Moraxellaceae and *Moraxella* interact with the altered airway mycobiome in asthma

2 Hai-yue Liu[a,b,c]*, Chun-xi Li[a]*, Zhen-yu Liang[d], Shi-yu Zhang[a], Wan-ying Yang[a],

Yan-mei Ye[a], Yan-xia Lin[a], Rong-chang Chen[d], Hong-wei Zhou[b,c#], Jin Su[a#]

4 Affiliations: Department of Respiratory and Critical Care Medicine, Chronic Airways

Diseases Laboratory, Nanfang Hospital, Southern Medical University, Guangzhou,

6 China[a]; State Key Laboratory of Organ Failure Research, Division of Laboratory

Medicine, Zhujiang Hospital, Southern Medical University, Guangzhou, China[b];

8 Microbiome Medicine Center, Zhujiang Hospital, Southern Medical University,

Guangzhou, China[c]; and State Key Laboratory of Respiratory Disease, National Clinical

10 Research Center for Respiratory Disease, Guangzhou Institute of Respiratory Health, the

First Affiliated Hospital of Guangzhou Medical University, Guangzhou, China[d]. *These

12 authors contributed equally to this work. [#]Both authors contributed equally.

14 Correspondence: Dr. Jin Su, Department of Respiratory and Critical Care Medicine,

Chronic Airways Diseases Laboratory, Nanfang Hospital, Southern Medical University,

16 Guangzhou, China. E-mail: drsujin@126.com. Telephone: +8613711193283.

Dr. Hong-wei Zhou, State Key Laboratory of Organ Failure Research, Division of

18 Laboratory Medicine, Zhujiang Hospital, Southern Medical University, Guangzhou,

China. E-mail: biodegradation@gmail.com. Telephone: +8618688489622.

20

Running title: Moraxellaceae, *Moraxella* and airway mycobiome

ABSTRACT Airway microbial-microbial interaction plays an important role in chronic airway inflammatory diseases such as asthma. *Moraxella* is widely regarded as a human respiratory tract pathogen. We aimed to investigate the interactions among *Moraxella*, Moraxellaceae (to which *Moraxella* belongs) and the airway microbiome in asthma. Induced sputum samples were obtained from 116 asthma patients and 29 healthy individuals, and the bacterial/fungal communities were profiled using 16S rRNA and ITS1 DNA gene sequencing. We found that asthma patients harboured significantly greater airway bacterial and fungal α-diversity than that of healthy individuals. Moraxellaceae, *Moraxella* and *Moraxella* otu19 (bacteria), and Schizophyllaceae, Polyporaceae, *Aspergillus*, *Schizophyllum* and *Candida* (fungi) were increased in the airway of asthma. Moreover, Moraxellaceae, Schizophyllaceae, Polyporaceae and *Candida* were positively associated with airway fungal α-diversity. Correlation networks revealed Moraxellaceae and *Moraxella* as microbial "hubs" in asthma that had significant negative connections with multiple bacterial communities, such as Leptotrichiaceae, Lachnospiraceae, Peptostreptococcaceae, Porphyromonadaceae, *Prevotella*, *Veillonella, Rothia* and *Leptotrichia*, but positive interactions with fungal communities such as Schizophyllaceae, Polyporaceae, *Candida* and *Meyerozyma*. Together, our finding revealed an altered microbiome and complex microbial-microbial interactions in the airway of asthma. Moraxellaceae and *Moraxella* showed significant interactions with the airway mycobiome, providing potential insights into the novel pathogenic mechanisms of asthma.

IMPORTANCE With the advent of culture-independent techniques, growing evidence suggests that the airway microbiome is closely correlated with chronic respiratory diseases such as asthma. The complex microbial-microbial interaction exists in the airways of both healthy individuals and patients with respiratory diseases, which is of great significance for the pathogenesis and disease progression of asthma. In this study, we evaluated the airway dysbiosis in asthma patients, described the interaction between Moraxellaceae, *Moraxella* and airway bacterial/fungal communities, and it contributes to further understanding the pathogenic mechanisms of asthma.

KEYWORDS: asthma, airway microbiome, mycobiome, interaction, 16S rRNA, ITS

INTRODUCTION

Asthma is a common airway condition characterized by chronic airway inflammation that affects approximately 300 million people worldwide (1). Recently, new culture-independent techniques have revealed that human airways harbour unique microbial communities (including bacteria, fungi and viruses) that are closely correlated with chronic lung diseases such as asthma (2-4). The airway bacterial community in asthma patients differs significantly from that of healthy individuals, with increased bacterial diversity, and more Proteobacteria, especially *Moraxella*, and fewer Bacteroidetes (2).

Increasing evidence indicates that the airway bacterial microbiome plays an important role in asthma. In a study of more than 600 infants at 1 month of age, *Moraxella*

64   *catarrhalis*, *Haemophilus influenzae* or *Streptococcus pneumoniae* cultured from the

oropharynx was correlated with increased risks of later wheezing and asthma (5).

66   Culture-independent techniques have suggested that airway bacteria may cause asthma

and affect asthma activity (6). Several studies have indicated that airway bacteria are

68   correlated with disease-related features and with the severity and therapeutic response of

asthma (7-10). *Moraxella* is currently regarded as an important and common pathogen in

70   the human respiratory tract and is associated with a range of diseases such as sinusitis,

tracheitis, bronchitis and pneumonia in children and bronchitis, pneumonia and

72   nosocomial infections in adults (11), as well as with exacerbation of asthma (12). In many

asthma patients, *Moraxella catarrhalis* is a dominant pathogen in the airway bacterial

74   community (10) and is one of the most frequently isolated bacteria from sputum cultures

from patients with asthma exacerbations (13).

76   Recently, the presence of the fungal microbiome (mycobiome) in both healthy and

abnormal lungs has been increasingly recognized, and this mycobiome may impact the

78   clinical course of chronic pulmonary diseases such as asthma (14). One previous study

identified differences in the airway mycobiome between asthma patients and healthy

80   controls by using standard PCR techniques and pyrosequencing (15).

Airway bacterial-bacterial interactions are known to be associated with immune

82   responses and airway inflammatory processes (16), thereby influencing the therapeutic

response and clinical outcome of lung diseases (17). Recent findings have provided

84   insights on the airway bacterial-fungal interaction, which may contribute to the decline in

4

lung function and to disease progression (18) and is important in respiratory disease

86   settings (19). However, fungal-bacterial interactions in asthma have not been studied

extensively using culture-independent methods, and relatively little is known about the

88   interaction among *Moraxella*, Moraxellaceae (to which *Moraxella* belongs) and the

airway mycobiome in asthma.

90   Recognizing that complex airway microbial communities and microbial-microbial

interactions play an increasingly important role in the development and progression of

92   asthma (16, 19-21), we conducted a cross-sectional study to explore the alterations of

Moraxellaceae and *Moraxella* in asthma airways as well as the interactions among

94   Moraxellaceae*, Moraxella* and the airway mycobiome. We aimed to provide new insights

on airway microbial characteristics, bacterial-fungal interactions, and the potential

96   pathogenic mechanisms of asthma.

98   **RESULTS**

Clinical characteristics of the subjects. A total of 145 sputum samples were

100   obtained from 116 asthma patients (55 stable asthma patients and 61 exacerbated asthma

patients) and 29 healthy subjects. After the sequencing analysis, samples from 128 and

102   145 participants were analysed for bacterial and fungal community composition data,

respectively. No significant differences were found in age, gender or smoking history

104   between the healthy and asthmatic groups. Patients with stable asthma had more ICS

usage than patients with exacerbated asthma (*P*=0.017) (Table 1).

5

106

Airway bacterial and fungal communities in asthma patients differed from

108 **those in healthy individuals.** Compared with healthy individuals, asthma patients

showed a significantly greater airway α-diversity (calculated using the Shannon index

110 and PD_whole_tree index) (Fig. 1) and distinct differences in the community

compositions (β-diversity) (Fig. S1A) of both bacterial and fungal communities.

112 In addition, we compared the airway bacterial and fungal community compositions

(relative abundance >1%) between asthma patients and healthy individuals (Table S1).

114 Asthma patients harboured a slightly higher abundance of Proteobacteria (P=0.222) and

an extremely increased abundance of Moraxellaceae, *Moraxella* and *Moraxella* otu19 in

116 the airway than healthy individuals (Fig. 2). Among these selected bacterial and fungal

communities, more than 70% of the fungal community differed significantly between

118 asthma patients and healthy individuals, especially Ascomycota, Basidiomycota,

Schizophyllaceae, Polyporaceae, *Aspergillus, Schizophyllum, Candida* and *Meyerozyma*,

120 which were more prevalent in asthma patients, while only 20-45% of the bacterial

community differed significantly between the two groups, suggesting that the airway

122 fungal community had a more varied and complex dysbiosis than the bacterial

community in asthma.

124 Bronchial hyperresponsiveness in outpatients with asthma may affect the airway

microbiome (22). Accordingly, we divided the asthmatic population into two

126 subgroups—stable asthma and exacerbated asthma—according to disease activity.

However, both the microbial α-diversity (Shannon index, bacteria: P=0.554, fungi:

128   P=0.316, respectively) and β-diversity (bacteria: R^2=0.009, P=0.269, fungi: R^2=0.008,

P=0.549, respectively) were similar, and no significant differences in either bacterial or

130   fungal community composition were found between the two subgroups (Fig. S2).

Although not statistically significant, the phylum that exhibited the greatest increase

132   in asthma was Proteobacteria. We therefore evaluated the overall families belonging to

Proteobacteria. We found that the relative abundance of Moraxellaceae was significantly

134   higher in asthma patients than in healthy individuals (80.5-fold, P=0.007) (Fig. 2).

However, the abundances of other families (average abundance >1%), such as

136   Neisseriaceae and Pasteurellaceae, were not significantly increased, and some were even

decreased; for example, Burkholderiaceae was lower in asthma patients (P=0.041) (Fig.

138   S1B). Focusing on the distinct difference in Moraxellaceae between asthma patients and

healthy individuals, we further compared the different microbes at the genus level and

140   OTU level. In agreement with the results for Moraxellaceae, *Moraxella* and *Moraxella*

otu19 were significantly increased in asthma patients (314.7-fold, P=0.027; not detected

142   in healthy individuals, P=0.029, respectively) (Fig. 2).

144   **Relationship among Moraxellaceae, *Moraxella* and airway bacterial/fungal**

diversity. As shown above, the airway microbial diversity in asthma patients differed

146   from that in healthy individuals. Thus, we explored whether there was a relationship

among the increased Moraxellaceae, *Moraxella* and airway bacterial/fungal α-diversity in

7

148   asthma.

We first focused on the bacterial diversity and found no significant relationships

150   among Proteobacteria, Moraxellaceae, *Moraxella, Moraxella* otu19 and bacterial

diversity. However, a significantly positive correlation was found between Moraxellaceae

152   and fungal diversity (Fig. 3). Next, we investigated whether the airway fungal community

was related to fungal diversity and found that Basidiomycota, Schizophyllaceae,

154   *Schizophyllu*m, *Schizophyllum commune* otu6, *Aspergillus* otu5, Polyporaceae and

Candida were positively correlated with fungal diversity (all *P* values<0.001) (Fig. S3).

156   Our findings suggested that Moraxellaceae had a greater influence on the fungal

community than the bacterial community in the asthma airway.

158

Interaction among Moraxellaceae, *Moraxella* and the airway mycobiome. To

160   further explore the interaction among the increased Moraxellaceae, *Moraxella* and the

airway microbiome in asthma, we used Spearman correlation analysis to build correlation

162   networks (Fig. 4).

Examination of the microbial network revealed a few microbial "hubs" that were

164   negatively connected with other portions of the airway microbiome. At the phylum level,

Proteobacteria was significantly negatively associated with Firmicutes, Bacteroidetes and

166   Actinobacteria but significantly positively correlated with Ascomycota and

Basidiomycota. At the family level, Moraxellaceae showed 9 negative correlations with

168   bacterial families, including with Veillonellaceae, Lachnospiraceae, Leptotrichiaceae,

8

Prevotellaceae, Peptostreptococcaceae and Porphyromonadaceae, but 2 positive

170   correlations with Schizophyllaceae and Polyporaceae. Moreover, Schizophyllaceae,

Polyporaceae and Trichocomaceae exhibited strong positive correlations with each other

172   and were negatively correlated with Leptotrichiaceae, Lachnospiraceae,

Peptostreptococcaceae and Mogibacteriaceae. At the genus level, *Moraxella* was

174   negatively associated with 7 bacterial genera (*Veillonella, Leptotrichia, Rothia,*

Streptococcus, Peptostreptococcus, Prevotella an*d Lautropia*) and positively associated

176   with 2 fungal genera (*Candida* and *Meyerozyma*). *Candida* was positively related to

Aspergillus and *Schizophyllum* but negatively linked to *Peptostreptococcus* and

178   *Streptococcus. Meyerozyma* was negatively associated with *Prevotella* and *Veillonella.* At

the OTU level, *Moraxella* otu19 was significantly negatively associated with *Veillonella*

180   *parvula* otu22, *Streptococcus* otu5, *Peptostreptococcus* otu16 and Unclassified Fungi

otu20 but significantly positively associated with *Schizophyllum commune* otu6 and

182   Unclassified Fungi otu12. Additionally, *Schizophyllum commune* otu6 was negatively

correlated with *Neisseria* otu1 and Unclassified Fungi otu1. Taken together, these results

184   suggested complex and close interactions among Moraxellaceae, *Moraxella* and airway

microbial communities (12 out of 21 communities and 10 out of 18 communities,

186   respectively), especially the fungal community (Schizophyllaceae, Polyporaceae,

Candida and *Meyerozyma*).

188

DISCUSSION

9

190   Asthma is a chronic airway inflammatory disease associated with altered microbial

communities in the airway, and these communities are closely related to airway

192   inflammation. Recent studies have highlighted the importance of the complex

microbial-microbial interactions in asthma (16, 18, 19). In addition to bacterial dysbiosis

194   (23), we concurrently identified fungal dysbiosis characterized by alterations in diversity

and composition in the asthma airway. We found that Moraxellaceae was positively

196   associated with airway fungal diversity. Moraxellaceae and *Moraxella* showed positive

interactions with Schizophyllaceae, Polyporaceae, *Candida* and *Meyerozyma* but negative

198   interactions with Leptotrichiaceae, Lachnospiraceae, Burkholderiaceae, Mogibacteriaceae,

Peptostreptococcaceae,   Porphyromonadaceae,   *Prevotella*,   *Veillonella,   Rothia,*

200   *Leptotrichia* and *Streptococcus,* which were enriched in healthy individuals, indicating

that Moraxellaceae and *Moraxella* interact with the altered airway mycobiome in asthma.

202   Our findings revealed significant differences in airway microbial diversity and

community composition between asthma patients and healthy individuals. In agreement

204   with previous studies, we found that asthma patients harboured a higher bacterial

α-diversity (22, 23) and greatly increased proportions of Moraxellaceae and *Moraxella* in

206   the airway compared with healthy individuals. Airway dysbiosis in asthma was evident

not only in the bacterial community but also in the fungal community. Our study is the

208   first to report that asthma patients have greater fungal α-diversity (1.6-fold greater

Shannon index and 1.2-fold greater PD_whole_tree compared with healthy individuals)

210   and more complicated fungal composition dysbiosis compared with bacterial dysbiosis

(71.4-100% vs. 20-45%) in the airway.

212   *Moraxella* is widely recognized as a pathogen in the airway and is associated with several respiratory diseases such as asthma (11, 12). Multiple studies have reported

214 altered airway microbial dysbiosis, with pathogenic Proteobacteria more frequently found in asthma (2, 22-25), and we found that this increase occurred mainly because of the

216 increases in Moraxellaceae and *Moraxella*, which were 80.5-fold and 314.7-fold more abundant than in healthy individuals, respectively.

218   Previous studies demonstrated bacterial-bacterial interactions in chronic airway diseases such as asthma (16, 19). Our results showed unique microbial networks in

220 asthma. Moraxellaceae and *Moraxella* were negatively correlated with 9 and 7 bacterial communities respectively, most of which included Leptotrichiaceae, Lachnospiraceae,

222 Burkholderiaceae, Peptostreptococcaceae, Porphyromonadaceae, Mogibacteriaceae, *Prevotella*, *Veillonella, Rothia, Leptotrichia* and *Streptococcus.* These communities have

224 been more prevalently found in healthy airways either in our findings or in previous studies (9, 23, 26, 27), suggesting that Moraxellaceae and *Moraxella* drive airway

226 dysbiosis by inhibiting the commensal microbiome.

      In the present study of airway microbial composition, Moraxellaceae had a greater

228 influence on the α-diversity of fungi than bacteria. The dynamic interaction between bacteria and fungi may have dramatic effects on airway inflammatory processes (18, 28).

230 The correlation network showed positive correlations connecting Moraxellaceae and *Moraxella* with Schizophyllaceae, Polyporaceae, *Candida* and *Meyerozyma*.

232   Additionally, *Schizophyllum*, which represents the vast majority of the family

Schizophyllaceae, is reported as a respiratory pathogen. *Schizophyllum* appears to

234   enhance both the severity and the exacerbation frequency of asthma (29); sensitization to

Schizophyllum is an important risk factor for exacerbation frequency and a rapid decline

236   in lung function in asthma (30). A previous study suggested that *Candida* may be

pathogenically important in patients with asthma (31) and associated with severe

238   exacerbations of asthma requiring hospital admission (32). We found that these

pathogenic fungi were positively associated with Moraxellaceae and *Moraxella* but

240   negatively associated with healthy enriched communities such as Leptotrichiaceae and

Leptotrichia, which were more common in the asthma airway. These results suggest that

242   Moraxellaceae and *Moraxella* have more significant effects on airway fungal

communities than on bacterial communities. Moraxellaceae and *Moraxella* may influence

244   the airway microbiome via interactions with the airway mycobiome, thus acting

synergistically with pathogenic fungi to potentially drive the disease.

246   However, no significant differences were found between stable asthma and

exacerbated asthma, suggesting that our outpatients with exacerbated asthma had disease

248   that was extremely mild in severity without dyspnoea and easily controlled during

exacerbation (33). Consequently, the microbial composition was similar between patients

250   with exacerbated asthma and those with stable asthma.

Our study is strengthened by its relatively large sample size in China (9, 34) and by

252   the combined exploration of airway bacterial and fungal communities. A limitation is the

cross-sectional design, which cannot capture the dynamic changes in the airway

254   microbiome during the progression of asthma.

In conclusion, we have revealed for the first time an increase in fungal α-diversity

256   and more severe dysbiosis of fungi than of bacteria in the asthma airway. Importantly, our

study explored the composition of Moraxellaceae and *Moraxella*, as well as the

258   microbial-microbial interactions in the airway of asthma, and our results suggest that the

interaction among Moraxellaceae, *Moraxella* and the airway mycobiome is important for

260   further understanding airway dysbiosis in asthma. Our findings may provide a foundation

for new insights on the pathogenic mechanisms of asthma. Further studies are needed to

262   confirm the cause-effect relationship underlying the interaction of Moraxellaceae,

Moraxella and the airway mycobiome in asthma.

264

MATERIALS AND METHODS

266   **Study design and subjects.** This study was approved by the ethics committee of

Southern Medical University (Permit No. 2012-072). All subjects have provided written

268   informed consent, in accordance with the Declaration of Helsinki. A total of 145 sputum

samples were collected from 116 asthma patients and 29 healthy subjects enrolled at

270   Nanfang Hospital, Southern Medical University (Guangzhou, China), between June 2015

and December 2016. Subjects provided clinical information including age, gender, forced

272   expiratory volume in 1 second (FEV1), forced vital capacity (FVC), smoking history and

use of inhaled corticosteroids (ICS).

274   Exacerbation of asthma is characterized by a progressive increase in symptoms of

dyspnoea, cough, wheezing or chest tightness and a progressive decline of lung function,

276   which represent changes in the patient's usual state.

The inclusion criteria for asthma patients included age >15 years; initial diagnosis

278   based on the Global Initiative for Asthma (GINA) guidelines (35); and a positive

bronchodilator reversibility test result (FEV1 increased by >12% and 200 mL after

280   inhaling 400 mg of salbutamol) or positive methacholine provocation test result. All

subjects were free of clinical bacterial infection at the time of the study. Exclusion criteria

282   included respiratory tract infection diagnosed by chest X-ray (every patient received a

chest X-ray) within the past 4 weeks; the presence of any airway disease other than

284   asthma; and peripheral white blood cell (WBC) counts outside the normal range.

Sample collection, processing, DNA extraction and 16S rRNA/ITS1 gene

286   **amplification.** For sputum induction and processing, the recommendations of the Task

Force on Induced Sputum of the European Respiratory Society were used as the

288   guidelines (36, 37). All samples were immediately stored at -80°C for subsequent DNA

extraction after collection. The sputum samples were thawed under ventilation for 15

290   minutes, and genomic DNA extraction was performed using a total genomic DNA

Nucleic Acid Extraction Kit (Bioeasy Technology, Inc., China) according to the

292   manufacturer's instructions.

The bacterial 16S rRNA genes and fungal ITS1 genes were amplified using the V4

294   region and ITS1 region of the genes, respectively. The amplicons were sequenced on an

14

iTorrent sequencing platform. Detailed information on the DNA amplification and

296   purification steps was described in our previous studies (38, 39).

         Statistical analysis. The raw sequence files for both the 16S rRNA V4 amplicons

298   and fungal 18S–28S rRNA gene internally transcribed spacer regions ITS1 amplicons

         were processed by a barcoded Illumina paired-end sequencing (BIPES) pipeline (40).

300   First, the barcode primers were trimmed and filtered if they contained ambiguous reads or

         mismatches in the primer regions according to the BIPES protocol. Then, we removed

302   sequences that had more than one mismatch in the 40–70 bp regions. Next, we screened

         and removed chimaeras using UCHIME in de novo mode (41), and finally we generated

304   the high-quality sequence reads of the 16S rRNA genes or ITS1 DNA genes.

         We used the software pipeline "Quantitative Insights into Microbial Ecology"

306   (QIIME) (1.9.1) to conduct subsequent analyses (42). Operational taxonomic units

         (OTUs) were clustered by using USEARCH with the default parameters and a threshold

308   distance of .03. The Greengenes 13_8 database was used as a template file, and multiple

         alignments of QIIME-based sequences were performed using Python Nearest Alignment

310   Space Termination (PyNAST) (43); representative sequences for the ITS1 gene were

         assigned with reference to the QIIME_ITS database (version information:

312   sh_qiime_release_s_28.06.2017) (42); unclassified sequences were further defined by

         BLAST according to the closest shared taxonomic level. The representative 16S rRNA

314   gene sequences were classified into specific taxa using the Ribosome Database Project

         (RDP) classifier (44). The sequences were deposited in the European Nucleotide Archive

316   (ENA) under accession number PRJEB28853.

Alpha diversity metrics were analysed by the Shannon index, which indicates the

318   evenness and richness of the airway microbial community, and PD_whole_tree, which

reflects the phylogenesis of species. Beta diversity distances were calculated by principal

320   coordinate analysis (PCoA) using unweighted_uniFrac distances, and statistical values

were evaluated with the Adonis method. Differential features between groups were

322   identified using a linear discriminant analysis (LDA) effect size (LEfSe) method with a

threshold cut-off value for the logarithmic LDA score of 2.0. Because of the limitation of

324   statistical power to detect uncommon OTUs, we selected the OTUs with an average

relative abundance of >1% in any group. Statistical analyses of clinical parameters were

326   performed using IBM SPSS version 20.0. The graphical representations were generated

using GraphPad Prism 5, and the interaction network was constructed using Cytoscape

328   (v3.6.0) (45).

336   The authors' responsibilities were as follows. H.L., C.L., H.Z., and J.S. designed the

16

experiments. H.L., C.L., Z.L., S.Z., M.Y., M.Z., W.Y., and Y.L. collected samples and

338   performed the experiments. H.L., C.L., and Z.L. analyzed the data. H.L., C.L., H.Z., and

J.S. prepared the manuscript and had primary responsibility for final content. All authors

340   read and approved the final manuscript. None of the authors reported a conflict of

interest.

342   We thank the patients, clinical doctors and microbiology staff who supported this

work. We thank American Journal Experts (AJE) for English language editing.

344

REFERENCES

346   1.   Bousquet J, Mantzouranis E, Cruz AA, Ait-Khaled N, Baena-Cagnani CE, Bleecker ER, Brightling CE, Burney P, Bush A, Busse WW, Casale TB, Chan-Yeung M, Chen R, Chowdhury B, Chung KF, Dahl R,
348   Drazen JM, Fabbri LM, Holgate ST, Kauffmann F, Haahtela T, Khaltaev N, Kiley JP, Masjedi MR, Mohammad Y, O'Byrne P, Partridge MR, Rabe KF, Togias A, van Weel C, Wenzel S, Zhong N,
350   Zuberbier T. 2010. Uniform definition of asthma severity, control, and exacerbations: document presented for the World Health Organization Consultation on Severe Asthma. J Allergy Clin
352   Immunol 126:926-938.

2.   Hilty M, Burke C, Pedro H, Cardenas P, Bush A, Bossley C, Davies J, Ervine A, Poulter L, Pachter L,
354   Moffatt MF, Cookson WO. 2010. Disordered microbial communities in asthmatic airways. PLoS One 5:e8578.

356   3.   Erb-Downward JR, Thompson DL, Han MK, Freeman CM, McCloskey L, Schmidt LA, Young VB, Toews GB, Curtis JL, Sundaram B, Martinez FJ, Huffnagle GB. 2011. Analysis of the lung
358   microbiome in the "healthy" smoker and in COPD. PLoS One 6:e16384.

4.   Charlson ES, Bittinger K, Haas AR, Fitzgerald AS, Frank I, Yadav A, Bushman FD, Collman RG. 2011.
360   Topographical continuity of bacterial populations in the healthy human respiratory tract. Am J Respir Crit Care Med 184:957-963.

362   5.   Bisgaard H, Hermansen MN, Buchvald F, Loland L, Halkjaer LB, Bonnelykke K, Brasholt M, Heltberg A, Vissing NH, Thorsen SV, Stage M, Pipper CB. 2007. Childhood asthma after bacterial
364   colonization of the airway in neonates. N Engl J Med 357:1487-1495.

6.   Beigelman A, Weinstock GM, Bacharier LB. 2014. The relationships between environmental
366   bacterial exposure, airway bacterial colonization, and asthma. Curr Opin Allergy Clin Immunol 14:137-142.

368   7.   Huang YJ, Boushey HA. 2015. The microbiome in asthma. J Allergy Clin Immunol 135:25-30.

8.   Taylor SL, Leong LEX, Choo JM, Wesselingh S, Yang IA, Upham JW, Reynolds PN, Hodge S, James AL,
370   Jenkins C, Peters MJ, Baraket M, Marks GB, Gibson PG, Simpson JL, Rogers GB. 2018. Inflammatory phenotypes in patients with severe asthma are associated with distinct airway
372   microbiology. J Allergy Clin Immunol 141:94-103 e115.

9.   Li N, Qiu R, Yang Z, Li J, Chung KF, Zhong N, Zhang Q. 2017. Sputum microbiota in severe asthma
374   patients: Relationship to eosinophilic inflammation. Respir Med 131:192-198.

10.   Green BJ, Wiriyachaiporn S, Grainge C, Rogers GB, Kehagia V, Lau L, Carroll MP, Bruce KD, Howarth
376   PH. 2014. Potentially pathogenic airway bacteria and neutrophilic inflammation in treatment resistant severe asthma. PLoS One 9:e100645.

378   11.   Verduin CM, Hol C, Fleer A, van Dijk H, van Belkum A. 2002. Moraxella catarrhalis: from Emerging to Established Pathogen. Clin Microbiol Rev 15:125-144.

380   12.   Bisgaard H, Hermansen MN, Bonnelykke K, Stokholm J, Baty F, Skytt NL, Aniscenko J, Kebadze T, Johnston SL. 2010. Association of bacteria and viruses with wheezy episodes in young children:
382   prospective birth cohort study. BMJ 341:c4978.

13.   Cazzola M, Matera MG, Rossi F. 1991. Bronchial hyperresponsiveness and bacterial respiratory
384   infections. Clin Ther 13:157-171.

14. Aliouat-Denis CM, Chabe M, Delhaes L, Dei-Cas E. 2014. Aerially transmitted human fungal pathogens: what can we learn from metagenomics and comparative genomics? Rev Iberoam Micol 31:54-61.

15. HC. vW, Gregory. C, Brown. R, Marchesi. JR, Hoogendoorn. B, Matthews. IP. 2013. Differences in fungi present in induced sputum samples from asthma patients and non-atopic controls. BioMed Central 13:69-74.

16. Kyda J, Krishnamurthya A, McGratha J, Brownea J, Vahedia F. 2011. Airway Bacterial Interactions and Impact on Host Immune Responses. Adv Otorhinolaryngol 72:116–120.

17. Twomey KB, O'Connell OJ, McCarthy Y, Dow JM, O'Toole GA, Plant BJ, Ryan RP. 2012. Bacterial cis-2-unsaturated fatty acids found in the cystic fibrosis airway modulate virulence and persistence of Pseudomonas aeruginosa. ISME J 6:939-950.

18. Nguyen LD, Viscogliosi E, Delhaes L. 2015. The lung mycobiome: an emerging field of the human respiratory microbiome. Front Microbiol 6:89-97.

19. Short FL, Murdoch SL, Ryan RP. 2014. Polybacterial human disease: the ills of social networking. Trends Microbiol 22:508-516.

20. Lu J, Xiong L, Zhang X, Liu Z, Wang S, Zhang C, Zheng J, Wang G, Zheng R, Simpson JL, Wang F. 2017. The Role of Lower Airway Dysbiosis in Asthma: Dysbiosis and Asthma. Mediators Inflamm 2017:3890601.

21. Singanayagam A, Ritchie AI, Johnston SL. 2017. Role of microbiome in the pathophysiology and disease course of asthma. Curr Opin Pulm Med 23:41-47.

22. Huang YJ, Nelson CE, Brodie EL, Desantis TZ, Baek MS, Liu J, Woyke T, Allgaier M, Bristow J, Wiener-Kronish JP, Sutherland ER, King TS, Icitovic N, Martin RJ, Calhoun WJ, Castro M, Denlinger LC, Dimango E, Kraft M, Peters SP, Wasserman SI, Wechsler ME, Boushey HA, Lynch SV, National Heart L, Blood Institute's Asthma Clinical Research N. 2011. Airway microbiota and bronchial hyperresponsiveness in patients with suboptimally controlled asthma. J Allergy Clin Immunol 127:372-381 e371-373.

23. Marri PR, Stern DA, Wright AL, Billheimer D, Martinez FD. 2013. Asthma-associated differences in microbial composition of induced sputum. J Allergy Clin Immunol 131:346-352 e341-343.

24. Goleva E, Jackson LP, Harris JK, Robertson CE, Sutherland ER, Hall CF, Good JT, Jr., Gelfand EW, Martin RJ, Leung DY. 2013. The effects of airway microbiome on corticosteroid responsiveness in asthma. Am J Respir Crit Care Med 188:1193-1201.

25. Huang YJ, Nariya S, Harris JM, Lynch SV, Choy DF, Arron JR, Boushey H. 2015. The airway microbiome in patients with severe asthma: Associations with disease features and severity. J Allergy Clin Immunol 136:874-884.

26. Durack J, Lynch SV, Nariya S, Bhakta NR, Beigelman A, Castro M, Dyer AM, Israel E, Kraft M, Martin RJ, Mauger DT, Rosenberg SR, Sharp-King T, White SR, Woodruff PG, Avila PC, Denlinger LC, Holguin F, Lazarus SC, Lugogo N, Moore WC, Peters SP, Que L, Smith LJ, Sorkness CA, Wechsler ME, Wenzel SE, Boushey HA, Huang YJ, National Heart L, Blood Institute's A. 2017. Features of the bronchial bacterial microbiome associated with atopy, asthma, and responsiveness to inhaled corticosteroid treatment. J Allergy Clin Immunol 140:63-75.

19

27.   Denner DR, Sangwan N, Becker JB, Hogarth DK, Oldham J, Castillo J, Sperling AI, Solway J, Naureckas ET, Gilbert JA, White SR. 2016. Corticosteroid therapy and airflow obstruction influence the bronchial microbiome, which is distinct from that of bronchoalveolar lavage in asthmatic airways. J Allergy Clin Immunol 137:1398-1405 e1393.

28.   Wargo MJ, Hogan DA. 2006. Fungal--bacterial interactions: a mixed bag of mingling microbes. Curr Opin Microbiol 9:359-364.

29.   Ogawa H, Fujimura M, Takeuchi Y, Makimura K. 2011. The influence of Schizophyllum commune on asthma severity. Lung 189:485-492.

30.   Ogawa H, Fujimura M, Takeuchi Y, Makimura K. 2013. Impact of Schizophyllum sensitization on decline of lung function in asthma. J Asthma 50:764-768.

31.   Khosravi AR, Bandghorai AN, Moazzeni M, Shokri H, Mansouri P, Mahmoudi M. 2009. Evaluation of Candida albicans allergens reactive with specific IgE in asthma and atopic eczema patients. Mycoses 52:326-333.

32.   O'Driscoll BR, Hopkinson LC, Denning DW. 2005. Mold sensitization is common amongst patients with severe asthma requiring multiple hospital admissions. BMC Pulm Med 5:4-13.

33.   Demoly. P, Crestani. B, Leroyer. C, Magnan. A, Mounedji. N, Humbert M. 2004. Control and exacerbation of asthma:a survey of more than 3000 French physicians. Allergy 59:920–926.

34.   Zhang Q, Cox M, Liang Z, Brinkmann F, Cardenas PA, Duff R, Bhavsar P, Cookson W, Moffatt M, Chung KF. 2016. Airway Microbiota in Severe Asthma and Relationship to Asthma Severity and Phenotypes. PLoS One 11:e0152724.

35.   Accordini S, Cappa V, Braggion M, Corsico AG, Bugiani M, Pirina P, Verlato G, Villani S, de Marco R. 2011. The impact of diagnosed and undiagnosed current asthma in the general adult population. Int Arch Allergy Immunol 155:403-411.

36.   Chanez P, Holz O, Ind PW, Djukanovic R, Maestrelli P, Sterk PJ. 2002. Sputum induction. Eur Respir J 20:3S-8s.

37.   Djukanovic R, Sterk PJ, Fahy JV, Hargreave FE. 2002. Standardised methodology of sputum induction and processing. Eur Respir J 20:1S-2s.

38.   He Y, Zhou BJ, Deng GH, Jiang XT, Zhang H, Zhou HW. 2013. Comparison of microbial diversity determined with the same variable tag sequence extracted from two different PCR amplicons. BMC Microbiol 13:208-215.

39.   Su J, Liu HY, Tan XL, Ji Y, Jiang YX, Prabhakar M, Rong ZH, Zhou HW, Zhang GX. 2015. Sputum Bacterial and Fungal Dynamics during Exacerbations of Severe COPD. PLoS One 10:e0130736.

40.   Zhou HW, Li DF, Tam NF, Jiang XT, Zhang H, Sheng HF, Qin J, Liu X, Zou F. 2011. BIPES, a cost-effective high-throughput method for assessing microbial diversity. ISME J 5:741-749.

41.   Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27:2194-2200.

42.   Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335-336.

466   43.   Al-Hebshi NN, Nasher AT, Idris AM, Chen T. 2015. Robust species taxonomy assignment algorithm for 16S rRNA NGS reads: application to oral carcinoma samples. J Oral Microbiol 7:28934.

468   44.   Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73:5261-5267.

470   45.   Paul Shannon, Andrew Markiel, Owen Ozier, Nitin S. Baliga, Jonathan T. Wang, Daniel Ramage, Nada Amin, Benno Schwikowski, Ideker T. 2003. Cytoscape: A Software Environment for

472   Integrated Models of Biomolecular Interaction Networks. Genome Res 13:2498-2504.

474

Figure Legends

476
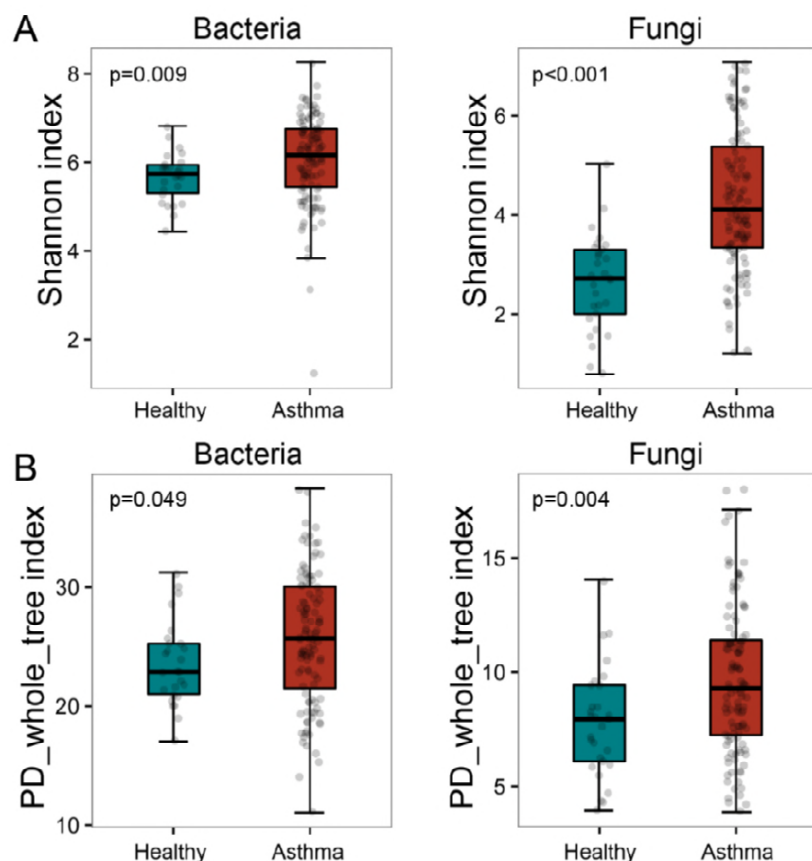
FIG 1 α-Diversity of the bacterial and fungal communities in healthy individuals and

478   asthma patients. The Shannon index (A) indicates the evenness and richness diversity of

the airway microbial community, and PD_whole_tree index (B) reflects the phylogenesis

480   diversity of the airway microbial community. P values are presented using the Wilcoxon

rank-sum test.

482
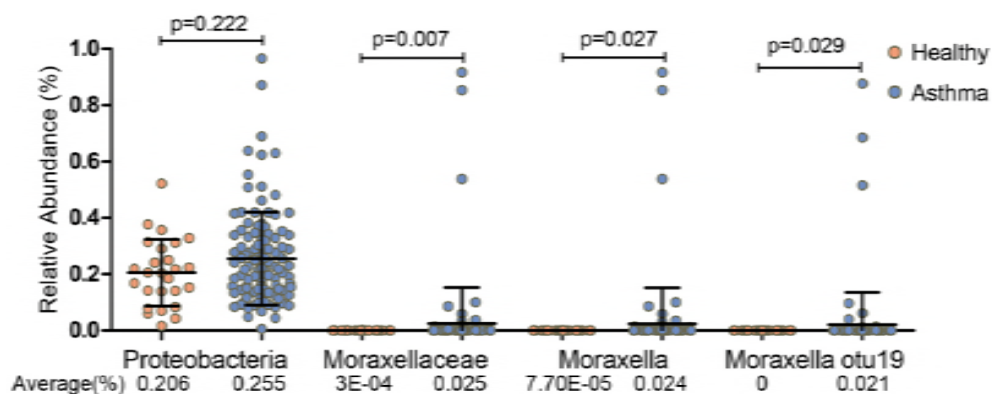
484 FIG 2 Comparison of the relative abundance of Proteobacteria, Moraxellaceae, *Moraxella*

and *Moraxella* otu19 between healthy individuals and asthma patients. P values

486 (Wilcoxon rank-sum test) and average relative abundances are presented.

488
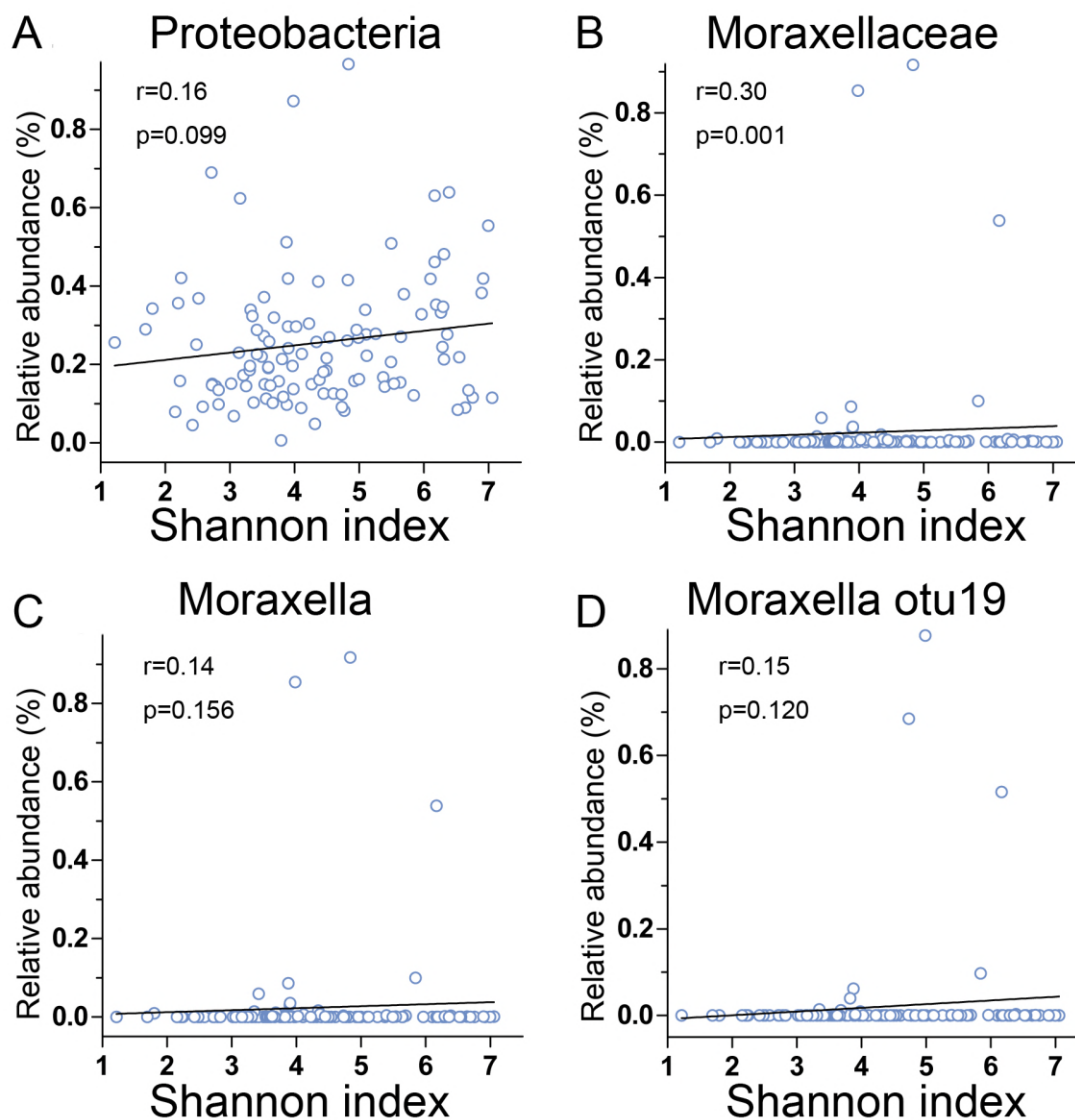
FIG 3 The relationships between Proteobacteria, Moraxellaceae, *Moraxella* and

490   *Moraxella* otu19 and fungal α-diversity. R (cut-off of over 0.3) and P values are presented

using Spearman rank correlation analysis.
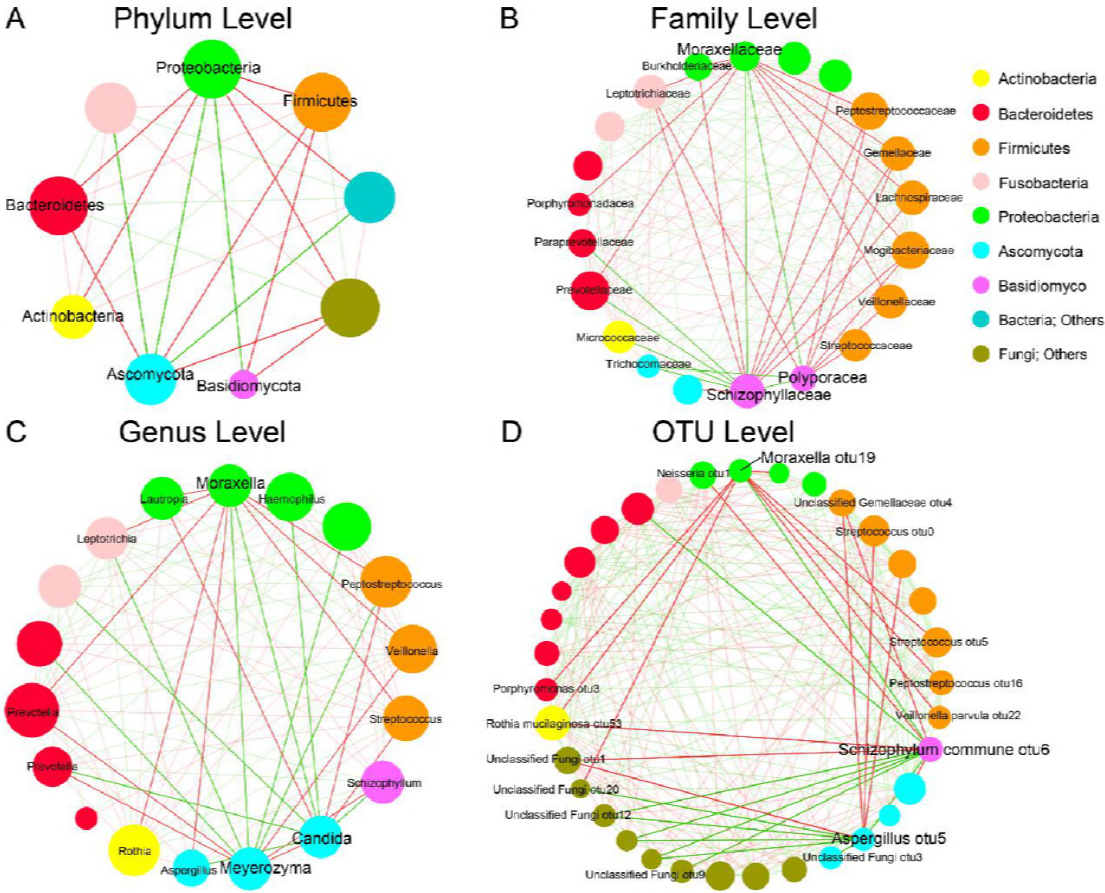
FIG 4 Correlation networks between airway microbial communities (microbial communities with a relative abundance of >1% are included) at the phylum (A), family (B), genus (C), and OTU (D) levels. Each node represents a microbial community, and its diameter is proportional to the degree of connectivity. The microbial communities are coloured by the phylum level. The red edges represent negative correlations, and the green edges represent positive correlations. The darker grey lines connect "hub" microbial communities that may play an important role in the asthmatic airway, and the lighter grey lines connect other airway microbial communities. Correlations were performed with Spearman rank correlation with a correlation cut-off P value of less than 0.05.
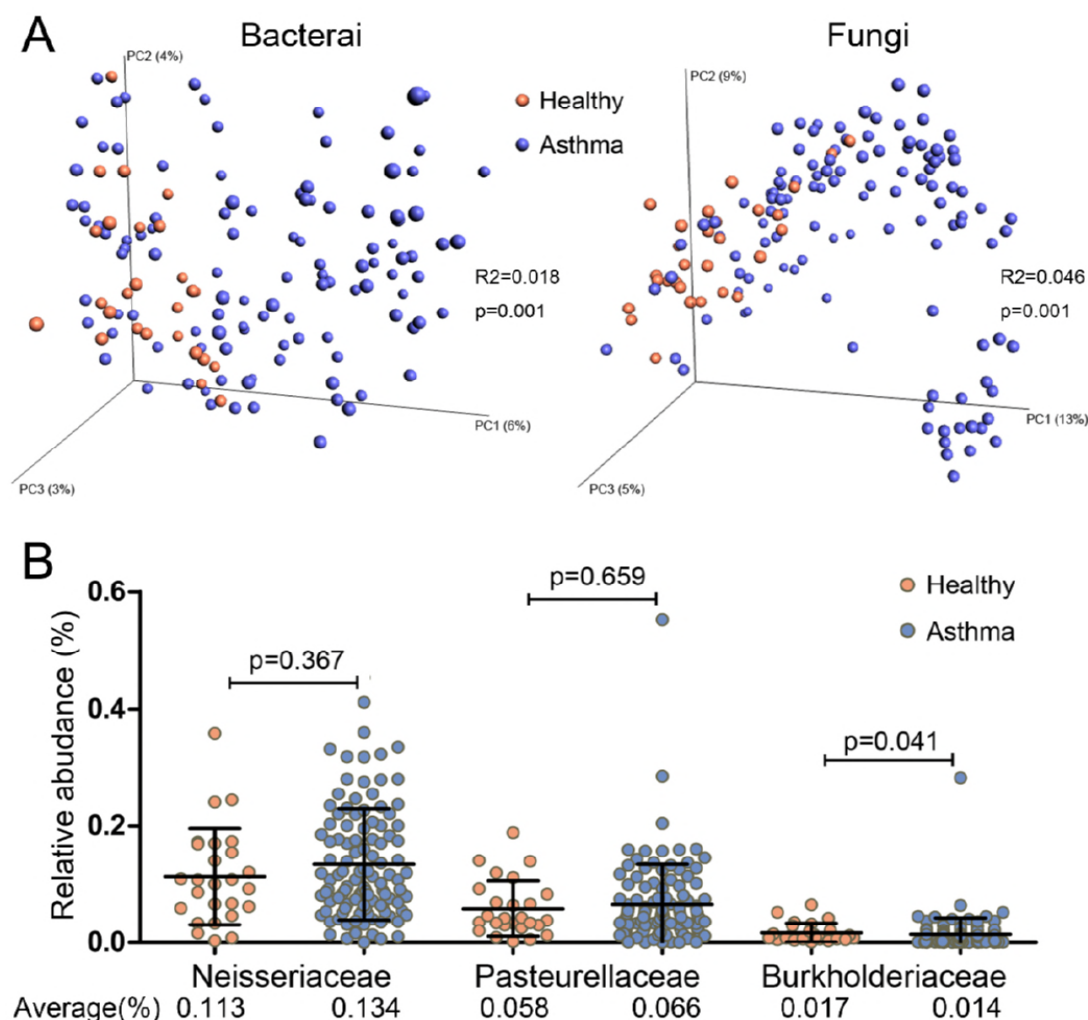
FIG S1 (A) Principal coordinate analysis (β-diversity) based on the unweighted_uniFrac distance of each sample of bacterial and fungal community between healthy individuals and asthma patients. (B) The relative abundances of the other families belonging to Proteobacteria compared between asthma patients and healthy individuals. P values (Wilcoxon rank-sum test) and the average relative abundance are presented.
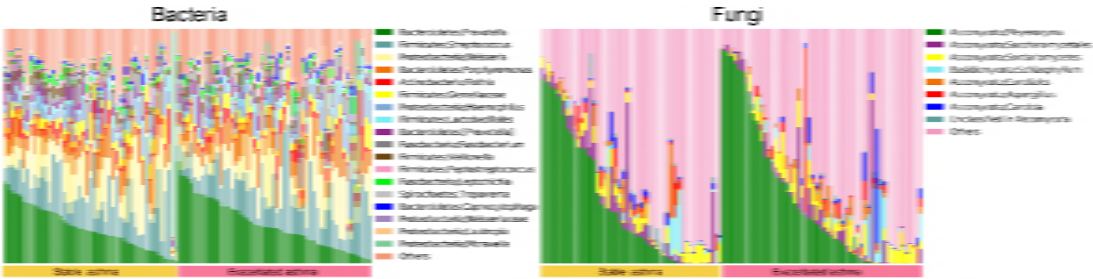
510   FIG S2 Taxonomic profiles of the most abundant airway bacterial and fungal genera in

each sample from patients with stable asthma and exacerbated asthma. Genera with a

512   relative abundance higher than 1% in any group are presented; other genera are classified
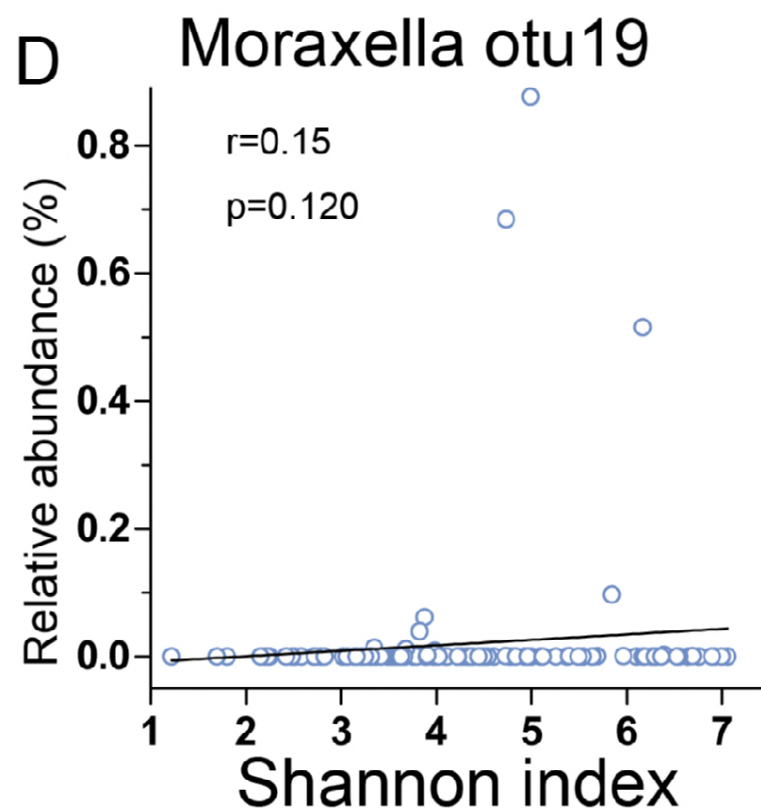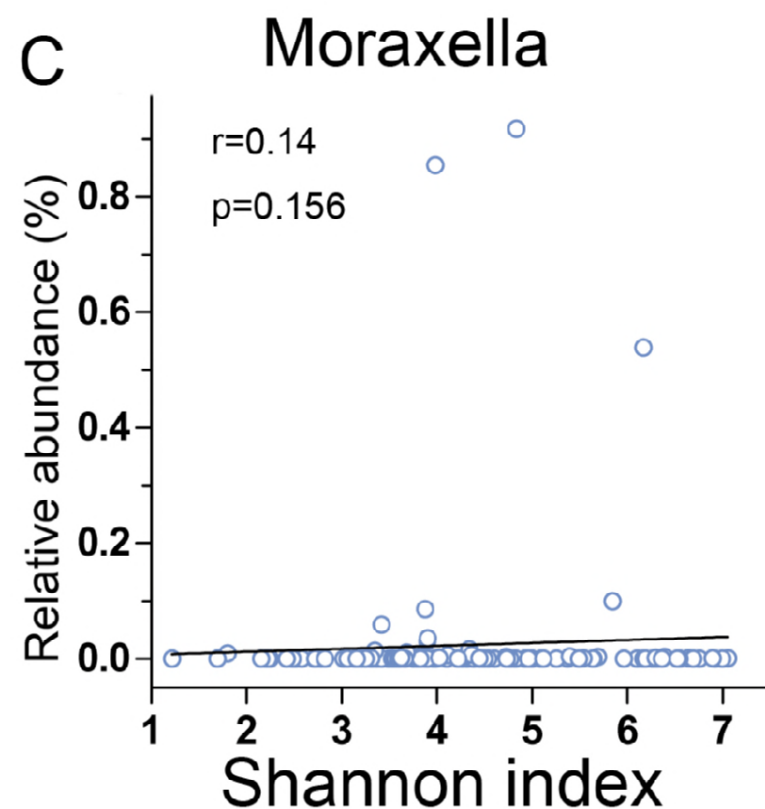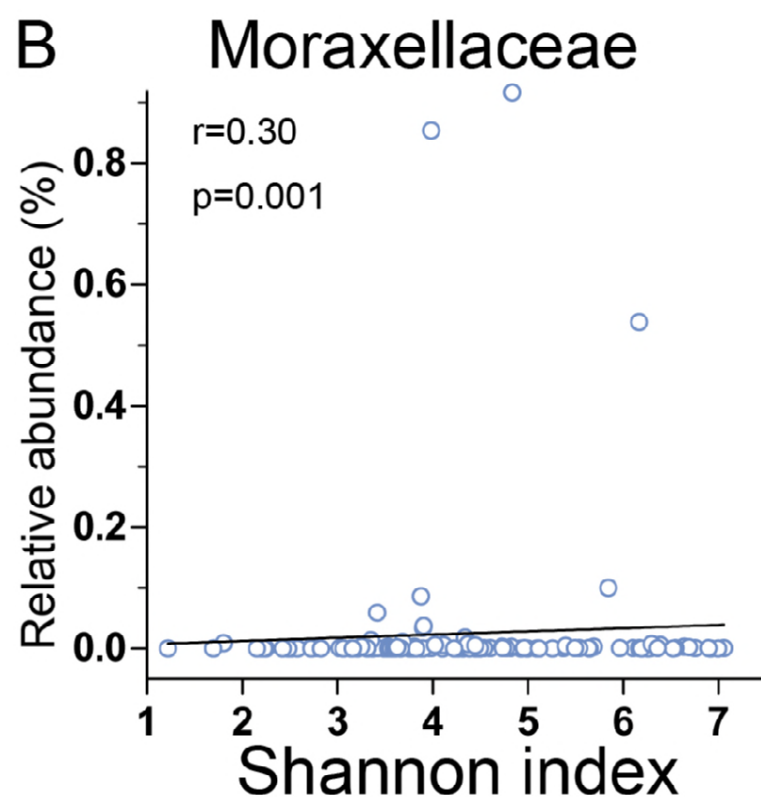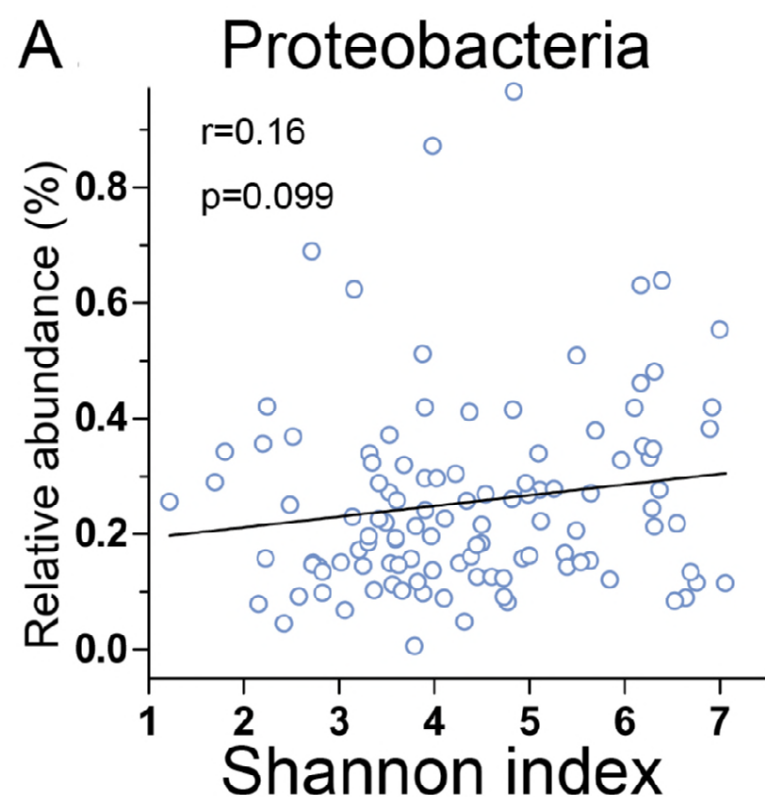
as "Others".

514
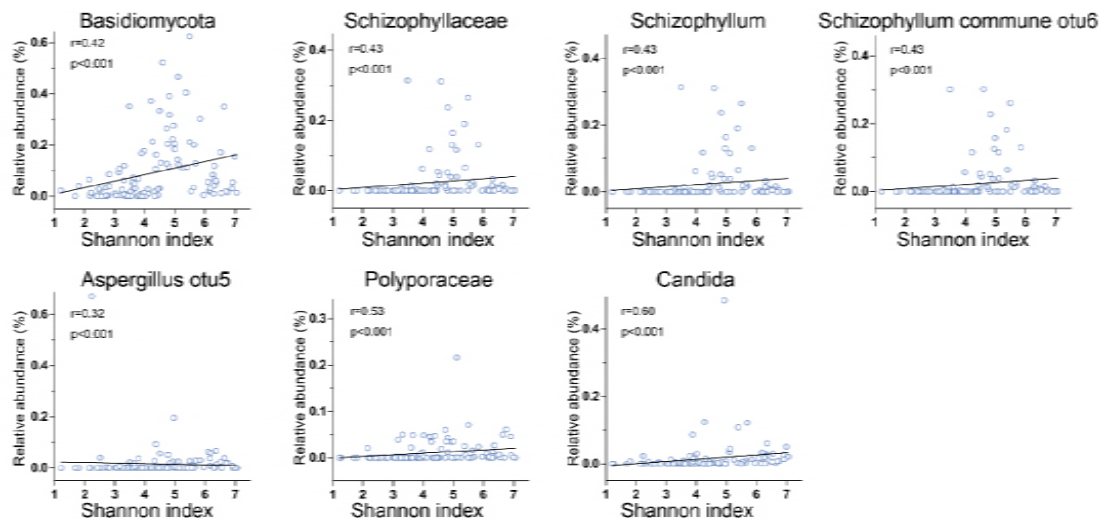
FIG S3 The relationship between fungal communities and fungal α-diversity. R (cut-off of

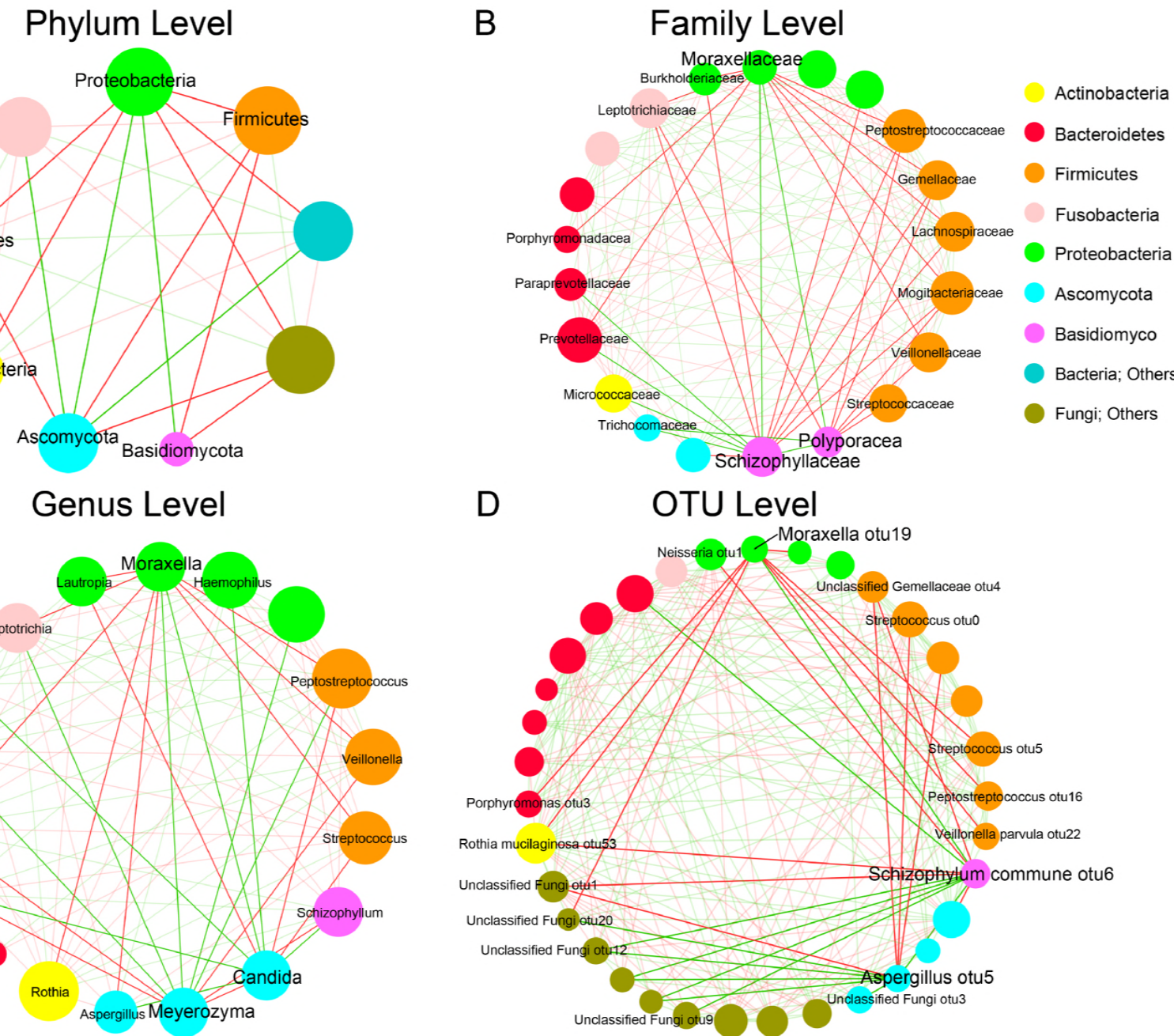516   over 0.3) and P values are presented using Spearman correlation analysis.

	Proteobacteria		Moraxellaceae		Moraxella		Moraxella otu19	
Average(%)	0.206	0.255	3E-04	0.025	7.70E-05	0.024	0	0.021
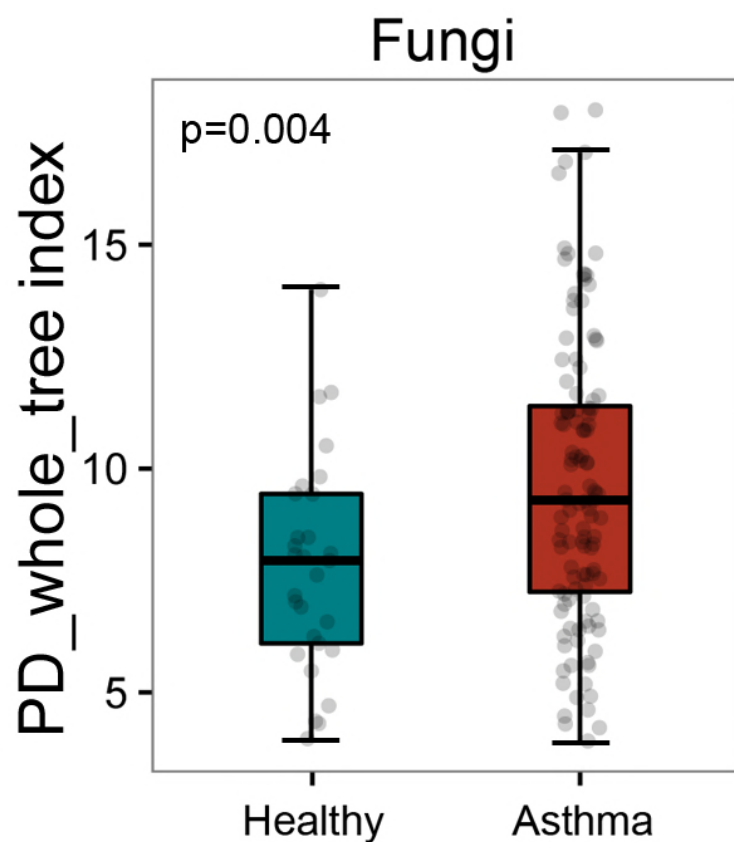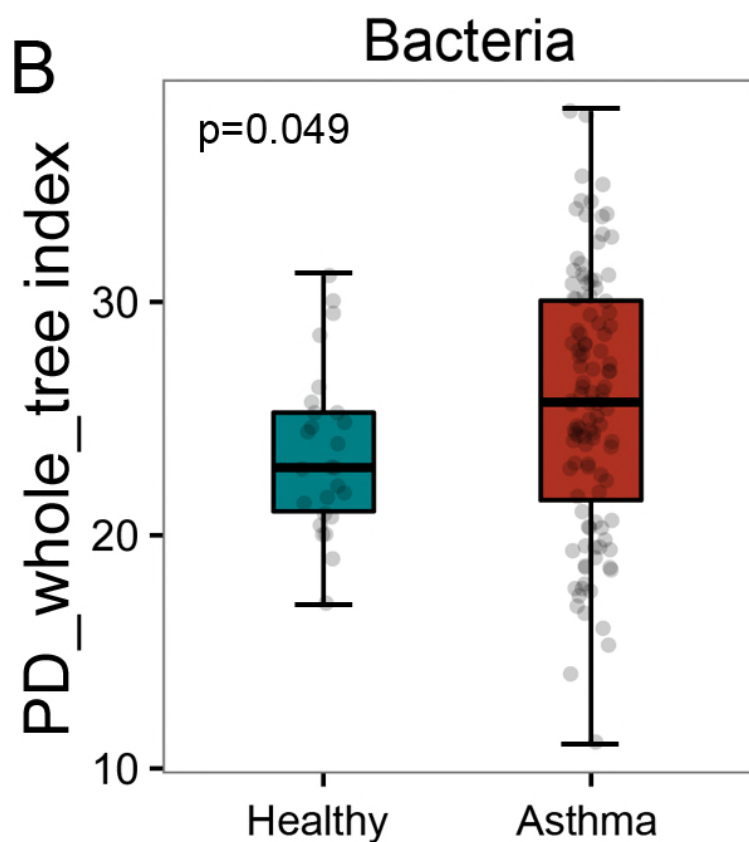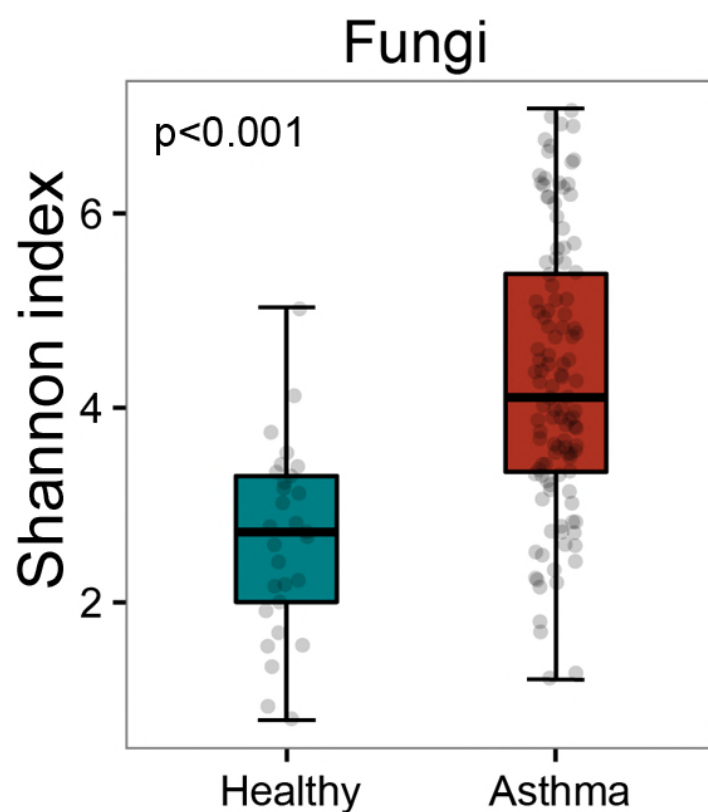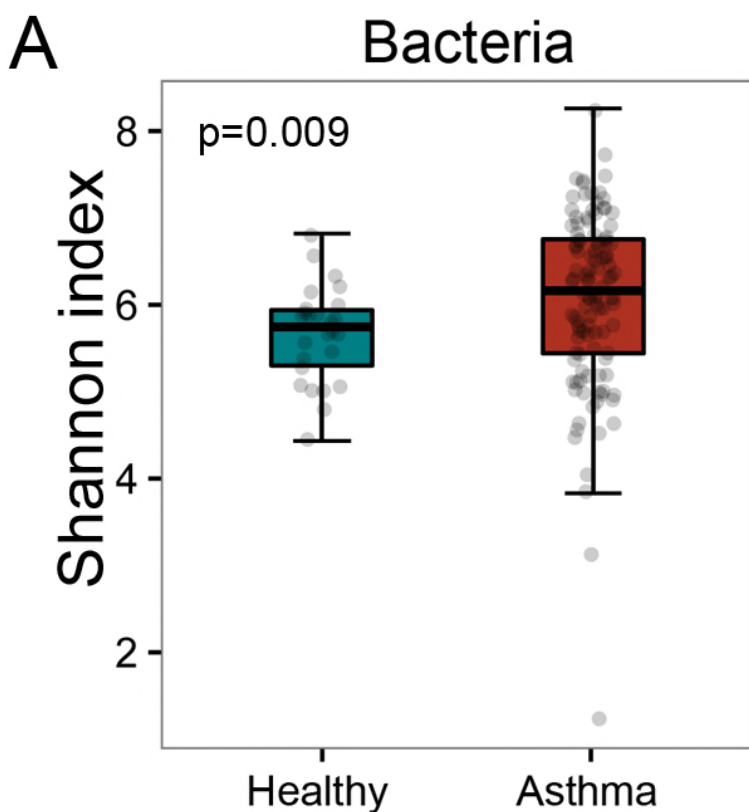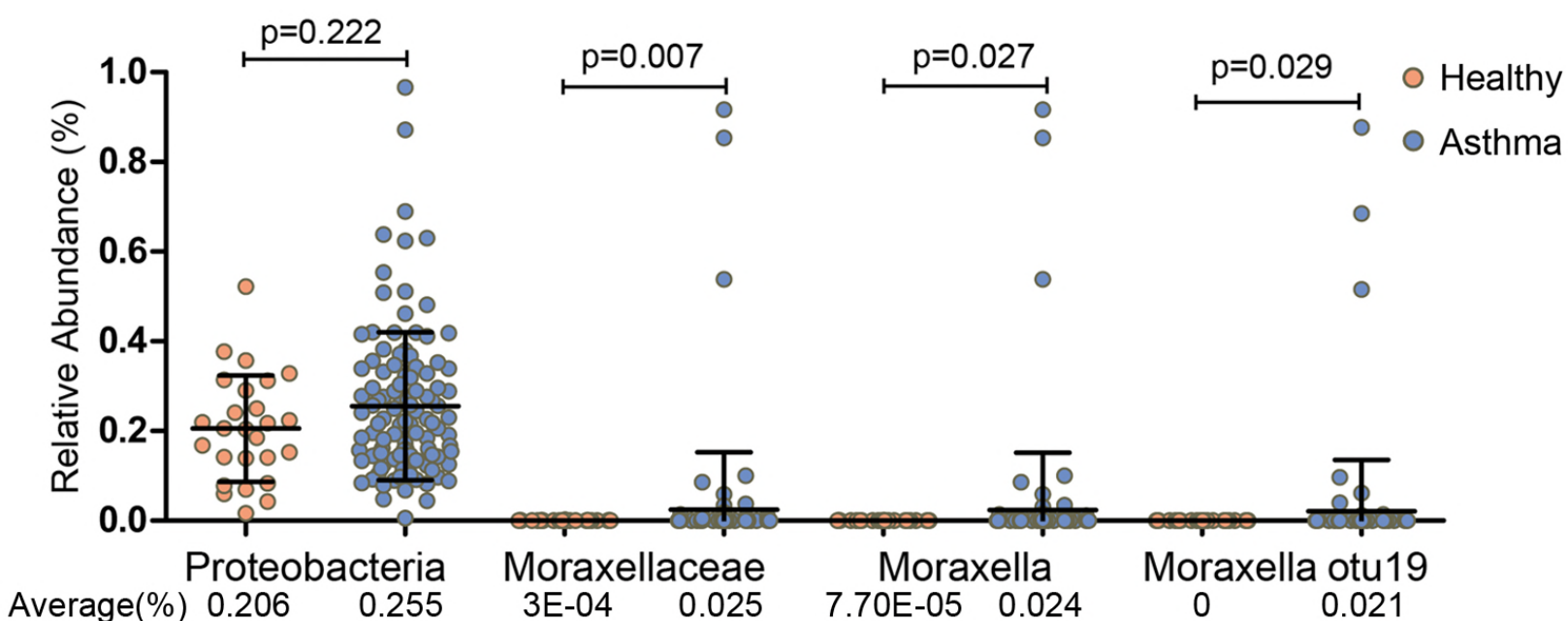
TABLE 1 Clinical characteristics of the subjects

Parameters	Healthy (n=29)	Asthma (n=116)	*P* value	Stable asthma (n=55)	Exacerbated asthma (n=61)	*P* value
Age (years)	44.3±23.1	42.7±14.0	.733	41.4±12.8	44.0±15.1	.480
Gender, n (male:female)	21:8	62:54	.065	28:27	34:27	.603
FEV1 (% pred)	NA	87.9±18.8	NA	89.6±16.9	86.5±2.4	.934
FVC (% pred)	NA	101.9±16.4	NA	102.3±15.6	101.5±17.2	.506
FEV1/FVC (%)	NA	86.7±14.6	NA	87.9±12.6	85.7±16.2	.771
Smoking, n (yes:no)	9:20	24:91	.244	11:44	13:47	.826
ICS, n (yes:no)	NA	78:38	NA	43:12	35:26	.017*

FEV1: forced expiratory volume in 1 second, FVC: forced vital capacity, ICS: inhaled

corticosteroids.

The data are presented as n (chi-square test) or as the mean±standard deviation (independent t-test * represents *P*<.05).