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Characterization of microbiota in bronchiectasis patients with different disease severities

Short title: microbiota in bronchiectasis

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ABSTRACT

The application of 16S rRNA gene pyrosequencing has expanded our knowledge about the respiratory tract microbiome originally obtained using conventional, culture-based methods. In this study, we employed DNA-based molecular techniques for examining the sputum microbiome in bronchiectasis patients in relation to disease severity.

Of 63 study subjects, 42 had mild and 21 had moderate or severe bronchiectasis, which was classified by calculating the FACED score based on FEV_1 (forced expiratory volume in 1 s, %) (F, 0–2 points), age (A, 0–2 points), chronic colonization by *Pseudomonas aeruginosa* (C, 0-1 point), radiographic extension (E, 0–1 point), and dyspnoea (D, 0–1 point). Bronchiectasis was defined as mild at 0–2 points, moderate at 3–4 points, and severe at 5–7 points.

The mean age was 68.0 ± 9.3 years; 33 patients were women. *Haemophilus* (p=0.005) and *Rothia* (p=0.043) were significantly more abundant in the mild bronchiectasis group, whereas *Pseudomonas* (p=0.031) was significantly more abundant in the moderate or severe group. However, the alpha or beta diversity did not significantly differ among sputum microbiota, i.e. the same dominating genera were found in all samples. Further large-scale studies are needed to investigate the sputum microbiome in bronchiectasis.

Key Words: Bronchiectasis, FACED score, microbiome

INTRODUCTION

Bronchiectasis is a chronic, irreversible airway disease with abnormal dilatation of one or more bronchi, causing chronic cough and purulent sputum production. Impaired mucociliary clearance in bronchiectasis patients is associated with continuous or repeated respiratory infection inducing a vicious cycle of blockage, inflammation, exacerbation, and damage in affected bronchi. [1] Bronchiectasis is associated with extended hospitalizations and high mortality, causing a significant economic burden. [2,3]

Prevention of exacerbation, reduction of respiratory symptoms, and stopping the progression of the disease are important to the management of bronchiectasis. [4] By improving bronchial hygiene and decreasing bronchial inflammation, recurrent infection and frequent exacerbation can be prevented. [5] Therefore, the precise identification of colonizing bacterial species including potential pathogens is important for the clinician who treats a bronchiectasis patient.

Conventional, culture-based microbiological analysis identified multiple bacterial pathogens in bronchiectasis patients, such as *Pseudomonas aeruginosa, Haemophilus influenzae, Streptococcus pneumoniae, Staphylococcus aureus,* and *Moraxella catarrhalis*. Importantly, previous studies showed that colonizing *P. aeruginosa* in bronchiectasis is linked to clinical, functional, and radiographic deterioration. Although culture-based diagnostic standard methods are widely used, chronic infections caused by anaerobes or certain bacterial species that barely grow under standard conditions are difficult to diagnose using these methods. [6] The application of new generation sequencing (NGS) using 16S rRNA gene pyrosequencing has expanded our understanding of the pathogenesis of bronchiectasis and is helping physicians to select appropriate antibiotic treatments. [7]

Martínez-García *et al.* used five dichotomised variables to develop a scoring system for non-cystic fibrosis bronchiectasis, the "FACED score", which considers lung function, age,

colonization by *P. aeruginosa*, radiographic extension, and dyspnoea. [8] The authors conducted a multicentre, observational study with 819 bronchiectasis patients who were classified for disease severity in relation to the 5-year all-cause mortality.

In this study, we employed culture-independent, DNA-based molecular techniques for examining the composition of bacterial microbiota in sputum samples in relation to disease severity, which we derived using the FACED scoring system.

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METHODS

Study population

Bronchiectasis was diagnosed by high-resolution computed tomography (HRCT). Patients who have active tuberculosis, or trauma/tuberculosis-related destroyed lungs were excluded from the study. Figure 1 shows the patient flow chart. Initially, from Apr 1, 2017 to Aug 31, 2017, a total of 70 patients with bronchiectasis agreed to participate in this prospective study, but 7 patients were excluded from the study because of incomplete data (n=6), and low amount of extracted DNA (n=1) for analysis. Therefore, a total of 63 patients with bronchiectasis were investigated in this study.

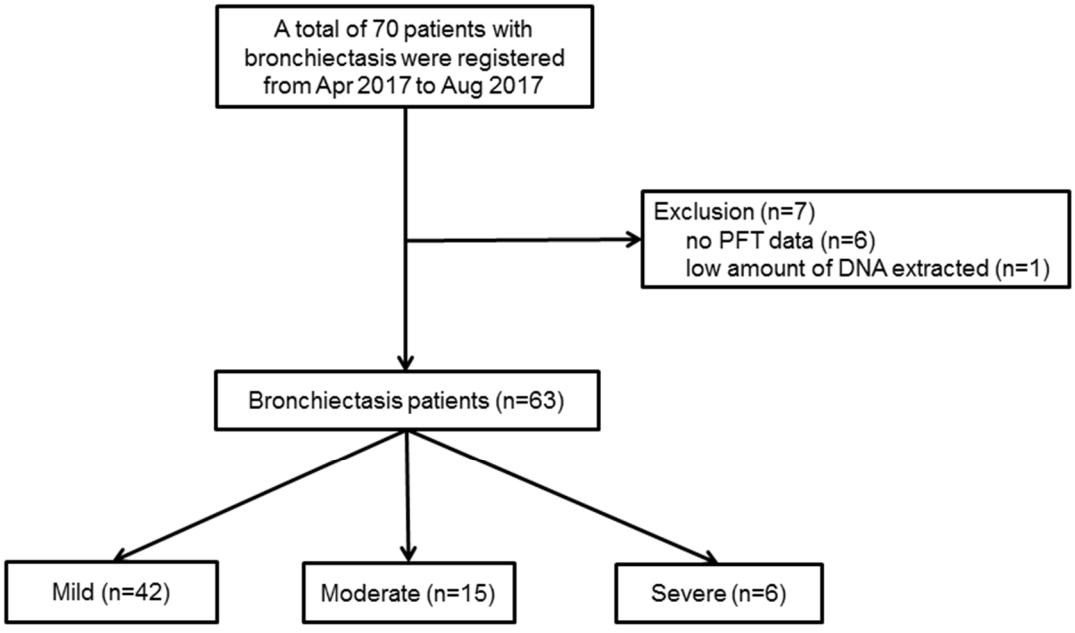

Figure 1. Patient flow chart. From April 1, 2017 to August 31, 2017, a total of 70 patients with bronchiectasis agreed to participate in this prospective study, but 7 patients were excluded from this study because of incomplete data (n=6) and too low amount of extracted DNA (n=1) for analysis.

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Severity of bronchiectasis was classified using the FACED score as follows; forced expiratory volume in 1 second % predicted (FEV_1 in %) (F, cut-off 50%, 0–2 points); age (A, cut-off 70 years, 0–2 points); presence of chronic colonisation by *P. aeruginosa* (C, dichotomic, 0–1 point); radiographic extension (E, number of lobes affected, cut-off two lobes, 0–1 point); and dyspnoea (D, cut-off grade II on the Medical Research Council scale, 0–1 point). Mild bronchiectasis was defined as 0–2 points, moderate was 3–4 points, and severe was 5–7 points. [8] Out of 63 patients, 42 had mild bronchiectasis, and 21 had moderate (n=15) or severe (n=6) bronchiectasis. Demographic data and clinical measurements were collected, including age, sex, body mass index (BMI), smoking status and amount, respiratory symptoms, pulmonary function test (PFT), CT findings, sputum culture study, and comorbidities.

Sputum sample acquisition method

Before sputum acquisition, patients were asked to rinse their mouth with sterile saline and to breathe deeply five times. After 30 minutes, patients produced the sputum (≥1 mL) by repeated deep breaths and coughing into a sterile container. [9] Acquired sputum samples were stored at -70 °C in a freezer, and DNA extraction was performed within 24 hours after sputum acquisition. DNA extraction was performed with a commercial DNA extraction kit (PowerSoil DNA isolation kit, Mo Bio Laboratories, Inc. Carlsbad, USA). Extracted DNA samples were stored at -20 °C in a freezer before analysis by polymerase chain reaction (PCR).

PCR amplification and sequencing

Purified DNA was used as template for PCR amplification with primers targeting the V3

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and V4 regions of the 16S rRNA gene. The primers were 341F (5′-TCGTCGGCAGCGTC-AGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GTCTCGTGGGCTCGG-AGATGTGTATAAGAGACAG-GACTACHVGGGTATCTAATCC-3′). The amplification program was as follows. First, denaturation at 95 °C for 3 minutes, then 25 cycles of denaturation at 95 °C for 30 seconds. Primers were annealed at 55 °C for 30 seconds, and extended at 72 °C for 30 seconds, using a final elongation at 72 °C for 5 minutes. To attach the Illumina NexTera barcode, a secondary amplification was carried out with the i5 forward primer (5′-AATGATACGGCGACCACCGAGATCTACAC-XXXXXXXX-TCGTCGGCAGCGTC-3′; X indicates the barcode region) and the i7 reverse primer (5′-CAAGCAGAAGACGGCATACGAGAT-XXXXXXXX-AGTCTCGTGGGCTCGG-3′). The program for secondary amplification was same as described above except the amplification cycle was set to 8.

Using 2% agarose gel electrophoresis and a Gel Doc system (BioRad, Hercules, CA, USA), the PCR amplification products were confirmed and then purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). Short fragments (non-target products) were removed by Ampure beads kit (Agencourt Bioscience, MA, USA). The products were assessed on a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) for quality and size using a DNA 7500 chip.

Mixed amplicons were pooled and an Illumina MiSeq Sequencing system (Illumina, USA) was used for sequencing at Chunlab, Inc. (Seoul, Korea) according to the manufacturer's instructions.

Miseq pipeline method

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To remove low-quality reads, quality check and filtering of raw reads was performed by Trimmomatic 0.32[1]. After quality control, PANDAseq was used for merging the paired-end sequence data. With the help of ChunLab's program, primers were trimmed (cut off value: 0.8). Using the HMMER's hmmsearch program, non-specific amplicons, which do not encode 16S rRNA, were detected. The process of denoising sequences was performed with DUDE-Seq, and non-redundant reads were extracted by UCLUST-clustering. Taxonomic assignments were obtained using USEARCH (8.1.1861_i86linux32) in EzBioCloud database.

UCHIME[7] and the non-chimeric 16S rRNA database from EzBioCloud were used to find chimeras on reads that have a best hit similarity rate of less than 97%. Sequence data were clustered using CD-HIT[8] and UCLUST[5]. The alpha diversity indices and rarefaction curves were estimated using an in-house code.

Ethics statement

The Institutional Review Board (IRB) of Seoul National University Bundang Hospital reviewed and approved this prospective study protocol (IRB approval number: B-1703/386-301). Informed written consent was obtained from the patient on the sputum collection day. All procedures were performed in accordance with the Declaration of Helsinki.

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RESULTS

The baseline characteristics of the study population are presented in Table 1. The age was higher (74.5 ± 5.9 years vs 64.8 ± 9.0 years) and there were more cases of dyspnoea (33.3% vs 7.1%) among the patients of the moderate/severe bronchiectasis group than among those in the mild bronchiectasis group ($p<0.001$ and $p=0.012$, respectively). Although the percentage of men and smokers was higher in the moderate/severe group, the difference was not significant ($p=0.285$, and $p=0.114$). Sputum was the most common respiratory symptom in the study population. Table 2 lists the comorbidities and results of pulmonary function test. There was no significant difference in comorbidities between the two study groups. Non-tuberculosis mycobacterium (NTM) disease, which was included in the diagnosis in 2007 by the American Thoracic Society (ATS)/Infectious Diseases Society of America (IDSA), was the most common comorbidity in both groups, but there was no significant difference in NTM disease between the two groups ($p=0.721$). The percentage of NTM was 52.4% in the mild bronchiectasis group, and 57.1% in the moderate/severe group. The moderate/severe group showed significantly decreased lung function. FVC (forced vital capacity, %) was 75.3 ± 19.8 in the moderate/severe group and 88.4 ± 16.5 in the mild group ($p=0.007$). FEV_1 (%) was 66.7 ± 24.5 in the moderate/severe group and 88.0 ± 21.1 in the mild group ($p=0.001$). The ratio of FEV1/FVC was also significantly lower in the moderate/severe group ($p=0.001$). The value of DL_{CO} (diffusing capacity for carbon monoxide) was within the normal range in both groups.

The dominating bacteria among the patients of the two study groups are shown in Table 3 and Figure 2. Proteobacteria and Firmicutes are most common phyla. Although the percentage of Proteobacteria was higher in the moderate/sever bronchiectasis group and the

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percentage of Actinobacteria was higher in the mild bronchiectasis group, there was no significant difference in the relative abundance at the phylum level between the two study groups (Figure 2A). At genus level, *Haemophilus* and *Rothia* were significantly more abundant in the mild bronchiectasis group than in the moderate/severe bronchiectasis group (p=0.005, and p=0.043, respectively), whereas *Pseudomonas* was significantly more common in the moderate/severe group (p=0.031) (Figure 2B). *Mycobacterium* was detected in a few patients by 16S rRNA gene sequencing analysis; *Mycobacterium*_uc_s was detected in three patients, and the *Mycobacterium abscessus* and the *Mycobacterium bisbanense* complex was detected in one patient each.

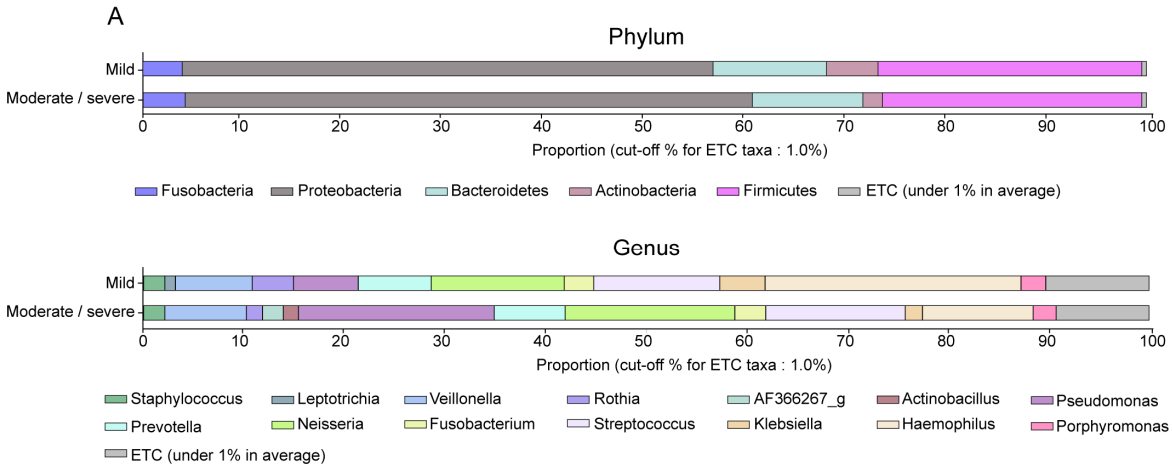

Figure 2. Abundance of dominant bacteria in patients with bronchiectasis according to disease severity: (A) phylum level, and (B) genus level. Haemophilus and Rothia were significantly more abundant in the mild bronchiectasis group than in the moderate/severe bronchiectasis group (p=0.005, and p=0.043, respectively), and Pseudomonas was significantly more common in the moderate/severe group (p=0.031)

The operational taxonomic unit (OUT) value was 189 (132, Q1; 252, Q3) in the mild bronchiectasis group and 157 (112, Q1; 234, Q3) in the moderate/severe group; the difference was not significant (p=0.277) (Figure 3A). Species richness estimates were not significantly different between the two groups, as demonstrated by the abundance-based coverage

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estimator (ACE, in Figure 3B, p=0.274) and Chao 1 (Figure 3C, p=0.307). The Shannon diversity index was also not significantly different (Figure 3D, p=0.550).

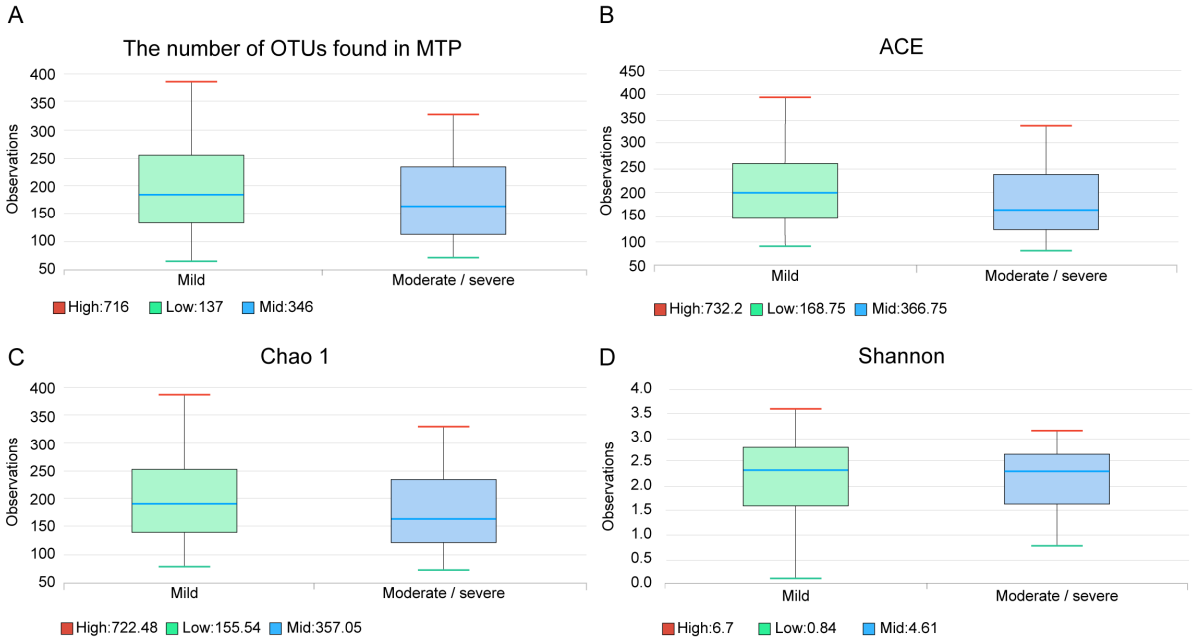

Figure 3. The number of operational taxonomic units (A, p=0.277) and the species richness estimate between two groups using ACE (B, p=0.274), Chao 1 (C, p=0.307), and Shannon diversity (D, p=0.550).

Figure 4 presents a principal coordinates analysis (PCoA) plot, which provides the beta diversity between the two study groups by estimating the distance; however, no significant difference was observed.

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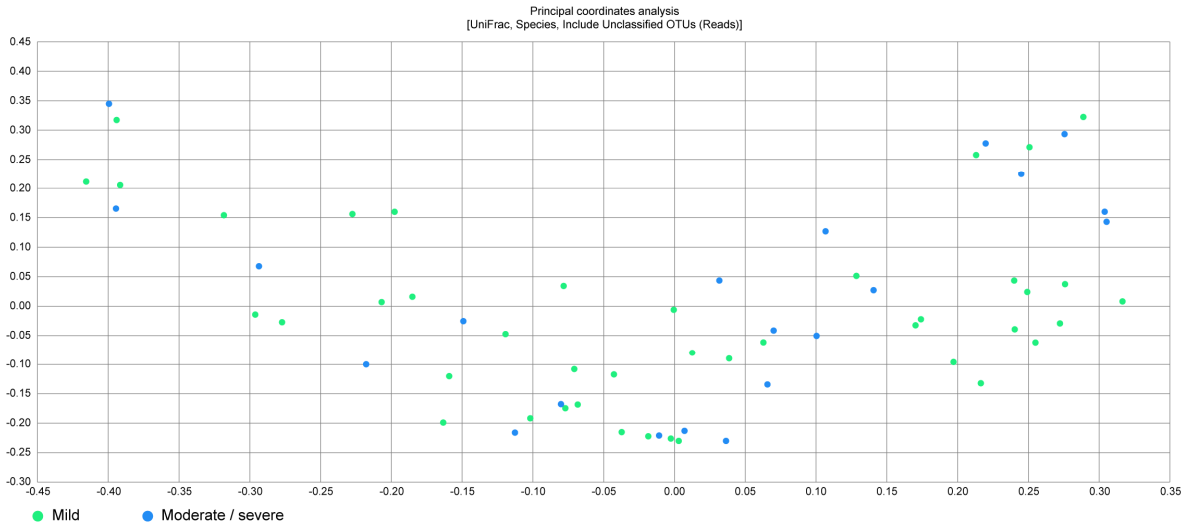

Figure 4. Principal coordinates analysis plot between the mild bronchiectasis group and the moderate/severe bronchiectasis group.

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DISCUSSION

In this study, we examined sputum microbiota using NGS for 16S rRNA gene pyrosequencing to determine their composition in relation to bronchiectasis severity. Overall, culture-independent, DNA-based molecular techniques did not identify significant differences between patients with mild bronchiectasis and moderate or severe bronchiectasis. The OTU values and species richness estimates were not significantly different between the two groups. Only the percentage of the genera *Pseudomonas*, *Haemophilus*, and *Rothia* were significantly different between two groups using the DNA sequencing method. Moreover, a significant difference was found in the detection of NTM using either NGS-based analysis or culture growth-based methods. However, neither *Rothia* nor NTM affected the severity of bronchiectasis.

P. aeruginosa is the most common pathogen in patients with NTM disease. [10] In our study, the relative abundance of *Pseudomonas* was significantly different at the genus level between the mild and the moderate/severe bronchiectasis group. Therefore, we hypothesized that the proportion of NTM would be significantly higher in the moderate/severe bronchiectasis group than in the mild bronchiectasis group, but this was not confirmed by our data. The observation suggests that bronchiectasis severity and progression are affected by the presence of *P. aeruginosa*, but NTM itself may not have an effect on bronchiectasis severity. Faverio *et al.* [11] compared bronchiectasis patients with pulmonary NTM and bronchiectasis patients with chronic *P. aeruginosa* infection in a prospective study. Patients with bronchiectasis and pulmonary NTM tended to have cylindrical bronchiectasis and low disease severity. Interestingly, NTM were rarely found using NGS-based analysis in our study. This may be due to the sensitivity of method for detecting NTM; the NGS-based analysis might not yet be

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optimized for NTM detection, whereas in a study on acid-fast bacilli (AFB), a microbiologist is focused on finding NTM or tuberculosis using optimized growth conditions. The lack of optimization for NTM detection might be responsible for the difference between the conventional culture method and an NGS-based analysis.

Haemophilus was the most common genus in our study, and its relative abundance was significantly higher in the mild bronchiectasis group, whereas the abundance of *Pseudomonas* was significantly higher in the moderate/severe bronchiectasis group. King *et al*. [12] studied the longitudinal change in microbial organisms in 89 patients with bronchiectasis over 5.7 years. In their study, the percentage of *H. influenza* was initially 47%, but the percentage decreased to 40% during the follow-up examination, whereas the percentage of *P. aeruginosa* increased from 12% to 18%. In addition, the authors showed that the clinical severity of bronchiectasis was higher in patients with *P. aeruginosa* than in patients with *H. influenza*. The authors suggested that the disease progresses from no pathogen to *Haemophilus* to *Pseudomonas*.

Rothia was originally proposed and classified as a member of the Micrococcaceae family by Georg & Bronwn in 1967. [13] Lim *et al.* [14] found that *Rothia mucilaginosa* was prevalent in patients with cystic fibrosis that carried *P. aeruginosa*. Interestingly, there is no obvious pattern of synergy or competition between the two organisms. Previous studies showed that *R. mucilaginosa* could be a lower respiratory pathogen in both immunocompetent and immunocompromised patients. [15-17] *Rothia*, mostly *R. mucilaginosa*, was also a predominant organism in bronchiectasis in our study. Although the proportion of *Rothia* was significantly higher in the mild bronchiectasis group, the abundance of *R. mucilanginosa* was not significantly different between two groups (p=0.064), similar to the study published by Lim *et al*.

Recently, Byun *et al*, [5] reported the characterization of the lung microbiome in stable or

exacerbated bronchiectasis using bronchoalveolar fluid samples from 14 patients. The authors found that *H. influenza*, *P. aeruginosa*, *M. catarrhalis*, and *Prevotella* spp. were common organisms. Specifically, they suggested that *Prevotella* and *Veillonella* could be potent anaerobic pathogens. In our study, although *Prevotella* and *Veillonella* were common in both the mild and the moderate/severe bronchiectasis group, the abundance of the two pathogens was not significantly different. It may indicate that *Prevotella* and *Veillonella* are risk factors for exacerbation of bronchiectasis, but they are not significantly associated with bronchiectasis severity. The authors also showed that the species richness estimate, the Simpson index, and the Shannon index were not different at the genus and family level between the clinically stable bronchiectasis group and the exacerbated bronchiectasis group. Similar to our study results, the OTU value, ACE, Chao 1, Shannon index, and PCoA plot were not significantly different between the mild bronchiectasis group and the moderate/severe bronchiectasis group.

There are some limitations in our study. First, although we used a previously validated method to acquire high-quality samples, any of the sample could become contaminated while passing through the oral space. Second, although DNA sequencing 16S rRNA analysis is sensitive and more informative than conventional, culture-based methods, it is limited with regard to the amplification primer. Only well-known binding sites can be used for pyrosequencing platforms. Third, daily diet and antibiotic use of patients was not investigated in this study. If this information would be available, results of this study would be more informative with respect to patient history and the dynamics of the lung microbiome. [18]

In conclusion, although the abundance of *Haemophilus* and *Rothia* differed significantly in relation to the severity of bronchiectasis, the NGS-based technique did not identify significant differences between the alpha diversity and beta diversity of the lung microbiomes of the mild bronchiectasis group and the moderate/severe bronchiectasis group. Respiratory

microbial community in bronchiectasis consisted of several abundant genera that did not significantly differ in relation to disease severity. Further prospective large-scale studies are needed to investigate the microbiome in bronchiectasis.

Acknowledge

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Author Contributions

S.H.L. and J.H.L. drafted the manuscript and revised it critically for important intellectual content. All authors made substantial contributions to the conception and design of the study as well as the acquisition or analysis and interpretation of the data. The authors agreed to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors approved the final version of the manuscript.

Additional Information

Competing Interests: The authors declare no competing interests.

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Table 1. Baseline characteristics of study population according to the severity of bronchiectasis

	Mild (n=42)	Moderate or severe (n=21)	p-value
Age (years)	64.8 ± 9.0	74.5 ± 5.9	<0.001
Sex, male (%)	18 (42.9)	12 (57.1)	0.285
BMI (kg/m^2)	22.0 ± 3.2	22.3 ± 3.5	0.783
Smoking status			0.604
Never smoker	29 (69.0)	13 (61.9)	
Ex-smoker	12 (28.6)	8 (38.1)	
Current smoker	1 (2.4)	-	
Smoking amount (pack-year)	4.3 ± 11.1	10.7 ± 19.4	0.114
Respiratory symptom			
Dyspnea	3 (7.1)	7 (33.3)	0.012
Cough	14 (33.3)	9 (42.9)	0.580
Sputum	25 (59.5)	8 (38.1)	0.108
Hemoptysis	1 (2.4)	2 (10.0)	0.241

Abbreviations: BMI; body mass index,

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Table 2. Comorbidities and clinical data according to the severity of bronchiectasis

	Mild (n=42)	Moderate or severe (n=21)	p-value
Comorbidities			
Diabetes mellitus	1 (2.4)	3 (14.3)	0.104
Hypertension	10 (23.8)	6 (28.6)	0.682
GERD	8 (19.0)	4 (19.0)	1.000
Sinusitis	5 (11.9)	2 (9.5)	1.000
Cardiovascular disease	1 (2.4)	1 (4.8)	1.000
Stroke	3 (7.1)	0 (0.0)	0.545
Liver disease	2 (4.8)	2 (9.5)	0.595
Renal disease	1 (2.4)	1 (4.8)	1.000
Non-tuberculosis mycobacterium	22 (52.4)	12 (57.1)	0.721
Pulmonary function			
FVC (%)	88.4 ± 16.5	75.3 ± 19.8	0.007
FEV_1 (%)	88.0 ± 21.1	66.7 ± 24.5	0.001
FEV_1/FVC ratio	0.71 ± 0.09	0.60 ± 0.16	0.001
DL_{CO} (%)	103.3 ± 22.9	92.8 ± 21.7	0.170

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Table 3. Abundance of specific bacteria in bronchiectasis by severity

Classification	Mild (n=42)			Moderate or severe (n=21)			p-value
	Total reads	%	Occurred	Total reads	%	Occurred	
Phyllum							
Proteobacteria	32780	50.9	42	31405	57.0	21	0.814
Firmicutes	16119	25.1	42	13090	23.8	21	0.431
Bacteroidetes	8300	12.9	42	6555	11.9	21	0.499
Actinobacteria	3893	6.1	42	1311	2.4	21	0.099
Fusobacteria	2726	4.2	42	2346	4.3	21	0.722
Saccharibacteria_TM7	293	0.5	36	137	0.2	18	0.259
Spirochaetes	146	0.2	35	128	0.2	17	0.862
Tenericutes	48	0.1	24	41	0.1	12	0.842
Synergistetes	10	0.0	24	43	0.1	15	0.227
Genus							
Haemophilus	15663	24.3	42	5767	10.5	21	0.005
Neisseria	8828	13.7	42	10405	18.9	21	0.618
Streptococcus	7439	11.6	42	6830	12.4	21	0.817
Pseudomonas	3082	4.8	39	9408	17.1	18	0.031
Veillonella	5496	8.5	42	4150	7.5	21	0.444
Prevotella	5243	8.1	42	3830	7.0	21	0.395
Rothia	3181	4.9	42	1089	2.0	21	0.043
Klebsiella	3279	5.1	28	933	1.7	11	0.386
Fusobacterium	1865	2.9	42	1928	3.5	21	0.950
Porphyromonas	1744	2.7	41	1640	3.0	20	0.926
Actinobacillus	544	0.8	34	1265	2.3	19	0.441
Staphylococcus	766	1.2	26	1077	2.0	15	0.810
Leptotrichia	798	1.2	39	404	0.7	19	0.079
AF366267_g	12	0.0	27	1736	3.2	13	0.325

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FIGURE LEGENDS

Figure 1. Patient flow chart. From April 1, 2017 to August 31, 2017, a total of 70 patients with bronchiectasis agreed to participate in this prospective study, but 7 patients were excluded from this study because of incomplete data (n=6) and too low amount of extracted DNA (n=1) for analysis.

Figure 2. Abundance of dominant bacteria in patients with bronchiectasis according to disease severity: (A) phylum level, and (B) genus level.

Haemophilus and *Rothia* were significantly more abundant in the mild bronchiectasis group than in the moderate/severe bronchiectasis group (p=0.005, and p=0.043, respectively), and *Pseudomonas* was significantly more common in the moderate/severe group (p=0.031)

Figure 3. The number of operational taxonomic units (A, p=0.277) and the species richness estimate between two groups using ACE (B, p=0.274), Chao 1 (C, p=0.307), and Shannon diversity (D, p=0.550).

Figure 4. Principal coordinates analysis plot between the mild bronchiectasis group and the moderate/severe bronchiectasis group.