7	0.98	30.0	30.9	0.38
Allergic rhinitis	110	31.7	31.0	32.1		40.0	31.5	
Non-allergic rhinitis	130	37.5	38.0	37.2		30.0	37.6	

Table 2. Associations between bacterial and fungal richness (represented as number of observed OTUs) and allergic and non-allergic rhinitis among students (N = 347) in junior high school, Terengganu. Taxonomical classes with the number of OTUs > 200 were included in the analysis. Cyanobacteria were not resolved at the class level, and thus the calculation was conducted at the phylum level. Odds ratio (OR) and 95% confidence interval (CI) were calculated by 3-level hierarchic linear regression models adjusted for gender, smoking and parental asthma. Odds ratio was calculated for 100 OTU increase. P values < 0.05 were formatted with bold font.

Kingdom	Phylum	Class	Number of OTUs	Allergic rhinitis	Allergic r
				OR (95% CI)	p-value
Bacteria	Proteobacteria	Alphaproteobacteria	999	1.00 (1.00,1.01)	0.39
		Gammaproteobacteria	307	**0.96 (0.93,0.99)**	**0.02**
		Betaproteobacteria	226	1.02 (0.99,1.06)	0.19
	Actinobacteria	Actinobacteria	593	1.01 (0.98,1.04)	0.49
	Cyanobacteria		937	0.99 (0.99,1.00)	0.28
	Firmicutes	Bacilli	287	0.99 (0.97,1.03)	0.93
	Deinococcus-Thermus	Deinococci	230	1.01 (0.98,1.03)	0.61
Total bacterial OTUs			4977	1.00 (1.00,1.01)	0.37
Fungi	Ascomycota	Dothideomycetes	803	1.01 (0.99,1.02)	0.47

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Kingdom	Phylum	Class	Number of OTUs	Allergic rhinitis	Allergic r
		Eurotiomycete	420	1.01 (0.99,1.03)	0.28
		Sordariomycetes	265	1.00 (0.97,1.03)	0.84
	Basidiomycota	Agaricomycetes	490	1.01 (1.00,1.02)	0.06
		Wallemiomycetes	274	1.00 (0.98,1.02)	0.82
Total fungal OTUs			2632	**1.01 (1.00,1.01)**	**0.01**

Table 3. Associations between the absolute concentration of indoor microbial species and allergic rhinitis in junior high school, Terengganu. Odds ratio and 95% confidence interval (CI) were calculated by 3-level hierarchic linear regression models adjusted for gender, smoking and parental asthma. The regression was conducted for species presented in at least 10 classrooms with mean relative abundance > 0.1%. In total, 170 bacterial and 89 fungal species were analyzed. Only associations with $p < 0.005$ are presented in the table. GM stands for geometric mean. The false discovery rate (FDR) was calculated by Benjamini-Hochberg (BH) procedure.

Kingdom	Phylum	Class	Species	Absolute abundance GM (95%C
				(copy / g dust)
Bacteria	Cyanobacteria		*Brasilonema bromeliae*	$4.1*10^5$ (1.1-14.7)
	Deinococcus-Thermus	Deinococci	*Deinococcus grandis*	$1.3*10^5$ (0.3-5.2)
	Proteobacteria	Gammaproteobacteria	*Aeromonas enteropelogenes*	$1.2*10^5$ (0.1-10.8)
			Escherichia fergusonii	$2.6*10^5$ (0.8-8.1)
fungi	Ascomycota	Sordariomycetes	*Chaetomium grande*	399 (183-871)

Table 4. Associations between the absolute concentration of indoor microbial species and non-allergic rhinitis in junior high school, Terengganu. Odds ratio and 95% confidence interval (CI) were calculated by 3-level hierarchic linear regression models adjusted for gender, smoking and parental asthma. The regression was conducted for species presented in at least 10 classrooms with mean relative abundance > 0.1%. In total, 170 bacterial and 89 fungal species were analyzed. Taxonomic information of the associated microbes is presented. Only significantly associated species ($p < 0.005$) are presented in the table. GM stands for geometric mean. The false discovery rate (FDR) was calculated by Benjamini-Hochberg (BH) procedure.

Kingdom	Phylum	Class	Species	Absolute abundance GM (95%C
				(copy / g dust)
Bacteria	Actinobacteria	Thermoleophilia	*Patulibacter minatonensis*	$5.4*10^5$ (1.0-30.6)
	Cyanobacteria		*Brasilonema bromeliae*	$4.1*10^5$ (1.1-14.7)
	Deinococcus-Thermus	Deinococci	*Deinococcus gobiensis*	$3.9*10^5$ (1.5-9.8)
	Firmicutes	Bacilli	*Streptococcus salivarius*	$4.0*10^5$ (1.0-15.7)
	Proteobacteria	Gammaproteobacteria	*Aeromonas enteropelogenes*	$1.2*10^5$ (0.1-10.8)
			Enterobacter xiangfangensis	$6.0*10^5$ (1.5-24.0)

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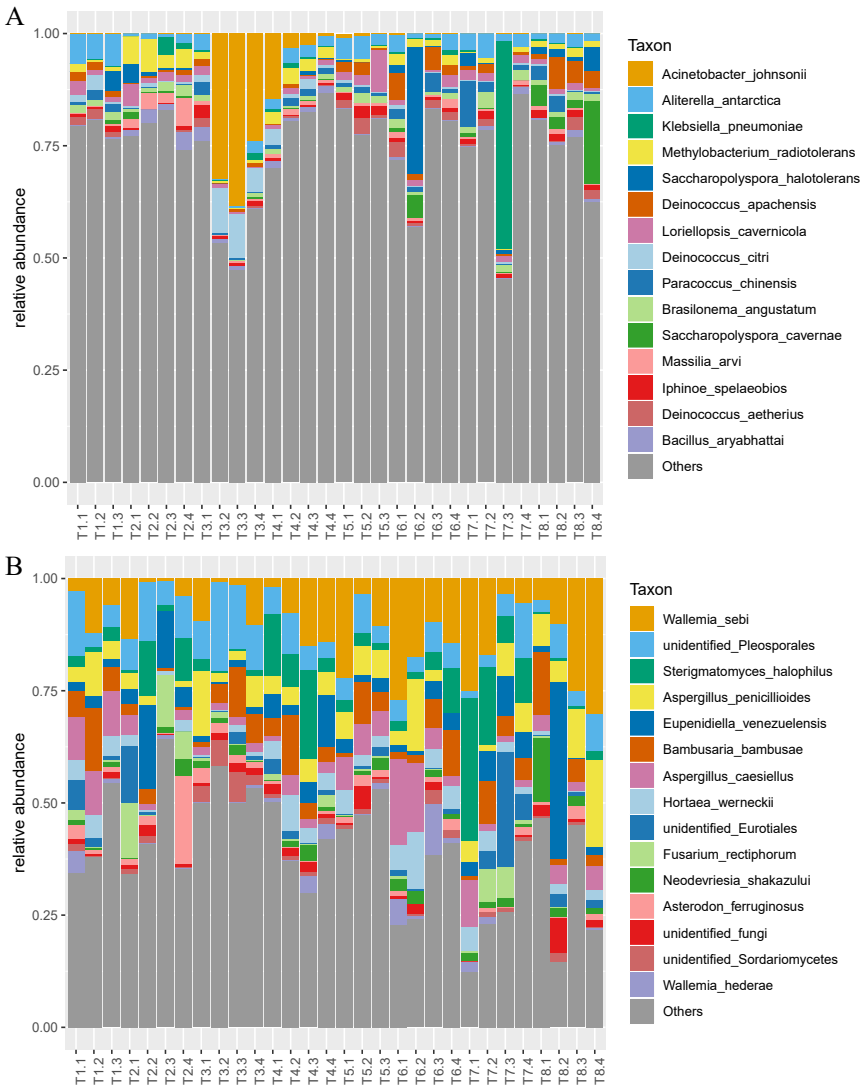

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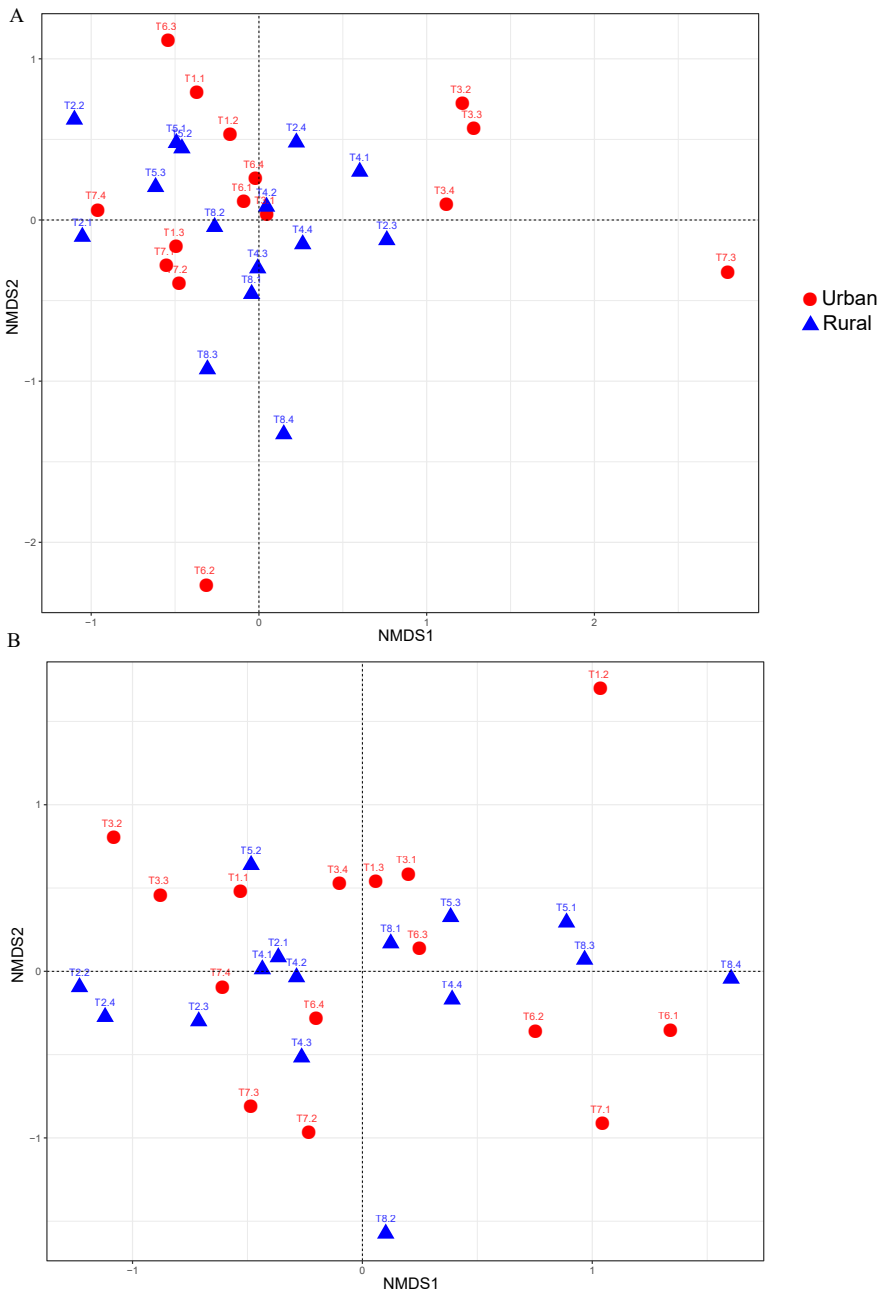

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