

Altered community compositions of *Proteobacteria* in adults with bronchiectasis

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Background: Bronchiectasis is a debilitating disease with chronic airway infection. *Proteobacteria*, the dominant phylum, can be detected with high-throughput sequencing.

Objective: To stratify *Proteobacteria* compositions according to culture findings in bronchiectasis.

Patients and methods: We sampled sputum, split for culture and 16srRNA sequencing, from 106 patients with stable bronchiectasis and 17 healthy subjects. Paired sputa from 22 bronchiectasis patients were collected during exacerbations and convalescence.

Results: Forty-five, 41, and 20 patients with clinically stable bronchiectasis had isolated *Pseudomonas aeruginosa* (PA), other potentially pathogenic microorganisms, and commensals at the initial visit, respectively. The PA group (but not other groups) demonstrated significantly greater relative abundance of *Proteobacteria*, and lower Shannon–Wiener Diversity Index, Simpson Diversity Index, and richness compared with healthy subjects. *Pseudomonas* was the dominant genus that discriminated bronchiectasis patients (particularly in the PA group) from healthy subjects. Compared with baseline levels, *Proteobacteria* community compositions in the PA group, but not in other groups, were more resilient during exacerbations and convalescence.

Conclusion: *Proteobacteria* community compositions could be partially reflected by conventional sputum bacterial culture. Significantly altered *Proteobacteria* community compositions – particularly, the increased relative abundance of *Pseudomonas* and diminished community diversity – represent critical targets for novel interventions to restore normal airway microenvironment in patients with bronchiectasis.

Keywords: bronchiectasis, *Proteobacteria*, *Pseudomonas aeruginosa*, culture, exacerbation

Introduction

Bronchiectasis is a debilitating disease characterized by disrupted airway microenvironment.^{1–3} Microbial dysbiosis plays pivotal roles in eliciting airway inflammation^{4,5} and in aggravating oxidative stress and excessive matrix metalloproteinase release.^{6,7} Identification of pathogenic microorganisms responsible for the vicious cycle may guide targeted therapy.⁸ *Proteobacteria* is the dominant phylum, comprising pathogens that are of clinical significance (eg, *Pseudomonas* spp., *Haemophilus* spp.).^{9,10} Although culture has been a “gold standard” for determining sputum bacteriology, limited categories of potentially pathogenic microorganisms (PPMs) could be detected,¹¹ raising concerns of under-detection in bronchiectasis.

Advances in molecular genotyping have enabled systematic exploration of microbial compositions in asthma,^{12,13} cystic fibrosis,^{14,15} interstitial lung disease,¹⁶ and bronchiectasis.^{17–21} In-depth analysis of microbial compositions in bronchiectasis is clinically significant because they correlate with lung function^{17,20,21} and change dynamically during exacerbations.¹⁸ Nevertheless, how *Proteobacteria* compositions correlate with culture findings remains unclear.

We hypothesized that sputum *Proteobacteria* compositions could be partially reflected by culture findings. We determined *Proteobacteria* compositions by stratifying culture findings in adults with clinically stable bronchiectasis, and evaluate the changes in *Proteobacteria* composition during bronchiectasis exacerbation and convalescence.

Patients and methods

Patients

Between March 2014 and November 2015, consecutive adult bronchiectasis patients (age >18 years) were enrolled from our outpatient clinics. Bronchiectasis was confirmed by high-resolution computed tomography (HRCT) of the chest, effective within 12 months. All patients could spontaneously expectorate at baseline visits, and remained exacerbation-free for 4 weeks. Exacerbations were defined as at least three criteria lasting for ≥ 2 days: significantly increased/aggravated sputum purulence/volume, tachypnea, chest pain, cough frequency, fever, wheezing, fatigue, hemoptysis, and exercise intolerance.^{11,22,23} We excluded patients with traction bronchiectasis, malignancy, acute respiratory tract infection, or antibiotic (excepting low-dose macrolides) use within the 4 weeks preceding study enrolment.

Healthy subjects (94% were never-smokers), recruited from a health check-up center, were aged 18–75 years and had no respiratory diseases, malignancy, or respiratory tract infection within 4 weeks.

The Ethics Committee of The First Affiliated Hospital of Guangzhou Medical University gave approval (Medical Ethics Year 2012 [The 33rd]). Written informed consent was obtained from all study participants (clinical trial registration number: NCT01761214).

Study design

At baseline, subjects underwent history inquiry, spirometry, and sputum sampling. Patients with bronchiectasis were followed-up every 3–4 months for repeated sputum cultures to determine isolation or colonization of *Pseudomonas aeruginosa* (see detailed definition in [Supplementary materials](#)).^{11,22} All patients were grouped according to their initial culture findings. In this study, bacterial isolation was defined as sputum culture positive to any of the PPM (mentioned above) at the initial visit. Bacterial colonization (referred to as infection for *P. aeruginosa*) was defined as sputum culture positive of an identical PPM for at least 2 occasions within 1 year, at least 3 months apart. Bacterial isolation denoted sputum culture positive of PPMs at baseline.

During follow-up, patients underwent reassessment of spirometry and sputum sampling once exacerbations were

confirmed, followed by antibiotic treatment.^{22,23} During exacerbation visits, sputum was sampled prior to initiation of antibiotics. We undertook convalescence visits at 1 week after 14-day antibiotic therapy. Only one sample at (typically, the first) exacerbation and convalescence hospital visits was collected for individual patients.

Clinical assessment

Bronchiectasis etiologies were determined using a validated protocol.²² We conducted spirometry with spirometers (QUARK PFT; COSMED Inc., Italy).²³ Key parameters reflecting chest radiography included bronchiectatic lobes and HRCT scores (Modified Reiff Score). The main variables extracted from history inquiry were exacerbation frequency within 2 years, duration of symptoms, and smoking history.

Sputum collection and bacteriology

Fresh sputum was sampled during hospital visits. To minimize oral contamination, patients thoroughly gargled their mouths with distilled water and took deep breaths before expectoration into sterile plastic container.²⁴ Hypertonic saline (3%–5%) induction, validated for microbiota assessment,¹⁸ was applied for healthy subjects (who cannot spontaneously expectorate sputum). Oral salivary contamination conferred limited impacts on sputum microbial compositions.^{10,18} Sputum was split for culture and sequencing and stored in -80°C freezers before biomarker measurement.

Conventional culture was applied for sputum. We stratified culture findings into a *P. aeruginosa*-positive (PA group), other potentially pathogenic microorganism-positive (PPM group), and culture-negative group (Comm group). PPM denoted *Haemophilus* spp., *Staphylococcus aureus*, *Klebsiella* spp. and miscellaneous bacteria that are of clinical significance.¹¹

DNA extraction and sequencing

Nucleic acids were extracted using physical disruption and a centrifugal absorption column, followed by Agarose gel electrophoresis and quality control with an ND-100 Nanodrop system (Thermo Fisher Scientific, Waltham, MA, USA). Samples that met quality-control criteria were subject to library construction, and barcoded before pooling.

DNA was sequenced using the Miseq System (Illumina Inc., USA). Raw reads were denoised, followed by chimera removal. We clustered the remaining high-quality sequences into operational taxonomic units (OTUs) at 97% similarity. We aligned sequences for individual OTU, and assigned taxonomic identities by defining 50% as the confidence threshold. We rarefied the denoised files to calculate the Shannon–Wiener Diversity Index, and ran analyses to

phyla and genera levels of taxonomic resolution. Sequences were deposited in GenBank under the accession number SAMN06768146-SAMN06768292. A flowchart demonstrating the main procedures associated with data processing is shown in [Figure S1](#).

Statistical analysis

Data were processed with the R statistics package (www.r-project.org) and GraphPad Prism (GraphPad Inc., San Diego, USA). Numerical data were presented as mean \pm SD for normal distribution, or median (interquartile range) for non-normal distribution. Between-group differences were compared with independent *t*-tests for unpaired data or Wilcoxon signed-rank tests for paired data. Multiple-group comparisons were made using analysis of variance or analysis of covariance. Paired *t*-tests were applied to pairwise samples. We analyzed κ statistics to evaluate the concordance between presence/absence of genera detected with culture and sequencing. Multiple-group comparison was subject to Bonferroni correction. We calculated the Shannon–Wiener Diversity Index, Simpson Diversity Index, and richness for reflecting community compositions. We used principal component analysis to assess the distinct bacterial phyla or genera when the patient remained clinically stable and during an exacerbation. We conducted linear multivariate regression

analysis to determine how key clinical variables correlated with the relative abundance of *Proteobacteria*, and Shannon–Wiener Diversity Index of different phyla. We applied Bray–Curtis metrics for pairwise comparisons of community compositions, which were compared at different visits using a nonmetric multidimensional scaling algorithm.

Results

Subject recruitment

Two hundred and thirty-two subjects underwent screening, and the baseline assessment included 106 bronchiectasis patients and 17 healthy subjects. Twenty-two patients were included in the exacerbation subgroup, of whom 19 completed convalescence visits (Figure 1).

Clinical characteristics

Patients who were included in baseline assessment ($n=106$) represented a cohort with predominantly moderate disease severity. Most patients had idiopathic and post-infectious bronchiectasis. At the initial visit, 45, 41, and 20 bronchiectasis patients had PA, other PPMs, and commensals isolation. Thirty-five of 45 (77.8%) patients had colonization by *P. aeruginosa* ([Table S1](#)). None of the patients had simultaneously isolated *P. aeruginosa* and other potentially

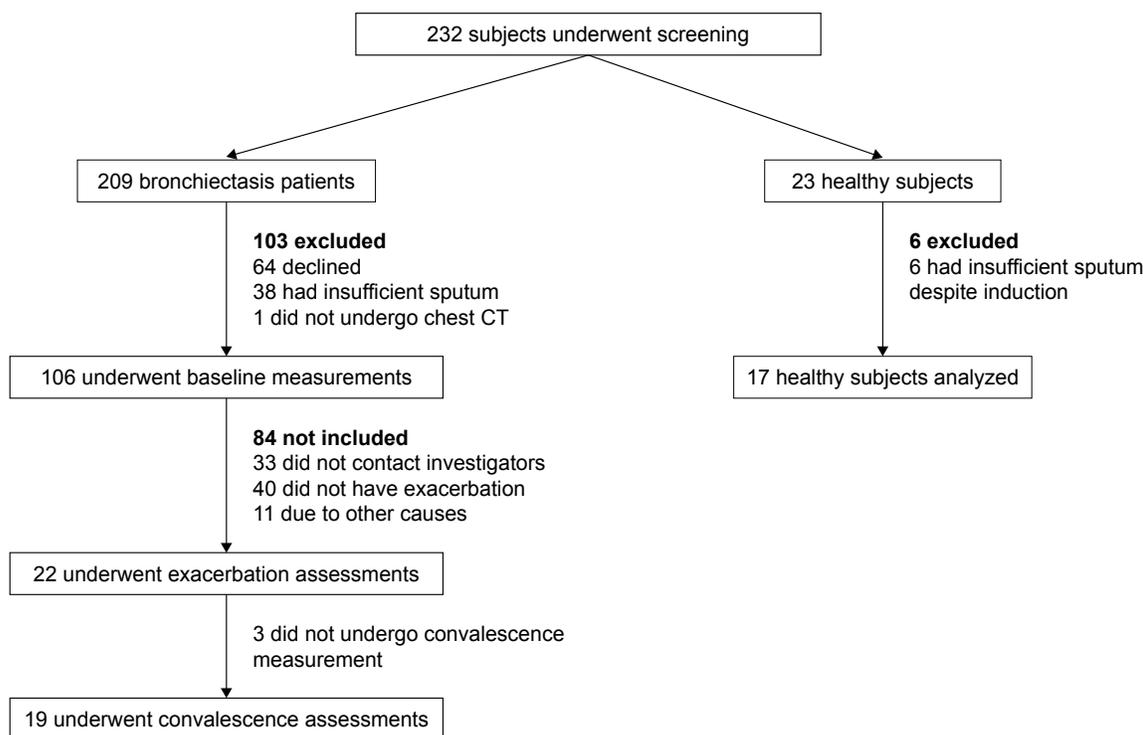


Figure 1 Subject enrollment flowchart.

Note: In total, 232 subjects (209 patients with bronchiectasis and 23 healthy subjects) underwent screening; the baseline assessment included 106 bronchiectasis patients and 17 healthy subjects; 22 patients were included in the exacerbation subgroup, of whom 19 completed convalescence visits.

pathogenic bacteria at any visit. Patients who were included in the baseline assessment (n=106) did not differ from those in the exacerbation subgroup (n=22) nor those excluded (n=103) in terms of demographics and the Bronchiectasis Severity Index (Table S2). A comparison of clinical characteristics of the PA, PPM, and Comm groups is presented in Table 1. Healthy subjects were older and had higher body mass index ($P<0.05$) compared with patients with bronchiectasis.

Sequencing results

Following filtering of low-quality reads and removal of reads not classified to the kingdom “Bacteria”, we collectively identified 45,331 OTUs at 97% identity, with 1,478 OTUs per sample among patients with bronchiectasis for all visits (Table S3). The median unique OTUs were not different in patients with bronchiectasis at clinical stability, exacerbation, and convalescence (43 [range 9–80], 37 [17–74], and 43 [15–67]; [$P=0.348$]). A median of 46, 41, and 29 unique OTUs was detected in the PA, PPM, and Comm groups ($P=0.007$), respectively.

Microbial compositions

The most common phylum among patients with clinically stable bronchiectasis and healthy subjects was *Proteobacteria* (mean relative abundance, 83.79% vs 75.33%, $P=0.017$), followed by *Firmicutes* (7.31% vs 10.49%, $P=0.022$) and *Bacteroidetes* (5.20% vs 8.97%, $P=0.005$; Figures 2A and S2). Compared with healthy subjects (mean, 75.33%), the relative abundance of *Proteobacteria* was significantly higher in the PA group (mean, 92.07%, $P<0.001$) but not in the PPM (82.83%, $P=0.056$) or Comm group (67.13%, $P=0.353$). The PA, PPM, and Comm groups differed considerably in the relative abundance of *Proteobacteria* ($P<0.001$; Figure S3A and C). Patients in the PA group had lower relative abundance of *Firmicutes* and *Bacteroidetes* than other groups. No significant difference was found in the relative abundance of major phyla between healthy subjects and the PPM group, or the Comm group (both $P>0.05$; Table S4, Figure S3B and D).

Genera compositions differed considerably. The relative abundance of *Serratia* increased progressively in the PA

Table 1 Comparison of demographic and clinical characteristics of patients with bronchiectasis when stratified by sputum culture findings

Parameters	PA group (n=45)	PPM group (n=41)	Comm group (n=20)	P-value
Anthropometry				
Age (years)	44.2±14.1	44.4±14.1	44.9±13.4	0.986
BMI (cm/kg ²)	20.1±3.5	21.0±3.1	20.7±3.5	0.497
Female no. (%)	27 (60.0%)	18 (43.9%)	14 (70.0%)	0.116
Never smokers no. (%)	40 (88.9%)	38 (92.7%)	19 (95.0%)	0.676
Disease-related parameters				
Duration of bronchiectasis (years)	15.0 (15.0)	16.0 (15.0)	8.5 (15.8)	0.167
Exacerbations within previous 2 years	2.0 (3.0)	2.0 (5.0)	3.5 (3.0)	0.495
No. of bronchiectatic lobes	5.0 (2.0)	4.0 (2.0)	4.0 (5.8)	0.052
HRCT score	10.0 (7.0)	7.4±3.5	8.0±4.6	0.013
Bronchiectasis Severity Index	7.5±3.7	4.0 (4.5)	3.5 (4.7)	0.001
24-hour sputum (mL)	30.0 (30.0)	30.0 (22.5)	20.0 (26.2)	0.250
Etiology				
Post-infectious no. (%)	18 (40.0%)	13 (31.7%)	3 (15.0%)	0.137
Immunodeficiency no. (%)	8 (17.8%)	6 (14.6%)	4 (0.0%)	0.857
Miscellaneous no. (%)	7 (15.6%)	7 (17.1%)	3 (15.0%)	0.972
Idiopathic no. (%)	13 (28.9%)	15 (36.6%)	11 (55.0%)	0.131
Spirometry				
FEV ₁ predicted%	51.1±22.3	64.8±20.1	68.6±27.3	0.004
FEV ₁ /FVC (%)	66.9±13.6	70.9±14.6	75.2 (24.5)	0.247
Medications used within 6 months				
Inhaled corticosteroids no. (%)	16 (35.6%)	8 (19.5%)	4 (20.0%)	0.186
Macrolides no. (%)	25 (55.6%)	16 (39.0%)	10 (50.0%)	0.304
Mucolytics no. (%)	35 (77.8%)	29 (70.7%)	16 (75.0%)	0.654

Notes: Numerical data were presented as mean ± SD for normal distribution or otherwise median (interquartile range). Categorical data were expressed as number (percentage) and compared using the chi-square test. No patient was receiving oral or inhaled antibiotics during the study. *P*-value represents the comparison among the three groups. *P*-value in bold indicated the comparison with statistical significance.

Abbreviations: BMI, body mass index; Comm, commensals; PA, *Pseudomonas aeruginosa*; PPM, potentially pathogenic microorganism; FVC, forced vital capacity; FEV₁, forced expiratory volume in one second; HRCT, high-resolution computed tomography.

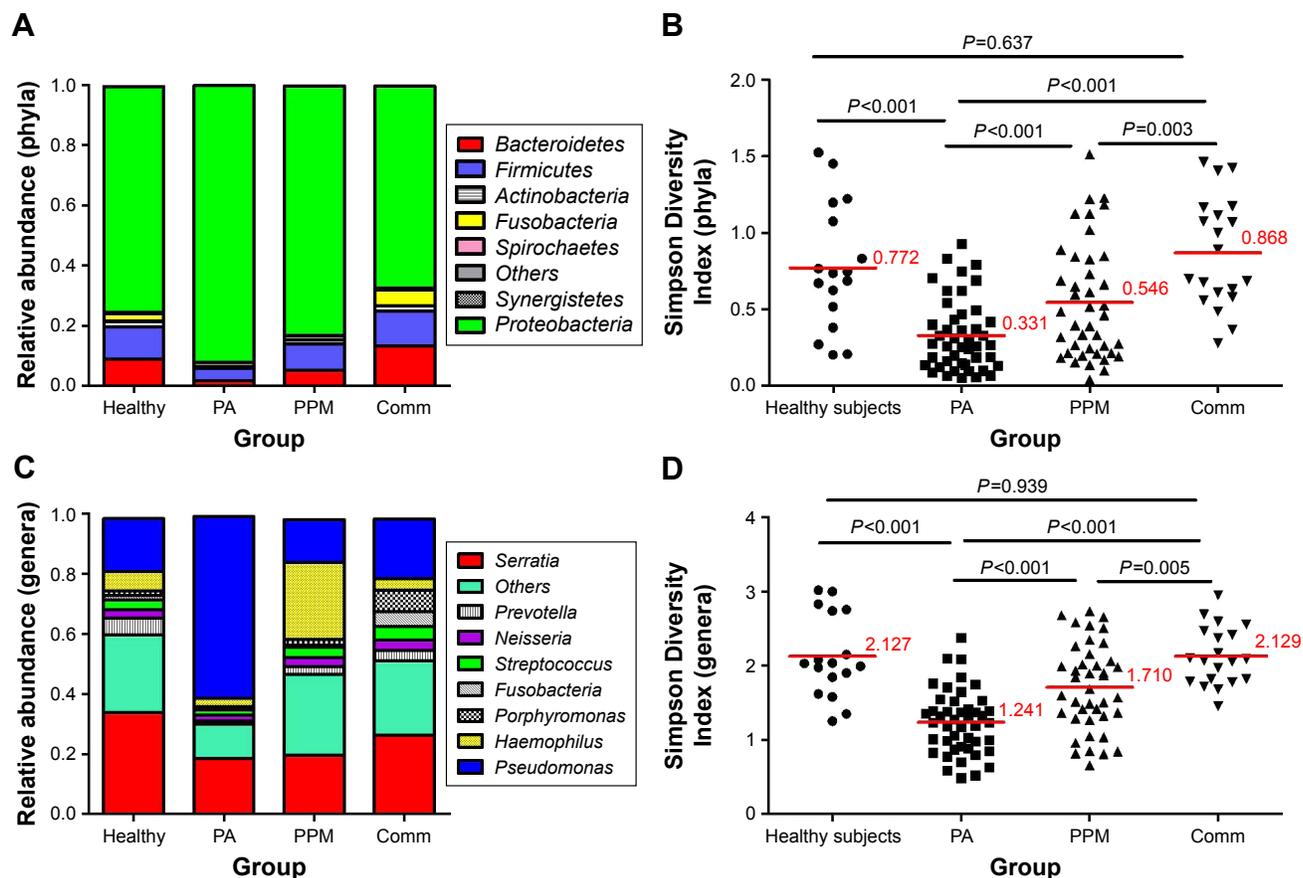


Figure 2 Microbial compositions at phyla and genera levels in patients with bronchiectasis and healthy subjects.

Notes: (A) Relative abundance of bacterial phyla in PA (n=45), PPM (n=41), and Comm (n=20) groups and in healthy subjects (n=17). (B) Comparison of microbial diversity (Shannon–Wiener Diversity Index) of bacterial phyla in PA (n=45), PPM (n=41), and Comm (n=20) groups and in healthy subjects (n=17). (C) Relative abundance of compositions of different bacterial genera in PA (n=45), PPM (n=41) and Comm (n=20) groups and in healthy subjects (n=17). (D) Comparison of microbial diversity (Shannon–Wiener Diversity Index) of different bacterial genera in PA (n=45), PPM (n=41), and Comm (n=20) groups and in healthy subjects (n=17) (B and D), greater values of the Shannon–Wiener Diversity Index represent greater magnitudes of bacterial diversity. The black lines within the boxes are the medians, whereas the ends of the whiskers correspond to the range. No significant difference in microbial community diversity is observed between the Comm group and healthy subjects, whereas microbial compositions differ considerably between the PA group and the rest of the groups.

Abbreviations: Comm, commensals; PA, *Pseudomonas aeruginosa*; PPM, potentially pathogenic microorganism.

(mean 18.48%), PPM (19.70%), and Comm groups (26.39%) and healthy subjects (33.79%; Figure 2C). Focusing on the phylum *Proteobacteria*, the *Pseudomonas* spp. dominated (mean relative abundance 60.46%) in the PA group, but not in other groups (14.28%–19.67%). Moreover, the relative abundance of *Pseudomonas* differed significantly among the PA, PPM, and Comm groups ($P<0.001$). However, the relative abundance of the *Pseudomonas* spp. was not different among the PPM group, Comm group, and healthy subjects. A greater relative abundance of *Haemophilus* (mean, 25.53%) was observed in the PPM group than in other groups (2.71%–6.30%). Genera composition was comparable between healthy subjects and the Comm group (Table S5; Figure S4A–D).

Furthermore, *Proteobacteria* and *Pseudomonas* spp. contributed the most (92.07% and 38.33%) to the similarity

of bacterial composition at phyla and genera levels, respectively (Tables 2 and S6).

Concordance between sequencing and culture

Concordance between culture and sequencing was good ($\kappa=0.75$, 95% CI 0.62–0.88) when stratifying bacteriology into PA vs non-PA category, but not for PPM (including *Pseudomonas* spp.) versus commensals ($\kappa=0.46$, 95% CI 0.30–0.62). The relative abundance of dominant genera correlated significantly with bacterial loads detected with culture ($r=0.51$, $P<0.001$).

Community diversity

At phyla levels, the PA group demonstrated substantially reduced bacterial diversity (mean 0.33), followed by the PPM

Table 2 The percentage similarity of microbial community (at genera levels) in patients with bronchiectasis when clinically stable

Groups	Genus	Mean abundance	Mean contribution	% contribution	Cumulative %
PA	<i>Pseudomonas</i>	0.60	49.27	68.91	68.91
	<i>Serratia</i>	0.18	11.39	15.92	84.83
	Other bacteria	0.12	8.31	11.62	96.45
PPM	Other bacteria	0.27	15.48	31.31	31.31
	<i>Serratia</i>	0.20	12.49	25.26	56.57
	<i>Haemophilus</i>	0.26	8.95	18.11	74.68
	<i>Pseudomonas</i>	0.14	7.81	15.81	90.48
Comm	Other bacteria	0.25	21.26	33.27	33.27
	<i>Serratia</i>	0.26	19.56	30.62	63.89
	<i>Pseudomonas</i>	0.20	13.25	20.73	84.62
	<i>Streptococcus</i>	0.05	2.86	4.47	89.09
	<i>Neisseria</i>	0.03	1.70	2.66	91.74

Note: Shown in the table are the major categories of genera that contributed most to the similarity in community composition when clinically stable.

Abbreviations: Comm, commensals; PA, *Pseudomonas aeruginosa*; PPM, potentially pathogenic microorganism.

group (mean 0.55), Comm group (mean 0.87), and healthy subjects (mean 0.77). There was no remarkable difference between healthy subjects and the Comm group ($P=0.637$; Figure 2B). Similar findings were replicated at genera levels, with the PA group demonstrating most dramatically reduced diversity (mean 1.24) and a lack of significant difference between healthy subjects and the Comm group ($P=0.939$; Figure 2D).

Compared with healthy subjects, both the Simpson Diversity Index and richness were substantially lower in bronchiectasis (both $P<0.001$; Table S7). The PA group demonstrated the lowest levels of Simpson Diversity Index and bacterial richness, followed by the PPM and Comm groups, at phyla (Figure S5A and B) and genera levels (Figure S5C and D).

At phyla levels, except for richness, we found comparable relative abundance of *Proteobacteria*, the Shannon–Wiener and Simpson Diversity Index (all $P>0.05$) between patients with colonization of *P. aeruginosa* and isolation of *P. aeruginosa* (transiently infected), and between regular users of macrolides and their counterparts (Table S7).

Microbial compositions during exacerbations and convalescence

We noted a disparate distribution of phyla *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* in the coordinate axis. *Proteobacteria* explained most of the dissimilarity for microbial compositions. *Pseudomonas* and *Haemophilus* contributed most to the community structural variation when remaining clinically stable and during exacerbations (Figure 3A and B). However, key clinical parameters (including the prior exacerbation frequency, HRCT scores, and lung function) did not correlate with the relative abundance of *Proteobacteria* and the Shannon–Wiener Diversity Index in clinically stable bronchiectasis. No correlation was found

between key clinical parameters and the Simpson Diversity Index and richness at phyla level, except that the Bronchiectasis Severity Index (which has taken into account colonization status of any potentially pathogenic bacteria) correlated with bacterial richness (Table S8).

In light of *Proteobacteria* predominance, we interrogated whether there would be greater changes in its relative abundance and bacterial diversity during bronchiectatic exacerbations and convalescence. Three patients (30.0%) in the PA group had converted from *P. aeruginosa* at baseline to miscellaneous bacteria during exacerbations. However, their data were still included in the PA group. Compared with the baseline, there were no significant differences in the total copies of *Proteobacteria* (Figure S6A–C), Shannon–Wiener Diversity Index (Figure S6D–F), Simpson Diversity Index (Figure S7A–C), or richness during exacerbations and convalescence (Figure S8A–C, all $P>0.05$). Stratification by baseline culture findings did not reveal differential changes during exacerbations and convalescence (Figures S6–S8).

Finally, we compared the pairwise Bray–Curtis distances for 22 sputa samples (exacerbation cohort) using a non-metric multidimensional scaling model. There were considerable overlaps in the dots representing different visits for individuals in PA group (Figure 4A), particularly the pairs for baseline and exacerbation visits. However, greater separations were observed in the PPM (Figure 4B) and Comm groups (Figure 4C), particularly when focusing on baseline and exacerbation visits.

Discussion

Our study revealed a differential relative abundance and diversity of *Proteobacteria* (particularly *Pseudomonas*) stratified by culture findings in bronchiectasis. The PA group distinguished considerably from other groups with regard to *Proteobacteria* compositions, which varied less significantly during exacerbations and convalescence compared with baseline.

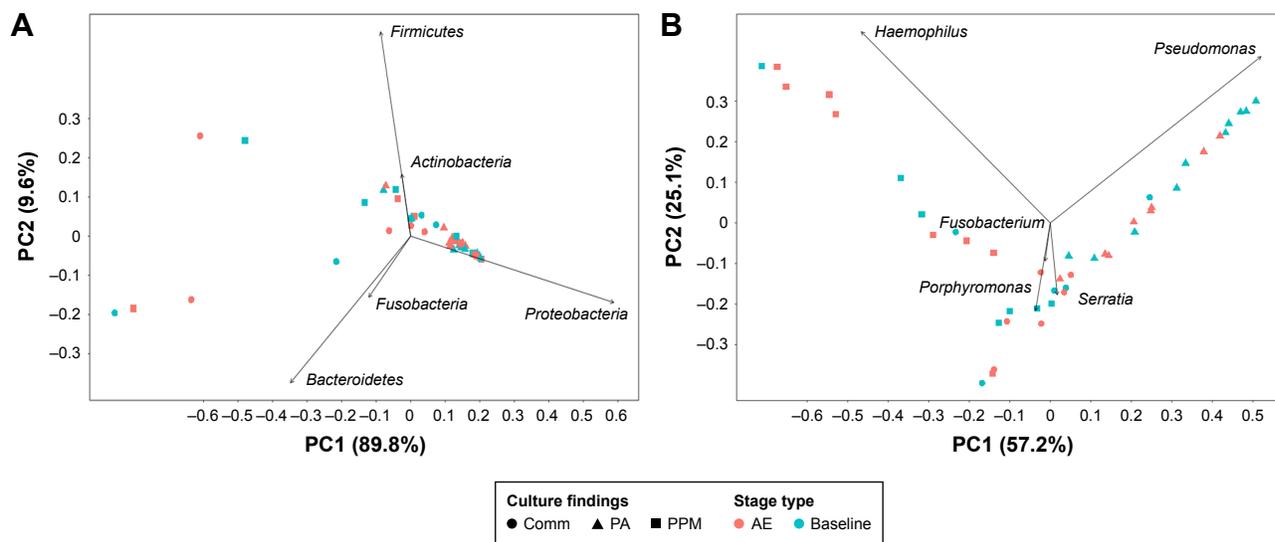


Figure 3 Dissimilarity of phylum *Proteobacteria* and its major genera in patients with bronchiectasis when clinically stable or during exacerbations. **Notes:** (A) Principal component analysis showing distinct phyla in PA (n=45), PPM (n=41), and Comm (n=20) groups. The first principal coordinate explains 89.8% of the variance in bacterial phyla compositions, whereas the second principal coordinate explains 9.6% of the variance in bacterial phyla compositions. (B) Principal component analysis showing distinct *Proteobacteria* species in PA (n=45), PPM (n=41), and Comm (n=20) groups. The first principal coordinate explains 57.2% of the variance in bacterial genera compositions, whereas the second principal coordinate explains 25.1% of the variance in bacterial genera compositions. Using principal coordinate analysis, we assign each item of high-dimensional data a location on a low-dimensional space according to the distance matrix. Presented in Figure 3 are the first principal coordinate (PC1), which accounts for the majority of the variability in the data, whereas the second principal coordinate (PC2) accounts for most of the residual variability. The greater proximity of individual points (which represent individual sputum samples) corresponds to a greater magnitude of similarity between different bacterial communities. Moreover, we present arrows which indicate the degree of correlation of the relative abundance of the dominant operational taxonomic units. Grouping was purely based on the sputum culture findings at baseline visits (when clinically stable). Therefore, sputum bacteriology was not applied for grouping during exacerbation visits. **Abbreviations:** Comm, commensals; PPM, potentially pathogenic microorganism; PA, *P. aeruginosa*; AE, acute exacerbation; PC, principal component.

A key question was whether airway *Proteobacteria* compositions differed considerably when stratified by culture findings. Our study has extended previous reports which confirmed the predominance of *Proteobacteria* in bronchiectasis regardless of culture findings.^{17–19,21,26,27}

Pseudomonas solely predominated in the PA group, albeit being detectable in other groups. The outgrowth of *Pseudomonas* was associated with greater relative abundance of *Proteobacteria*. These help to interpret the greater concordance between sequencing and culture findings

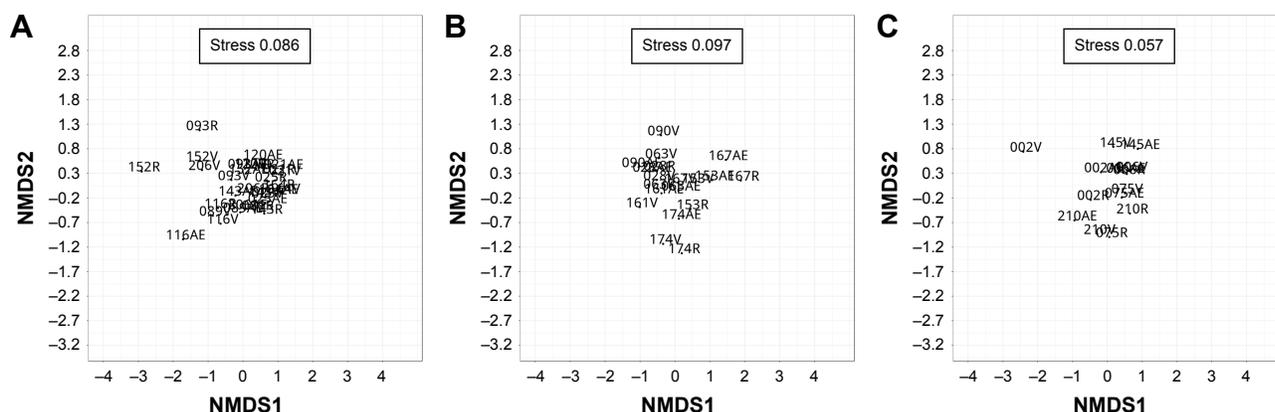


Figure 4 Pairwise comparison of Bray–Curtis distances in 17 patients with bronchiectasis (stratified by sputum culture findings) when clinically stable as well as during exacerbations and convalescence based on the non-metric multidimensional scaling analysis. **Notes:** (A) Pairwise comparison of Bray–Curtis distances in the PA group based on the non-metric multidimensional scaling analysis. (B) Pairwise comparison of Bray–Curtis distances in the PPM group based on the non-metric multidimensional scaling analysis. (C) Pairwise comparison of Bray–Curtis distances in the Comm group based on the non-metric multidimensional scaling analysis. There were considerable overlaps in the dots representing different clinical visits for individuals in PA group, particularly when comparing Bray–Curtis distances between clinically stable and exacerbation. However, there were significant separations in the PPM and Comm groups, particularly focusing on clinically stable and exacerbation visits. Grouping was purely based on the sputum culture findings at baseline visits (when clinically stable). Therefore, sputum bacteriology was not applied for grouping during exacerbation or convalescence. The number of patients included for analyses was ten in the PA group, seven in the PPM group (two patients did not undergo convalescence visits), and five in the Comm group (one patient did not undergo a convalescence visit, respectively). **Abbreviations:** Comm, commensals; PA, *Pseudomonas aeruginosa*; PPM, potentially pathogenic microorganism; NMDS, non-metric multidimensional scaling.

for *Pseudomonas*. According to the pathogen competition theory,²⁸ the predominance of *Pseudomonas* and other PPMs (eg, *Haemophilus*) is mutually exclusive. Greater abundance of *Pseudomonas*, which is frequently accompanied by reduced bacterial diversity, renders an outgrowth of *Pseudomonas* spp. that can be more readily identified with sputum culture. Nonetheless, *Haemophilus* reportedly dominated in the phylum *Proteobacteria*,^{17–19,22,27} which was not invariably observed herein. Differences in smoking status,^{21,26,27} concomitant use of medications (particularly inhaled corticosteroids), and disease severity might contribute to the disparity.^{21,27} For instance, patients regularly on macrolide therapy might demonstrate a predominance of *Pseudomonas*, but not *Haemophilus*.²⁷

In the Comm group, we unexpectedly observed a predominance of *Enterobacteriaceae* (eg, *Serratia* spp.), the relative abundance of which was comparable with that of healthy subjects. Most genera of *Enterobacteriaceae* are facultative anaerobes that cannot be readily identified with aerobic culture. In fact, *Enterobacteriaceae* was reportedly more prevalent in bronchiectasis patients with new-onset community-acquired pneumonia or recurrent infection.^{25,29} Our data suggested that outgrowth of *Pseudomonas* counteracted the growth of *Haemophilus* and *Enterobacteriaceae*.

We noted comparable *Proteobacteria* community compositions and Shannon–Wiener Diversity Index between the Comm group and healthy subjects. The magnitude of differences in *Proteobacteria* community compositions in the PPM group was greater when compared with the PA group. This might be related to the lower relative abundance of *Proteobacteria* and higher relative abundance of other phyla (eg, *Firmicutes*, *Bacteroidetes*), respectively. Acquisition of *Proteobacteria* might have imposed immense pressures of growth (eg, virulence factors) on the less pathogenic bacteria, including *Veillonella*, *Porphyromonas*, and *Prevotella*.^{17,18} Patients with bronchiectasis with isolated normal flora (commensals) demonstrated minor airway inflammation,¹¹ indirectly lending support to the aggravated inflammatory responses associated with airway dysbiosis – particularly, acquisition of *P. aeruginosa*.

No significant correlation was found between the relative abundance of *Proteobacteria* (including *Pseudomonas* spp.) and the key clinical parameters reflecting the disease severity except for bacterial richness, which partially echoed a recent study's findings.²⁶ From the data available, bacterial richness – but not relative abundance – preferentially reflected the severity of bronchiectasis.

The PA group demonstrated greater resistance to the changes at exacerbations and convalescence, compared

with the PPM and Comm groups. This finding mirrored that reported in Tunney et al's study,¹⁸ which included patients who had predominantly *Pseudomonas* isolation. Similarly, no substantial changes were observed in community compositions in patients with isolation of *P. aeruginosa* following long-term erythromycin treatment.²⁷ Therefore, community compositions might be more persistently disrupted following *Pseudomonas* acquisition. Unlike less (*Veillonella*, *Prevotella*)^{17,18} or more pathogenic genera (*Haemophilus*),^{18,19,21} the extensive resistance and biofilm formation of *P. aeruginosa* has rendered its eradication difficult despite exhaustive antibiotic therapy.³⁰ Our findings offer further evidence with regard to the differences in community compositions in PA group.

Despite a similar rate of colonization of *P. aeruginosa*, a previous study reported non-significant differences in sputum microbial compositions between the PA-colonized and non-PA-colonized groups in patients with COPD,³¹ which was in contrast to our main findings. Note that the percentage of patients regularly receiving inhaled corticosteroids and other inhaled bronchodilators was significantly greater among patients with severe COPD. Moreover, most patients in our study had moderate bronchiectasis, whereas Millares et al mainly recruited patients with severe COPD. Finally, the pathophysiology of bronchiectasis differs from that of COPD. These collectively indicated that the different sputum microbial compositions between bronchiectasis and COPD may have been affected by concomitant medications, disease severity, and underlying pathophysiology, or all of these factors. Nonetheless, no major difference in sputum microbial compositions was observed during exacerbations as compared with steady-state in both bronchiectasis and severe COPD, suggesting that sputum microbial compositions might not be the sole culprit responsible for exaggerated inflammatory responses and worsening of symptoms.

Apart from previous studies,^{17–19,21,26,27} we have focused on *Proteobacteria* compositions in bronchiectasis. Airway dysbiosis, measured with high-throughput sequencing which currently costs more than culture techniques, called for novel interventions (eg, probiotics) to restore the ecological balance. The resistance of *Proteobacteria* community composition after antibiotic therapy in the PA group highlighted an integration of novel interventions aside from antibiotics. The concordance between culture and sequencing findings in the PA group has justified our estimation of *Proteobacteria* compositions according to *Pseudomonas* predominance. A sequencing-based antibiotic selection strategy might be

preferable because of its greater ability in identifying the expansion of pathogenic *Proteobacteria* genera.

Some caveats deserve comments. Potential salivary contamination cannot be completely avoided. There have been notable differences in sputum microbial compositions sampled with hypertonic saline induction and spontaneous cough.³² Nonetheless, sputum sampling via spontaneous coughing would be preferred by most bronchiectasis patients, and sputum expectoration avoided an invasive approach (eg, bronchoscopy), rendering our findings clinically applicable. Sputum induction for healthy subjects, although valid for sputum collection, created a bias toward comparison of microbial compositions for spontaneous sputum. We sampled sputa once when clinically stable; however, repeat analysis suggested minimal variation in the relative abundance at phyla levels (Table S9). A plethora of microorganisms may have collectively shaped the airway niche; therefore, we failed to address the interactions between bacteria and viruses/fungi. The demographic characteristics and lung function differed considerably between healthy subjects and patients with bronchiectasis, which might have partially confounded our comparisons on sputum microbial compositions. The limited sample size for both exacerbation and convalescence cohorts could have accounted for the limited changes in sputum microbial compositions during bronchiectatic exacerbations and convalescence as compared with baseline levels. However, similar findings have been demonstrated in another cohort of patients with bronchiectasis, suggesting that changes in microbial compositions might have limited contribution in the exaggerated inflammatory responses.¹⁸ The current study design cannot fully address whether the expansion of *Fusobacteria* and *Porphyromonas* in the Comm group was responsible for the decline in the relative abundance of *Serratia*. Further investigations are warranted to determine whether changes in anaerobe compositions may indicate predisposition to the disease state. Finally, analysis on exacerbation and convalescence was biased by the antibiotics administered and symptoms which led to the heterogeneity of exacerbations.

Limitations

There remain some unanswered questions related to the sputum microbial compositions in patients with bronchiectasis. For instance, it is likely that an initial dysbiosis of commensal anaerobes could result in an increased likelihood of subsequent infection. In addition, it is unclear whether a broader killing of commensal microbiota by *Serratia* upon treatment would lead to a greater ability of opportunistic pathogens to invade and finally colonize bronchiectatic airways, which

needs to be addressed in future investigations. We only sampled sputa at a single time point among most patients (when clinically stable); however, repeat analysis suggested minimal variation in relative abundance at phyla levels (Table S9).

Conclusion

In summary, the significant alterations of *Proteobacteria* compositions indicate disrupted airway dysbiosis, calling for integrated management for normalizing microbial compositions in bronchiectasis.

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Author contributions

All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

Disclosure

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