# Lung Microbiota and Bacterial Abundance in Patients with Bronchiectasis when Clinically Stable and during Exacerbation

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*Rationale*: Characterization of bacterial populations in infectious respiratory diseases will provide improved understanding of the relationship between the lung microbiota, disease pathogenesis, and treatment outcomes.

*Objectives*: To comprehensively define lung microbiota composition during stable disease and exacerbation in patients with bronchiectasis.

*Methods*: Sputum was collected from patients when clinically stable and before and after completion of antibiotic treatment of exacerbations. Bacterial abundance and community composition were analyzed using anaerobic culture and 16S rDNA pyrosequencing.

*Measurements and Main Results*: In clinically stable patients, aerobic and anaerobic bacteria were detected in 40 of 40 (100%) and 33 of 40 (83%) sputum samples, respectively. The dominant organisms cultured were *Pseudomonas aeruginosa* (n = 10 patients), *Haemophilus influenzae* (n = 12), *Prevotella* (n = 18), and *Veillonella* (n = 13). Pyrosequencing generated more than 150,000 sequences, representing 113 distinct microbial taxa; the majority of observed community richness resulted from taxa present in low abundance with similar patterns of phyla distribution in clinically stable patients and patients at the onset of exacerbation. After treatment of exacerbation, there was no change in total (*P* = 0.925), aerobic (*P* = 0.917), or anaerobic (*P* = 0.683) load and only a limited shift in community composition. Agreement for detection of bacteria by culture and pyrosequencing was good for aerobic bacteria such as *P. aeruginosa* ($\kappa$ = 0.84) but poorer for other genera including anaerobes. Lack of agreement was largely due to bacteria being detected by pyrosequencing but not by culture.

*Conclusions*: A complex microbiota is present in the lungs of patients with bronchiectasis and remains stable through treatment of exacerbations, suggesting that changes in microbiota composition do not account for exacerbations.

**Keywords**: bronchiectasis; lung microbiome; exacerbation; bacteria

## AT A GLANCE COMMENTARY

### Scientific Knowledge on the Subject

Molecular techniques have emerged that can provide a comprehensive description of an entire population of bacteria at a body site. Despite the importance of the microbial–host relationship in the pathogenesis and progression of infectious diseases, these approaches have not been applied to define the lung microbiota in patients with bronchiectasis.

### What This Study Adds to the Field

Diverse polymicrobial communities are present in the lungs of patients with bronchiectasis when clinically stable and during an exacerbation. A surprising degree of stability was observed in both microbial load and community composition before and after antibiotic treatment of patients with acute pulmonary exacerbations. This finding suggests that changes in lung microbiota composition do not account for pulmonary exacerbations in patients with bronchiectasis.

Bronchiectasis is a chronic airway disease characterized by irreversible dilation of one or more bronchi (1). This results in poor mucus clearance and a vicious cycle of persistent bacterial colonization, airway obstruction, inflammation, and progressive tissue destruction (2). Patients with bronchiectasis frequently develop acute infective pulmonary exacerbations characterized by symptoms of fever, purulent sputum, and dyspnea (3), with attendant adverse effects on both their morbidity and health-related quality of life (4–6). Studies using classical aerobic-based microbial culture techniques to examine airway specimens from patients with stable bronchiectasis have reported *Haemophilus influenzae* (14–47% of cases), *Pseudomonas aeruginosa* (5–31%), and *Streptococcus pneumoniae* (2–14%) as the most frequently isolated pathogens (7–10). Significantly, in a large proportion of patients, no organisms were isolated from purulent sputum, even though repeated cultures were performed in patients not receiving antibiotics.

Using aerobic and strict anaerobic bacteriological techniques, we have detected a spectrum of potentially pathogenic anaerobic species in sputum samples from patients with cystic fibrosis (CF) when stable (11) and during antibiotic treatment of acute infective exacerbations (12). Furthermore, using a range of culture-independent methods, our group and others have shown that a much more complex and diverse polymicrobial community exists in the lungs of patients with CF than can be detected by culture (12–15).

Despite the emergence of DNA sequence–based molecular techniques, the role of the lung microbiota in the pathogenesis and progression of bronchiectasis remains poorly defined. Moreover, the British Thoracic Society guidelines for non-CF bronchiectasis highlighted that only a few studies offered a comprehensive cross-sectional analysis of bacterial isolation, and that these studies were all performed by routine diagnostic microbiology techniques (16). Clearly, a better understanding of airway infection in patients with bronchiectasis could inform studies that assess the impact of antimicrobial therapy and ensure that rational treatment strategies can be devised to treat colonizing bacteria.

In this study, we collected sputum samples from patients with clinically stable bronchiectasis and from patients receiving antibiotic treatment for acute infective exacerbations. Strict anaerobic bacteriological culture techniques and pyrosequencing were used to provide an in-depth analysis of the microbiome in sputum samples from these patients. Furthermore, we determined whether microbial load and community composition changed during and after antibiotic treatment of an exacerbation. We also assessed the relationship between pulmonary function and both microbial density and community diversity. Some of the results of these studies have been previously reported in the form of an abstract (17).

## METHODS

### Subjects and Sample Collection

Patients with confirmed disease were recruited during a 23-month period (July 2008 to May 2010). Additional details with respect to patient recruitment are provided in the online supplement. Patients recruited during stable disease (cross-sectional group) were sampled at a single time point with clinical stability defined as no exacerbation or antibiotic treatment of a respiratory infection in the previous 4 weeks. Patients presenting with an exacerbation (longitudinal group) were also recruited, with exacerbations defined according to the criteria of Fuchs and colleagues (18). For this longitudinal group, samples were collected at initiation (24 h before to a maximum of 48 h after first dose of antibiotics) and completion of antibiotic treatment (24 h before to a maximum of 48 h after last dose of antibiotics) and 4–8 weeks posttreatment when clinically stable.

A single expectorated or induced sputum sample was collected from each patient at each time point and transported to the laboratory for processing in an anaerobic cabinet. If sufficient sample weight was collected the sample was divided, with one aliquot immediately processed for culture and the second aliquot frozen at $-80^\circ$C for subsequent molecular analysis.

### Clinical Data

Demographic data (age, sex), clinical measures (lung function [$FEV_1$] and serum inflammatory markers [C-reactive protein, CRP; white cell count, WCC]), and details of chronic maintenance therapy were recorded from patient notes.

### Bacterial Isolation and Identification

Culture and subsequent detection of isolates in sputum samples were performed as previously described (11, 12), with all bacteria detected quantified (colony-forming units per gram sputum; cfu/g) by total viable count (TVC) and identified by PCR and Sanger sequencing of 16S ribosomal genes.

### Pyrosequencing

DNA was extracted from sputum samples as previously described (12). Bar-coded pyrosequencing of the 16S ribosomal RNA (rRNA) V1–V3 hypervariable region was performed at the University of North Carolina (Chapel Hill, NC) High-Throughput Sequencing Facility. A full description of the protocols used for pyrosequencing is provided by Fodor and colleagues (19; METHODS) with minor modifications described in the online supplement.

### Data Analysis

A detailed description of the statistical methods is provided in the online supplement. Briefly, in the cross-sectional group, aerobe and anaerobe viable counts were compared by Wilcoxon signed-rank test. In the longitudinal group, participant characteristics before and after antibiotic treatment were compared by paired-samples *t* test or Wilcoxon signed-rank tests as appropriate. Viable counts were compared at each time point and before and after antibiotic treatment, using Wilcoxon signed-rank tests. Comparison between three time points simultaneously was done by Friedman test.

Sample diversity metrics were assessed on the basis of the nonparametic Shannon-Wiener (SW) diversity index. Bray-Curtis (BC) metrics were used to generate pairwise comparison of community structure and plotted in two-dimensional space, using nonmetric multidimensional scaling (nMDS). Bacterial diversity (SW index) was compared in samples from clinically stable patients and those with an exacerbation, using the Mann-Whitney test. A Spearman rank correlation coefficient was used to measure the strength of the linear association between lung function and microbial load (aerobic, anaerobic, and total viable counts) and diversity (SW index). Diversity in samples grouped according to the predominant genera was compared by Kruskal-Wallis test. κ statistics were calculated to measure agreement between the presence or absence of bacteria detected by culture and pyrosequencing.

## RESULTS

### Subjects and Sputum Samples

Forty patients were enrolled in the cross-sectional study, with a single sample collected. According to the Global Initiative for Obstructive Lung Disease (GOLD) guidelines (20), patients were categorized as having either mild (GOLD 1, n = 17), moderate (GOLD 2, n = 17), or severe (GOLD 3, n = 6) disease. Patient characteristics for this cohort are presented in Table 1.

A further 14 patients were enrolled in a longitudinal study when they presented with an exacerbation. For these patients, samples were collected at both initiation and completion of antibiotic treatment and for 13 of 14 patients, 4–8 weeks posttreatment (mean, 45 d; range, 28–63 d) when clinically stable. For 11 of 14 patients, the initiation of treatment sample was collected before antibiotic treatment, starting with samples collected from the other 3 patients within 24 hours of commencing antibiotics. Patient characteristics for this cohort are presented in Table 2. Patients with an exacerbation were treated with either oral

TABLE 1. DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF PARTICIPANTS IN CROSS-SECTIONAL STUDY

|  | Mean (SD) |
|---|---|
| Number | 40 |
| Age, yr | 65.5 (7.3) |
| Sex | 13M/27F |
| $FEV_1$, L | 1.76 (0.63) |
| CRP, mg/L* | 4.0 (2.0–6.0) |
| WCC, $\times 10^9$/L* | 6.6 (5.43–8.60) |

*Definition of abbreviations*: CRP = C-reactive protein; F = female; M = male; WCC = white cell count.

*As CRP and WCC are skewed, the median and interquartile range are presented.

ciprofloxacin or cefaclor (n = 4 patients) or intravenous (n = 10 patients) antibiotic therapy (*see* Table E1 in the online supplement) for a mean of 14.5 days (range, 14–21) with all patients improving clinically. After antibiotic treatment, there was no change (P = 0.383) in lung function (FEV$_1$). However, CRP (P = 0.018) and WCC (P = 0.035) both decreased significantly by medians of 12 mg/L and 1.40 × 10$^9$/L, respectively (Table 2).

## Culture Analysis of Lung Microbiota Composition in Clinically Stable Patients

Bacteria were detected in high abundance (up to 1.3 × 10$^9$ cfu/g sputum) in sputum samples from clinically stable patients in the cross-sectional study. A mean (range) of 5.97 (2–12) different genera were cultured per sample with a median (range) total viable count of 3.93 × 10$^7$ (1.52 × 10$^4$ to 1.96 × 10$^9$) cfu/g sputum. Aerobic bacteria were isolated in high abundance (up to 1.3 × 10$^9$ cfu/g sputum) in all samples with *P. aeruginosa* (10 of 40 patients), *H. influenzae* (12 of 40 patients), and *S. aureus* (6 of 40 patients) the predominant species detected (*see* Table E2). Anaerobes from 5 different genera were also detected in high numbers (up to 1 × 10$^8$ cfu/g) from 33 of 40 samples (83%), with *Prevotella* (18 of 40 patients) and *Veillonella* (13 of 40 patients) most frequently isolated (*see* Table E2). Aerobic bacteria were present in significantly greater numbers than anaerobic bacteria (median difference in TVC, 1.36 × 10$^7$ cfu/g [95% confidence interval [CI], 5.53 × 10$^6$, 32.7 × 10$^6$]; Wilcoxon signed-rank test, P < 0.001). There was a positive correlation between CRP and both total viable count (ρ = 0.340, P = 0.032) and anaerobic viable count (ρ = 0.346, P = 0.020) but no significant correlation with aerobic viable count (ρ = 0.289, P = 0.070). Also, no correlations were apparent between viable counts and either FEV$_1$ or WCC.

Of the 40 patients enrolled in the cross-sectional study, 16 were prescribed chronic maintenance therapy (azithromycin and colistin, n = 2 patients; azithromycin, n = 9 patients; colistin, n = 5 patients). Lung function (FEV$_1$) was significantly less (P = 0.022, independent *t* test) in patients prescribed maintenance therapy (mean [SD], range: 1.94 [0.70], 0.79–2.27) compared with those not prescribed maintenance therapy (mean [SD], range: 1.49 [0.38], 0.80–3.54). However, although not statistically significant, total (P = 0.064, Mann-Whitney test), aerobic (P = 0.060), and anaerobic (P = 0.070) counts were less in patients receiving chronic maintenance therapy (Figure 1).

There was also no significant difference in total (P = 0.913, Kruskal-Wallis test), aerobic (P = 0.995), and anaerobic (P = 0.580) viable counts across the GOLD categories described previously. However, CRP was significantly related to GOLD category (P = 0.002).

## Culture Analysis of Lung Microbiota Composition in Patients with an Exacerbation

Aerobic bacteria were detected in high abundance (up to 4.6 × 10$^8$ cfu/g sputum) in all samples from patients with an exacerbation; those bacteria previously deemed to be pathogens were present in 9 of 14 patients at the start of treatment (*P. aeruginosa*, 5; *H. influenzae*, 4; *Strep. pneumoniae*, 1; methicillin-resistant *Staphylococcus aureus* [MRSA], 1), 7 of 14 patients at completion of treatment (*P. aeruginosa*, 1; *H. influenzae*, 4; *Strep. pneumoniae*, 1; MRSA, 1), and 5 of 13 patients when stable (*P. aeruginosa*, 3; *Strep. pneumoniae*, 2) (*see* Table E3). *H. influenzae* was detected in three patients at the start and end of treatment and in one patient at the start but not at the end of treatment. *P. aeruginosa* was only cultured in one patient at the start and end of treatment with an additional four patients positive by culture at initiation but not completion of treatment.

Anaerobic bacteria were also detected in high numbers (up to 7.5 × 10$^7$ cfu/g sputum) in 12 of 14 patients at the start of treatment (*Prevotella*, 6; *Veillonella*, 5), 9 of 14 patients at completion of treatment (*Prevotella*, 3; *Veillonella*, 3), and 7 of 13 patients when stable (*Prevotella*, 1; *Veillonella*, 3; *Actinomyces*, 3) (*see* Table E3).

Aerobic bacteria were present in significantly greater numbers than anaerobic bacteria at completion of antibiotic treatment (P = 0.001, Wilcoxon signed-rank test) and when the same patients were clinically stable (P = 0.001) but less so at the start of antibiotic treatment (P = 0.064) (Figure 1). However, there was no change in total (P = 0.925, Wilcoxon signed-rank test), aerobic (P = 0.917), or anaerobic (P = 0.683) viable counts with antibiotic treatment (Figure 2). Similarly, comparison of TVCs (Friedman test) for the 13 patients from whom 3 samples (start and end of antibiotic treatment, stable) were collected revealed no evidence of a difference in either aerobic (P = 0.097) or anaerobic (P = 0.869) counts between the 3 groups (Figure 2).

## Analysis of Lung Microbiota Composition by Pyrosequencing

The bacterial communities in 29 sputum samples (cross-sectional study, n = 10 samples; longitudinal study, n = 19 samples [start of treatment, n = 11; end of treatment, n = 5, stable after exacerbation, n = 3]) from 21 patients were profiled by pyrosequencing. In agreement with our culture results, a community of lung-resident bacteria was identifiable in all sputum samples. After quality control processing and removal of all sequences that were not at least classified to the kingdom "Bacteria," a total of 159,621 high-quality reads were generated from these samples with an average of 5,701 (range, 3,200–8,352) reads per sample. After normalization, on the basis of the sample with

## TABLE 2. DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF PARTICIPANTS RECEIVING ANTIBIOTIC TREATMENT FOR AN INFECTIVE EXACERBATION IN LONGITUDINAL STUDY

| | Start of IV Antibiotics Mean (SD) | Completion of IV Antibiotics | | | Stable | | |
|---|---|---|---|---|---|---|---|
| | | Mean (SD) | Mean Diff. from Start* (95% CI) | P Value | Mean (SD) | Mean Diff. from Start* (95% CI) | P Value |
| Number | 14 | 14 | | | 13 | | |
| Age, yr | 63.8 (6.2) | 63.8 (6.2) | | | 64.3 (6.1) | | |
| Sex | 6M/8F | 6M/8F | | | 5M/8F | | |
| FEV$_1$, L | 1.52 (0.44) | 1.58 (0.46) | +0.04 (−0.095 to 0.195) | 0.383 | 1.54 (0.38) | +0.21 (−0.035 to 0.41) | 0.186 |
| CRP, mg/L* | 19.0 (5.75 to 37.25) | 7.0 (3.75 to 11.75) | −12.0 (−22.0 to −1.0) | 0.035 | 4.0 (1.5 to 7.5) | −17.0 (−36.0 to −4.0) | 0.003 |
| WCC, × 10$^9$/L* | 8.55 (6.08 to 10.53) | 6.95 (5.18 to 8.45) | −1.40 (−3.5 to −0.2) | 0.018 | 6.8 (5.6 to 9.6) | −0.15 (−1.8 to 0.8) | 0.722 |

*Definition of abbreviations*: CI = confidence interval; CRP = C-reactive protein; Diff. = difference; F = female; IV = intravenous; M = male; WCC = white cell count.
   *As CRP and WCC are skewed, the median and interquartile range are presented, along with the median difference from start (95% CI) and the P value from the Wilcoxon signed-rank test.
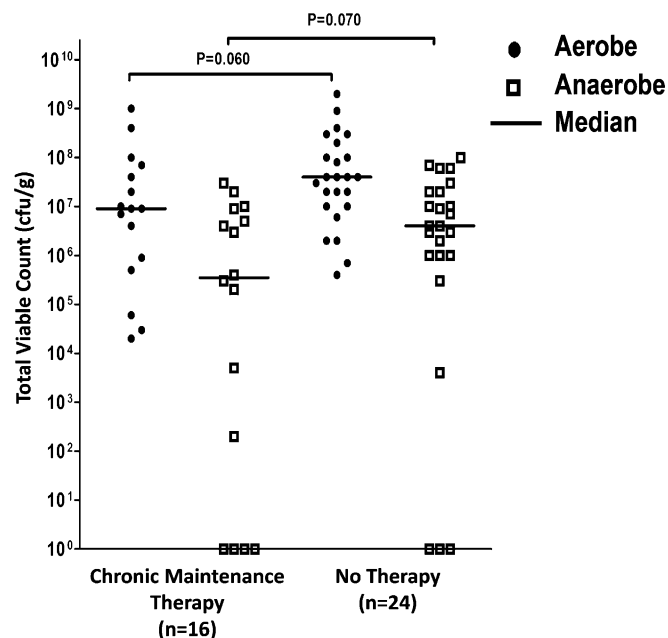
**Figure 1.** Comparison of total viable counts per gram sputum of aerobic and anaerobic bacteria cultured from sputum samples collected from patients with clinically stable bronchiectasis receiving (n = 16) and not receiving (n = 24) chronic maintenance antibiotic therapy. Although not statistically significant, total ($P = 0.064$, Mann-Whitney test), aerobic ($P = 0.060$), and anaerobic ($P = 0.070$) counts were less in patients receiving chronic maintenance therapy.

the fewest reads (3,200), a total of 92,800 reads were included in the analysis. From these sequences, we identified 113 operational taxonomic units (OTUs) at 97% identity (*see* Table E4). The mean (range) number of OTUs per sample was 29 (9–57).

Taxa from multiple phyla including Proteobacteria (60%), Firmicutes (24%), Bacteroidetes (5%), and Fusobacteria (1%) were detected (*see* Table E4); for each phylum present in a sample, there were typically one or two dominant genera including *Haemophilus* (30.7% of sequences), *Streptococcus* (14.8%), *Pseudomonas* (12.6%), and *Achromobacter* (8.8%). Furthermore, of the 113 OTUs identified, 10 accounted for more than 90% of the total sequences generated (*see* Table E4), indicating that the majority of the observed community richness resulted from taxa present in low abundance.

The agreement between the presence or absence of bacteria detected by culture and pyrosequencing was relatively good for aerobic bacteria such as *P. aeruginosa* ($\kappa = 0.84$) and *Achromobacter* ($\kappa = 0.87$) but poor for *H. influenzae* ($\kappa = 0.21$), *Staphylococcus* ($\kappa = 0.05$), and anaerobic genera such as *Prevotella* ($\kappa = 0.075$) and *Veillonella* ($\kappa = -0.070$). *Streptococcus* were detected in 16 of 29 (55%) samples by culture, but as they were detected in all 29 samples by pyrosequencing, a $\kappa$ coefficient could not be calculated. With the exception of *Staphylococcus*, where bacteria were detected in eight samples by culture but not pyrosequencing, lack of agreement between the methods was due to genera being detected by pyrosequencing but not culture (*see* Table E5).

## Community Structure Dynamics

Community structure was compared in samples from clinically stable patients (n = 10 samples) and in patients at the onset of an exacerbation (n = 11 samples) to determine whether clinical status affected community structure. Similar patterns of phyla distribution were observed in both groups (Figure 3a)

with no significant difference ($P = 0.105$, Mann-Whitney test) in microbial community diversity (SW index) apparent (Figure 3b). To enable further comparison, pairwise ecologic distances, calculated for all samples on the basis of the BC distance metric, were visualized by nMDS. There was considerable overlap between communities in samples from patients in each group, with differences in community composition not reflecting which cohort to which an individual patient/sample belonged (Figure 4). In addition, when we grouped all samples (n = 29), there was no correlation between lung function and microbial community diversity ($\rho = 0.123$, $P = 0.549$).

To further assess changes in microbial community structure after antibiotic treatment, we compared, by pyrosequencing, samples from five patients from whom sufficient sample was collected at both the start and end of treatment of an exacerbation. In general, abundance of the predominant bacteria decreased slightly after treatment, with a similar small increase in the abundance of unclassifiable bacteria and obligate anaerobes such as *Veillonella* and *Prevotella* (Figure 5). This limited shift in community composition within individuals after antibiotic treatment was confirmed when we compared BC distance metrics with samples from individual patients clustering closely together (Figure 4). To compare sequence similarities in samples collected from these 5 patients at various time points, we analyzed OTU clusters containing more than 10 sequences. In general, OTUs for the same sequence types were detected in matched samples from patients at different time points (*see* Table E6).
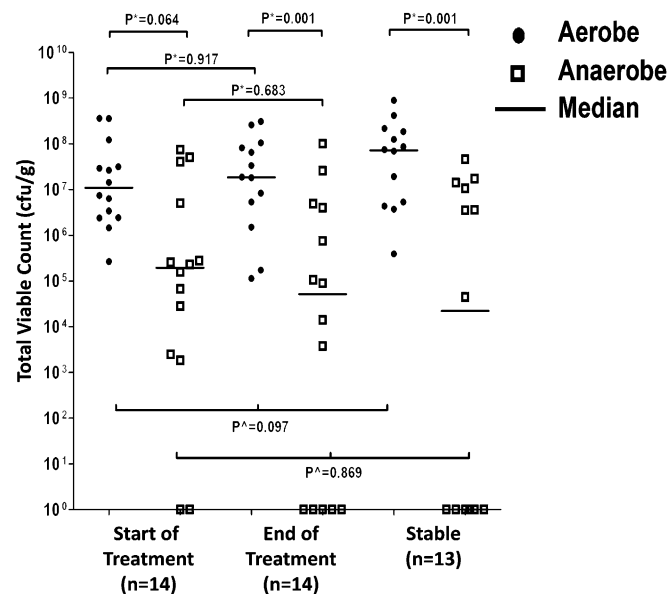


**Figure 2.** Total viable counts per gram sputum of aerobic and anaerobic bacteria cultured from sputum samples collected from patients with bronchiectasis at the start of treatment (n = 14) and end of treatment (n = 14) for an exacerbation and when clinically stable after exacerbation (n = 13). Aerobic bacteria were present in significantly greater numbers than anaerobic bacteria at the end of antibiotic treatment (median difference in total viable count, $1.9 \times 10^7$ cfu/g [95% CI, $1.41 \times 10^7$, $5.15 \times 10^7$]; $P = 0.001$, Wilcoxon signed-rank test) and when the same patients were clinically stable (median difference in total viable count, $6.0 \times 10^7$ cfu/g [95% CI, $3.2 \times 10^7$, $15.0 \times 10^7$]; $P = 0.001$) but less so at the start of antibiotic treatment (median difference in total viable count, $4.3 \times 10^6$ cfu/g [95% CI, $1.2 \times 10^7$, $2.0 \times 10^7$]; $P = 0.064$). *Comparing two groups using Wilcoxon signed-rank test; ^comparing three groups using Friedman test.
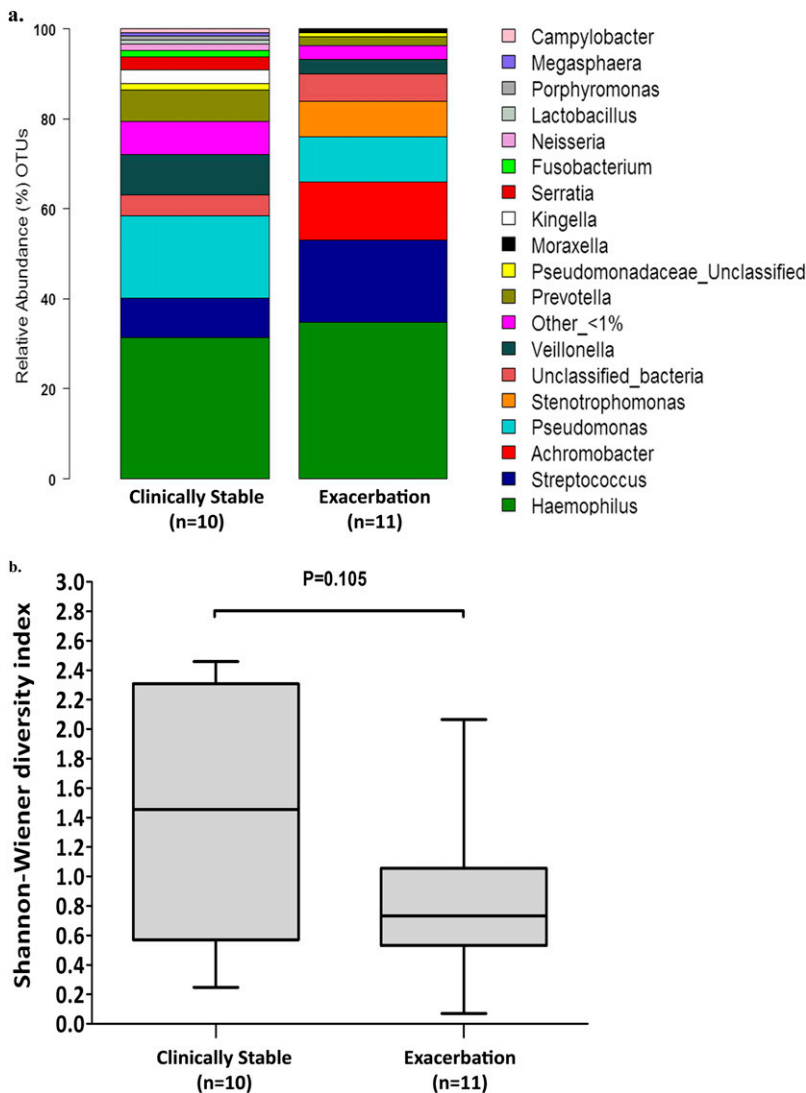
**Figure 3.** (*a*) Comparison of the percent abundance of the major identified phyla in pooled samples collected from patients when clinically stable (cross-sectional study, n = 10) and at the start of treatment for an exacerbation (longitudinal study, n = 11). Similar patterns of phyla distribution were observed in both groups. OTUs = operational taxonomic units. (*b*) Box plot comparison of microbial diversity (Shannon-Wiener diversity index) in samples from patients when clinically stable (cross-sectional study, n = 10) and at the start of treatment for an exacerbation (longitudinal study, n = 11), where higher values correspond to higher diversity. The top and bottom boundaries of each *box* indicate 75th and 25th quartile values, respectively, with the *black line* inside each *box* representing the median (50th quartile). The ends of the *whiskers* indicate the range. No significant difference ($P = 0.105$, Mann-Whitney test) in microbial community diversity is apparent between the two groups.

In our patient cohort, communities were most frequently dominated by *Haemophilus* (n = 10 samples), *Pseudomonas* (n = 8 samples), and *Streptococcus* (n = 6 samples), with these genera present as either a single dominant organism or sharing the majority of sequences with other genera. However, there was no significant difference ($P = 0.183$, Kruskal-Wallis test) in community diversity in samples within these three groups (Figure 6).

## DISCUSSION

In this study we used both quantitative aerobic/anaerobic culture and pyrosequencing to provide a comprehensive analysis of lung microbiota composition in patients with bronchiectasis when clinically stable and at initiation and completion of antibiotic treatment of acute pulmonary exacerbations. Previous studies of bronchiectasis have focused on patients in a stable state and used only routine aerobic selective culture. In the present study, bacteria such as *H. influenzae*, *P. aeruginosa*, and *S. pneumoniae* were detected by culture at similar rates to those reported in these studies (8–10, 21–25). However, additional aerobic and anaerobic bacteria from a wide range of genera were also detected in high abundance, highlighting that complex polymicrobial communities exist within the lungs of these patients. Aerobic genera detected included *Achromobacter*, *Stenotrophomonas*, and streptococci from the *Streptococcus milleri* group (SMG), which have

been reported to contribute to the onset of exacerbations in patients with CF (26). Although present in lower numbers than aerobes, anaerobes from genera including *Prevotella*, *Veillonella*, and *Actinomyces* were also detected by culture in more than 80% of samples. Only one previous study has reported detection of anaerobic bacteria in sputum from patients with bronchiectasis, with "anaerobic organisms" detected in only 2 of 123 (1.6%) samples and no details provided as to their identification (7). Similar aerobic and anaerobic genera have been detected, using both culture and molecular methods, in the lower airways of patients with a range of respiratory diseases including CF (11, 12, 19, 27–31), chronic obstructive pulmonary disease (COPD) (32, 33), and asthma (34) and also in healthy volunteers (32, 35). However, the densities detected here were more similar to that seen in CF samples.

High-throughput pyrosequencing revealed considerably greater bacterial diversity within sputa than detected by culture with multiple taxa from phyla including Proteobacteria, Firmicutes, and Bacteroidetes identified. Agreement between culture and pyrosequencing was relatively good for detection of abundant aerobic pathogens such as *P. aeruginosa* but was poor for detection of other genera including anaerobes present in lower abundance but greater diversity. In general, lack of agreement was due to bacteria being detected by culture-independent pyrosequencing but not culture, as has been previously reported for detection of bacteria in CF
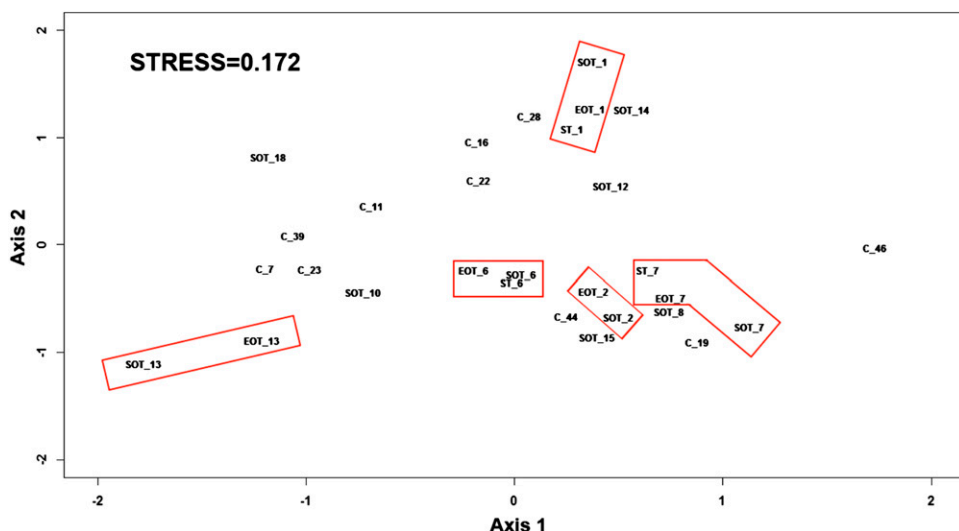
**Figure 4.** Pairwise comparison of Bray-Curtis distances for 29 sputum samples from 21 patients with bronchiectasis, using nonmetric multidimensional scaling (nMDS) analysis. Considerable overlap between communities in samples from patients in each group can be discerned, with differences in community composition not reflecting to which cohort an individual patient/sample belonged. A limited shift in community composition within individuals after antibiotic treatment was also apparent, with samples from individual patients clustering closely together. Clinically stable samples (C), n = 10; exacerbation samples, n = 19 (start of treatment [SOT], n = 11; end of treatment [EOT], n = 5; stable after exacerbation [ST], n = 3). *Red boxes* group together samples collected from the same patient at various time points.

sputum (12). Culture was more sensitive in detecting *Staphylococcus* than was pyrosequencing, a finding similar to that reported in a study examining CF respiratory specimens by a species-specific quantitative PCR assay (36). One study has raised concerns with respect to the efficient extraction of *Staphylococcus* DNA from sputum samples, using standard bacterial cell lysis buffer, similar to that employed in the present study, with more efficient extraction achieved by the addition of either lysostaphin or lysozyme (37). Our results suggests that pyrosequencing enables more comprehensive characterization of airway microbial community composition and is required to detect the presence of less abundant and potentially more difficult-to-culture bacterial genera. As has previously been described for CF, results from culture-independent pyrosequencing analysis could be potentially used to inform changes to culture protocols, thereby optimizing their sensitivity to detect difficult-to-culture bacteria (38).

Our results also support the observations in CF, that airway microbial communities can be partitioned into two groups (19, 39–41). One group is composed of a relatively small number of taxa that dominate the environment and in our patients with bronchiectasis, communities were most frequently dominated by *Haemophilus*, *Pseudomonas*, and *Streptococcus*. The second group consists of less commonly found taxa present in low abundance,

which account for the majority of the observed community richness. Furthermore, individual patients had unique microbiomes, with the composition of airway communities differing among patients. This finding of patient-specific community structures dominated by a single or few genera is consistent with studies of the lung microbiome in other respiratory diseases, which have shown a distinctive individual profile that is maintained over time (19, 32, 33).

Concerns have been expressed regarding contamination of sputum from sources outside the lung including the mouth, sinuses, and pharynx. However, a number of studies in CF have shown that sputum samples are not contaminated to a significant effect by bacterial species found within the oral cavity (11) and that anaerobic bacteria cultured from CF sputum samples were derived from the lungs and not the oral cavity (42). Furthermore, our finding that microbial communities were dominated by a single or few genera is similar to that of a study in CF that sampled secretions directly from the lobar airways of CF lungs, removed at the time of transplantation, and reported that lungs were dominated by one to three typical CF pathogens (43).

In patients with CF, it has also been suggested that decreased microbial diversity is associated with poorer lung function (13). However, in the patients with bronchiectasis included in this
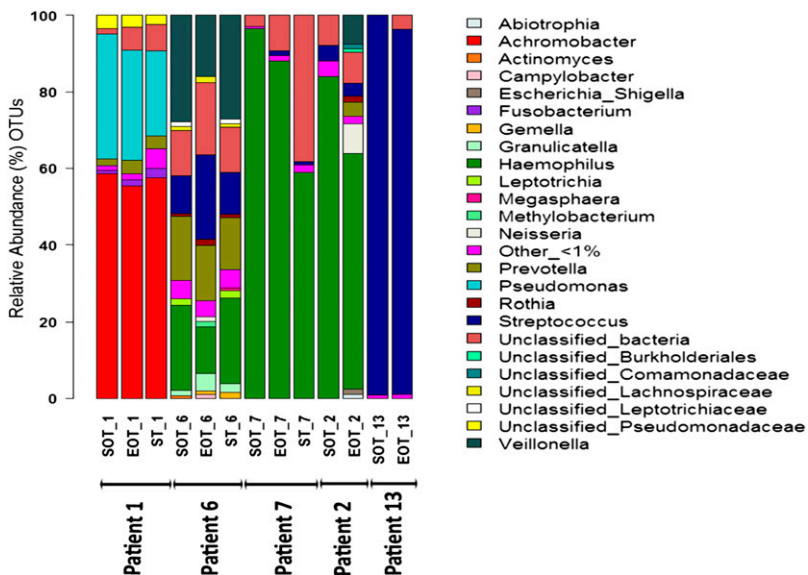


**Figure 5.** Comparison of the percent abundance of the major identified phyla in samples collected from patients at the start of treatment (SOT, n = 5) and end of treatment (EOT, n = 5) of an acute infective exacerbation and when clinically stable after exacerbation (ST, n = 3). Abundance of the predominant bacteria decreased slightly after treatment, with a similar small increase in the abundance of unclassifiable bacteria and obligate anaerobes such as *Veillonella* and *Prevotella*.
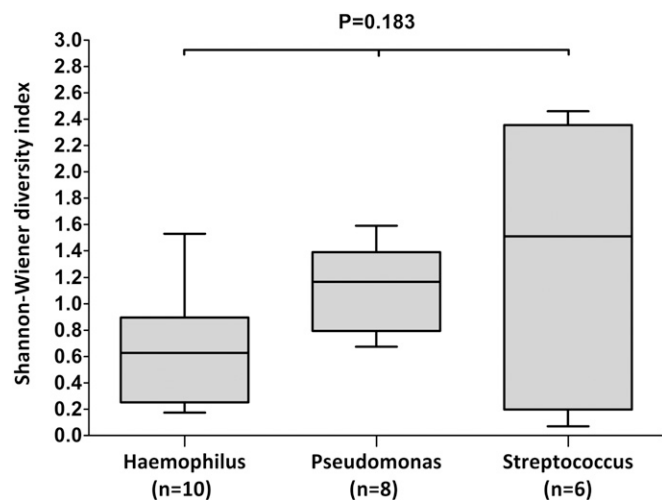
*Figure 6.* Box plot comparison of microbial diversity (Shannon-Wiener diversity index) in samples dominated by the genera *Haemophilus* (n = 10), *Pseudomonas* (n = 8), and *Streptococcus* (n = 6), where higher values correspond to higher diversity. The top and bottom boundaries of each *box* indicate 75th and 25th quartile values, respectively, with the *black line* inside each *box* representing the median (50th quartile). The ends of the *whiskers* indicate the range. There was no significant difference in diversity between the three groups (median Shannon-Wiener diversity index [interquartile range]: *Haemophilus*, 0.63 [0.25, 0.90]; *Pseudomonas*, 1.17 [0.79, 1.40]; *Streptococcus*, 1.51 [0.19, 2.35]; *P* = 0.183, Kruskal-Wallis test).

study, there was no correlation between diversity and lung function. This may be because patients with bronchiectasis do not receive the same intensive antibiotic therapy that has been shown to be the primary driver of decreasing diversity in patients with CF (31). A study by Sze and colleagues has reported a similar finding in that bacterial diversity in patients with very severe COPD (GOLD 4) was the same as that in nonsmoker and smoker control groups (33).

Pulmonary exacerbations are the single most important cause of morbidity in patients with bronchiectasis and are associated with disease progression (5, 6, 44), with bacterial colonization predicting future exacerbation risk (25). Although it is not clear what causes exacerbations, it has been suggested that onset may be triggered by changes in airway bacterial community composition (40), emergence of new strains or species (45), or spread of infection by the same species to new regions of the lung (19). Previous studies have shown that acquisition of a new strain of organisms including *H. influenzae* and *P. aeruginosa* is strongly associated with the occurrence of an exacerbation in COPD (46, 47). Similar to the reports for patients with CF (12, 31, 48, 49), we found by culture that total bacterial density did not increase significantly at the onset of exacerbation in patients with bronchiectasis, suggesting that an increase in bacterial load was unlikely to be a cause of exacerbation. However, there was an increase in anaerobic load relative to aerobic load and it is possible that this change may be a factor contributing to the onset of exacerbations. The majority of patients were treated with broad-spectrum antibiotics for 2 weeks and some effect on sputum bacterial density would have been expected. However, bacterial load did not decrease significantly with treatment. Sputum represents an average of inflammatory exudates produced in the lower respiratory tract and it is conceivable that heavily infected exudates produced during an infective exacerbation may remain trapped in the lower airways. Consequently, new airway infection caused by organisms present in low abundance may not always be detected or may be underrepresented in sputum

collected from the mouth, resulting in no significant change in bacterial load with treatment. Furthermore, there may be modest fluctuations in bacterial load over time that would not be detected by processing a single sputum sample. However, because of the technical challenges of collecting and immediately processing sputum samples under strict anaerobic conditions, it was not feasible to process 24-hour sputum samples in the current study.

A limitation of our study is that we had only a small number of patients (n = 5) from whom sufficient sputum was collected before and after antibiotic treatment to allow for pyrosequencing in addition to culture analysis. However, results from this data set provide further supportive evidence that microbial community composition remained relatively stable within individual patients, with only a limited reduction in abundance of the most abundant genera. Furthermore, OTUs for the same sequence types were determined in matched samples from patients at different time points. There are inherent limitations associated with the methods used to assign taxonomic ranking to data originating from pyrosequencing in that they can normally allow reliable taxon assignment only at the family or genus level (50). However, despite these limitations, our data illustrate that for the majority of genera, similar sequences were present pre- and postantibiotic treatment. Although changes in microbial community composition were not detected by culture and pyrosequencing, it is possible that exacerbations were driven by changes and/or adaptation in strains that would not be detected by pyrosequencing. For example, genomic differences have been shown to be associated with the ability of *H. influenzae* strains to cause exacerbations of COPD (51). Furthermore, adaptation within a species, such as mucoidy in *P. aeruginosa*, could also drive exacerbation (52) but would not be detected by sequence analysis. Further analysis of isolates, using typing methods such as pulsed-field gel electrophoresis and multilocus sequence typing, would be required to fully address this question.

Exacerbations are associated with increased airway inflammation, with the highest levels of inflammation associated with the acquisition of new bacterial pathogens (53). Similarly, in patients with COPD, it has been shown that disease progression is associated with an adaptive immune response that accounts for the increase in lymphocytes and their organization into lymphoid follicles (54). Therefore, it is possible that a similar type of immune response is present in the lungs of patients with bronchiectasis potentially driven by changes and/or adaptation in strains within the microbiome that would not be detected by sequence analysis. Determination of immune responses to bacteria is important in understanding the pathogenesis of infectious exacerbations. However, given the diverse polymicrobial communities present in the lungs of patients with bronchiectasis, it would be difficult to study the immune response to organisms by analyzing sputum directly. This further emphasizes the importance of culturing individual bacterial strains from all genera present, which can then be used to determine antibody response.

In four patients, *P. aeruginosa* was detected by culture before but not after antibiotic treatment; there was no significant change in total sputum bacterial density in these patients, suggesting that the reduction in some bacteria with antibiotic treatment may be offset by an increase in other bacteria. A previous study has reported that despite aggressive antibiotic treatment, only 1 of 15 patients with chronic *P. aeruginosa* colonization had the organism eradicated for more than 6 months (2). Therefore, further longitudinal samples would be required to determine whether *P. aeruginosa* had been truly eradicated in these patients or whether *P. aeruginosa* load had been transiently reduced to levels that could not be detected.

All patients with an exacerbation improved clinically with treatment, as shown by a decrease in markers of inflammation such as CRP and WCC. However, similar to other studies that have examined the effect of long-term inhaled antibiotic therapy in bronchiectasis (55, 56), there was no improvement in lung function after treatment of an infective exacerbation. This is in contrast to the marked improvements reported in lung function for patients with CF after both long-term inhaled antibiotic treatment (57, 58) and antibiotic treatment of acute infective exacerbations (12, 27).

Studies have also reported that treatment with long-term azithromycin decreases infective exacerbations in bronchiectasis and suppresses airway pathogens including *P. aeruginosa* (59, 60). Within our clinically stable patient cohort, lung function was significantly less in patients prescribed azithromycin; however, there was some evidence that bacterial density in sputum was less in these patients, suggesting that the antibacterial, antiinflammatory, and immunomodulatory effects of azithromycin had some effect on microbial load (40, 61). Furthermore, there was no relationship between bacterial load and lung function, with no difference in total viable counts across GOLD categories. Therefore, despite progression of lung disease, sputum bacterial density remains relatively stable as has been reported for patients with CF (31).

In summary, this study describes the composition of the lung microbiota in patients with bronchiectasis when clinically stable and during acute infective exacerbations and clearly shows that classical microbial culture techniques underestimate the richness and diversity of airway infection in patients with bronchiectasis. Although an array of bacterial genera including anaerobes was detected, communities were dominated by a small number of genera. Significant changes in sputum bacterial density and community diversity were not observed before and after treatment of pulmonary exacerbations, suggesting that changes in lung microbiota composition do not account for exacerbations in patients with bronchiectasis. Further studies of this complex microbial ecology are required to better determine the relationship between the airway microbiome in bronchiectasis and severity and progression of lung disease. Improved understanding of this relationship could enable more targeted use of antibiotics, particularly during exacerbations, which has the potential to enhance clinical efficacy and improve outcomes for patients.

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