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Predominant pathogen competition and core microbiota divergence in chronic airway infection

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Abstract

Chronic bacterial lung infections associated with non-cystic fibrosis bronchiectasis represent a substantial and growing healthcare burden. Where *Pseudomonas aeruginosa* is the numerically dominant species within these infections, prognosis is significantly worse. However, in many individuals, *Haemophilus influenzae* predominates, a scenario associated with less severe disease. The mechanisms that determine which pathogen is most abundant are not known.

We hypothesised that the distribution of *H. influenzae* and *P. aeruginosa* would be consistent with strong interspecific competition effects. Further, we hypothesised that where *P. aeruginosa* is predominant, it is associated with a distinct 'accessory microbiota' that reflects a significant interaction between this pathogen and the wider bacterial community. To test these hypotheses, we analysed 16S rRNA gene pyrosequencing data generated previously from 60 adult bronchiectasis patients, whose airway microbiota was dominated by either *P. aeruginosa* or *H. influenzae.* The relative abundances of the two dominant species in their respective groups were not significantly different, and when present in the opposite pathogen group the two species were found to be in very low abundance, if at all. These findings are consistent with strong competition effects, moving towards competitive exclusion. Ordination analysis indicated that the distribution of the core microbiota associated with each pathogen, readjusted after removal of the dominant species, was significantly divergent (ANOSIM, R = 0.07, P = 0.019). Taken together, these findings suggest that both interspecific competition and also direct and/or indirect interactions between the predominant species and the wider bacterial community, may contribute to the predominance of *P. aeruginosa* in a subset of bronchiectasis lung infections.

Introduction

The World Health Organization has reported that the global burden of diseases is shifting from communicable to non-communicable diseases, with chronic conditions such as heart disease, strokes, and lung diseases now being the chief causes of morbidity and mortality (Lopez *et al*., 2006). Among the great challenges in studying the causes and treatment of chronic lung infections, is a consequence of Koch's postulates and the subsequent concept of infection pathogenesis summarized by the expression 'one microbe, one disease' (Nelson *et al*., 2013). Koch's postulates have shaped our understanding of medical microbiology, as many important microbial diseases conform to them. However, that orthodoxy is being undermined by a growing recognition that diverse important diseases, including chronic lung infections, have a polymicrobial aetiology. This expanding understanding of chronic polymicrobial infections, originating from studies of cystic fibrosis airway microbiota, is beginning to be translated to other chronic lower respiratory diseases, including non-cystic fibrosis bronchiectasis (hereafter referred to as bronchiectasis).

Bronchiectasis is a chronic airway disease characterised by abnormal destruction and dilation of the large airways, bronchi and bronchioles (Cohen and Sahn, 1999). It is associated with chronic and frequently purulent expectoration, multiple exacerbations, and progressive dyspnoea that can become disabling (Ellis *et al*., 1981; Cohen and Sahn, 1999; Barker, 2002), and represents a substantial and growing healthcare burden. A recent US study demonstrated a marked increased prevalence in older populations varying from 4.2/100000 adults aged 18-34 years to 271.8/100000 aged ≥ 75 years (Weycker *et al*., 2005). Bronchiectasis often goes unrecognized or is misdiagnosed as asthma or chronic obstructive pulmonary disease (COPD), leading to an underestimated prevalence. Despite this, bronchiectasis is associated with substantial socioeconomic cost due to the frequent use of primary and secondary healthcare resources. An US epidemiological study of bronchiectasis-associated hospitalizations from 1993 to 2006 demonstrated an average annual hospitalization rate of 16.5/100000 population with a significant annual increase of 2.4% in men and 3.0% in women (Seitz *et al*., 2010), with the cost of managing bronchiectasis appearing to be rising (Joish *et al*., 2013).

Airway inflammation resulting from chronic bacterial infection is thought to be a significant contributory factor driving disease progression in bronchiectasis (Barker, 2002). The perceived importance of bacterial pathogens in airway disease progression is reflected in the use of antibiotics

(Serisier and Martin, 2011; Serisier *et al*., 2013a), both as maintenance therapy, and to treat episodes of acute exacerbation. However, our understanding of the mechanisms that underpin relationships between infection by particular bacterial taxa and clinical outcomes is currently poor. This situation undermines the development of rationales for the selection of particular antibiotic treatment regimes (Serisier, 2012) or potentially specific anti-inflammatory therapy (Visser *et al*, 2012), and achieving better insight into the manner in which treatments achieve beneficial outcomes. While bronchiectasis can result from a variety of recognised aetiologies, it is often considered idiopathic. Recent studies have revealed a substantial and diverse bacterial microbiota (Rogers *et al*., 2013b; Tunney *et al*., 2013; Rogers *et al*., 2014; van der Gast *et al*., 2014), which are typically dominated by either *Haemophilus influenzae* or *Pseudomonas aeruginosa*. Perhaps unsurprisingly given its colonisation of the upper airways in healthy individuals, *H. influenzae* is detectable in lower airway secretions from almost all bronchiectasis patients and is commonly the numerically dominant species (Rogers *et al*., 2013b; Rogers *et al*., 2014). In contrast, *P. aeruginosa* dominated infections occur in a smaller number of patients (Rogers *et al*., 2014), but, are associated with an accelerated decline in lung function, more frequent pulmonary exacerbations, greater sputum production, and a higher requirement for antibiotic therapy (Evans *et al*., 1996; Ho *et al*., 1998; Shoemark *et al*., 2007; Rogers *et al*., 2014).

A better understanding of the way in which *P. aeruginosa* interacts with the airway environment could provide important mechanistic insights into chronic infection in this patient group and in chronic respiratory infections more widely. Both the physiochemical characteristics of the airway environment, and the composition of the pre-existing lung microbiota, are likely to influence the likelihood of *P. aeruginosa* infection (Rogers *et al*., 2013a). Further, where *P. aeruginosa* dominates the infection microbiota, its growth is likely to further affect the composition of airway environment. This impact of colonisation could occur both directly through the metabolomic (Kozlowska *et al*., 2013) and secretomic (Bergamini *et al*., 2012) footprint of *P. aeruginosa*, and, in turn, indirectly by stimulating changes in the host immune response (Bergamini *et al*., 2012) and the activity of other co-colonising species (Bakkal *et al*., 2010; Tashiro *et al*., 2013). Whilst the causality in these interactions is difficult to demonstrate, were such relationships to exist, they would result in both an association between *P. aeruginosa* infection and measures of airway disease, and an association between *P. aeruginosa* infection and microbiota composition. The first of these associations has been well documented. However, to our knowledge, there have been no investigations to assess the second.

We hypothesised that (1) the distribution of *H. influenzae* and *P. aeruginosa* in airways samples would be consistent with strong interspecific competition effects; i.e. when *H. influenzae* is the dominant species in a bronchiectasis lung infection, the population size of *P. aeruginosa* will be negatively impacted and vice versa when *P. aeruginosa* is dominant. (2) Where *P. aeruginosa* or *H. influenzae* is dominant species in a bronchiectasis lung infection, they are associated with distinct 'accessory microbiota' that reflect a significant interaction between these pathogens and the wider bacterial community. To test these hypotheses, we analysed 16S rRNA gene pyrosequencing data, generated previously, from 60 adult bronchiectasis patients whose airway microbiota was dominated by either *P. aeruginosa* or *H. influenzae*. *H. influenzae*-dominated infections were chosen as a comparator group as they had been shown previously not to differ significantly in total bacterial load, dominant taxon relative abundance, or prior antibiotic burden (intravenous, oral, and combined), with those where *P. aeruginosa* was dominant (Rogers *et al*., 2014). Further, despite differences in disease course, these patients did not differ significantly in serum C-reactive protein (CRP) levels, or sputum IL-8 and IL-1β levels (Rogers *et al*., 2014) common markers of systemic and airway inflammation. To limit the potential effect of antibiotic therapy to influence microbiota composition (Serisier, 2013b), a sample set was chosen where there had been a four week period of clinical stability prior to collection, with no supplemental antibiotics administered (Serisier *et al*., 2013c).

Materials and methods

The analysis performed here was based on 16S ribosomal RNA gene pyrosequencing data generated from induced sputum samples from adult patients with bronchiectasis, as part of the BLESS trial (Serisier *et al*., 2013c). These data are available through the Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) under the accession number SRP0356. Details of patient recruitment, sample collection, nucleic acid extraction, PCR amplification pyrosequencing, and bioinformatics processing have been published previously (Rogers *et al*., 2013b; Serisier *et al*., 2013c; Rogers *et al*., 2014) and details of these processes are provided here as Supplementary Methods.

Of the 96 samples previously analysed, 26 were *P. aeruginosa*-dominated and 34 were *H. influenzae*-dominated. It is these 60 samples on which the analysis presented here is based. Patient details for these patients are shown in Table 1. As described previously, the species-level identification of *P. aeruginosa* and *H. influenzae* two species was confirmed using specific PCR-based

assays in all instances (Supplementary Methods), with identification by pyrosequencing treated as presumptive. Where species-level identities were not corroborated by specific assays, identities are presented at the genus level.

Bacterial taxa within each metacommunity were partitioned into core and satellite groups using the Poisson distribution test as previously described (van der Gast *et al*., 2011; Rogers *et al*., 2013c). One-way analysis of variance (ANOVA), regression analysis, coefficients of determination (r^2), residuals and significance (*P*) were calculated using Minitab software (version 16, Minitab, University Park, PA, USA) as described previously (van der Gast *et al*., 2011; Rogers *et al*., 2013c). Canonical correspondence analysis (CCA), analysis of similarity (ANOSIM), similarity of percentages (SIMPER) analysis were performed using the PAST (Palaeontological Statistics, version 2.17) program available from the University of Oslo website link (http://folk.uio.no/ohammer/ past) run by Øyvind Hammer. Mann Whitney tests were performed using GraphPad Prism (version 5.01, La Jolla, CA 92037 USA). Where predominant taxa were removed prior to analysis, the remaining relative abundance measures were rescaled and expressed as percentages.

Results and Discussion

P. aeruginosa and *H. influenzae* share certain similarities, for example, they are both Gram negative, rod-shaped, facultative anaerobic Gammaproteobacteria. However, these common opportunistic pathogens are associated with very different clinical courses when dominant in bronchiectasis lung infections. Here the relative abundances of the two predominant species in their respective groups were high and not significantly different (ANOVA, $F_{1,58}$ = 0.096, P = 0.758; *P. aeruginosa* mean abundance and standard deviation = 87.3 ± 13.4% and *H. influenzae* = 86.0 ± 17.9%) (Fig. 1). When present in the opposite dominated group the two species were found to be in only very low abundances (*P. aeruginosa* = 0.37 ± 1.3% and *H. influenzae* = 0.56 ± 0.77%; ANOVA, $F_{1,58}$ = 0.436, P = 0.511). Furthermore, *P. aeruginosa* was not detected in 12 from 34 samples of the *H. influenzae* group, and 2 from 26 for *H. influenzae* in the *P. aeruginosa* group (Fig. 1). This is consistent with strong competition effects between the two species, moving towards competitive exclusion of the inferior competitor species; and more so for *P. aeruginosa* within the *H. influenzae* group. If these patterns of dominance and suppression could be purely explained by the process of interspecies competition then no between group differences in accessory microbiota would be

176 expected. However, the analyses performed here demonstrate that taxa present in the microbiota
177 associated with *P. aeruginosa* and *H. influenzae* predominance are significantly divergent.

178 The distribution of the two sets of microbiota, as determined by direct ordination using Bray-
179 Curtis similarity measures, is shown in Fig. 2. Where the dominant taxa (*P. aeruginosa* or *H.*
180 *influenzae*) were included (Fig. 2a), divergence in the distribution of the microbiota was pronounced
181 and statistically significant (ANOSIM, $R = 1$, $P < 0.0001$). However, given the high proportion of total
182 bacterial abundance that these predominant taxa would account for within the microbiota (Fig. 1),
183 much of the variation between the two groups will result from their inclusion in the analysis. In order to
184 assess whether accessory microbiota composition differed significantly between the two groups, the
185 predominant taxa were removed and the relative abundances of the remaining taxa redistributed and
186 expressed as percentages. When subjected to ordination analysis, the distribution of the accessory
187 microbiota composition (Fig. 1b) were not found to be significantly divergent (ANOSM, $R = 0.036$, $P =$
188 0.11).

189 Many accessory microbiota taxa are of low relative abundance. The potential contribution to
190 accessory microbiota of transient bacterial populations within the airways, as opposed to populations
191 of chronically infective bacteria, is therefore high. In order to reduce the effect of these satellite taxa,
192 the core microbiota (composed of non-randomly distributed taxa) in each of the two groups was
193 determined (Fig. 3). This is an approach that has been applied successfully in the analysis of chronic
194 bacterial infections associated with cystic fibrosis (van der Gast *et al*., 2011; Rogers *et al*., 2013a). In
195 the *Haemophilus*-dominated group, 9 of the 92 taxa detected were classified as core and 83 as
196 satellite, and in the *Pseudomonas*-dominated group, 8 of the 70 taxa detected were classified as core
197 and 62 as satellite. In each case, the contribution of individual taxa to the core microbiota was
198 assessed by SIMPER analysis (Tables 2 & S1). Ordination analysis was then performed using the
199 core taxa, and again the difference between the distribution of the core microbiota that included the
200 predominant *P. aeruginosa* and *H. influenzae* populations was significant (ANOSM, $R = 1$, $P < 0.0001$)
201 (Fig.4a). Moreover, the divergence in the distribution of the core microbiota, readjusted after removal
202 of the dominant species, was also found to be significantly divergent (ANOSIM, $R = 0.07$, $P = 0.019$)
203 (Fig. 4b). Satellite taxa were not significantly different between the groups (ANOSIM, $R = 0.05$, $P =$
204 0.06).

To assess whether significant differences existed in the relative abundances of specific core taxa between the *P. aeruginosa*- and *H. influenzae*-dominated samples, Mann-Whitney tests were used and performed on readjusted core taxa abundance data after *P. aeruginosa* and *H. Influenzae* are removed. This process identified *Prevotella* spp. and *Flavobacterium* spp. as being significantly more abundant in the *P. aeruginosa*-dominated samples ($P < 0.0001$ and $P = 0.003$, respectively), while *Neisseria* spp. was significantly more abundant in *H. influenzae*-dominated samples ($P < 0.0001$). *Flavobacterium* is a genera that has been reported previously to contribute to bacterial communities present in chronic lung infections (Rogers *et al*., 2003; Rogers *et al*., 2004; van der Gast *et al*., 2011; Rogers *et al*., 2013a) although typically present at low relative abundances. In contrast, *Prevotella* spp. have been reported as both common, and often at high abundance in both bronchiectasis and CF lung infections (Tunney *et al*., 2008; Field *et al*., 2010; Stressmann *et al*., 2012), and otitis media (Brook, 2008). *Prevotella* spp. appear to be particularly prevalent when co-colonising with *P. aeruginosa*, a factor that has led to the previous suggestion that a synergistic relationship exists between *P. aeruginosa* and members of this genus (Su and Hassett, 2012). The genus *Prevotella* is composed of species that are obligate anaerobes. While *P. aeruginosa* and *H. influenzae* are both capable of fermentation and growth under anaerobic conditions (Schobert and Jahn, 2010; Langereis and Hermans, 2013), the contributions of thick mucoid secretions that *P. aeruginosa* can produce in the airways (Ma *et al*., 2012), a trait not demonstrated by *H. influenzae* (Langereis and Hermans, 2013), may contribute to reduced oxygen permeation, leading to the creation of greater opportunities for the growth of strict anaerobes, such as *Prevotella* spp. This model would be consistent with the association observed here between *H. influenzae* and *Neisseria*, a genus of typically aerobic species.

Canonical correspondence analysis was performed next to assess the extent to which variance in the microbiota distribution can be accounted for by variation in measures of disease severity, the presence of comorbidities, and non-antibiotic therapies. The results of these analyses are shown in Table 3, and additionally superimposed onto Figs 2 & 4. In keeping with previous reports (Rogers *et al*., 2014), the presence of *P. aeruginosa* in samples as the predominant taxon was associated with high pulmonary exacerbation frequency and poor lung function (low FEV_1 percent predicted), with these factors varying significantly with microbiota distribution. However, whilst such clinical measures are associated with the presence of *P. aeruginosa*, these analyses also show that a

235 significant relationship exists with the wider airway microbiota; a significant relationship was identified
236 here between the variance in core taxa and Leicester Cough Score (a measure of cough symptom
237 severity).

238 Here we observed distributions of predominant taxa consistent with strong interspecific
239 competition, supporting competitive exclusion in some instances. But we also observed, for instance,
240 that where *P. aeruginosa* is the numerically dominant species in a bronchiectasis lung infection it is
241 associated with a distinct accessory microbiota; suggesting in addition to interspecific competition
242 there are also direct and/or indirect interactions between the predominant species and the core
243 microbiota. To some extent, such an effect was also observed for *H. influenzae*, but was far less
244 pronounced. However, several different models could explain such interactions. For example, the
245 predominant species could influence the accessory microbiota composition through modification of the
246 airway environment and alteration of its selective properties (and vice versa); here, perhaps the fact
247 that *H. influenzae* is a common resident of the upper airways means that its presence is less
248 disruptive to the commonly occurring infective lower airway microbiota. Alternatively, the same change
249 in the characteristics of the airway could occur through intermediary interaction with the host that
250 results in an altered inflammatory profile, airway secretion composition, or secretion clearance rate.
251 Here, a wide array of virulence factors and pro-inflammatory traits possessed by *P. aeruginosa*
252 (Sadikot *et al*., 2005) may contribute to the magnitude of the effect of its predominance on the
253 accessory airway microbiota. Finally, external influences, such as antibiotic therapy, are likely to
254 contribute to selective pressures in the airway environment. While there was no significant difference
255 in historical antibiotic burden in the *H .influenzae*- and *P. aeruginosa*-dominated groups, the
256 contribution of more subtle differences in treatment history cannot be excluded. We suggest that no
257 single process is responsible for the associations observed, and rather a dynamic interaction between
258 many different factors give rise to the various types of microbiological scenario seen *in vivo*. The
259 potential complexity of these interactions makes their elucidation challenging. However, discerning
260 their basis is important given that a number of important clinical questions arise from the findings we
261 present here. These questions include; (1) Could accessory microbiota composition predict
262 subsequent *P. aeruginosa* predominance and its associated poor prognosis? (2) Could intervention
263 aimed at altering the characteristics of the airway environment, or the composition of the accessory
264 microbiota, reduce the likelihood of *P. aeruginosa* infection and predominance? (3) What are the

mechanisms to promote the dominance of *H. influenzae,* and as a consequence competitively exclude or suppress *P. aeruginosa*?

In each case, addressing these questions will require *in vitro* competition experiments between the two dominant bacterial species and members of the core microbiota that we have identified as having likely interactions with *P. aeruginosa* and *H. influenzae*. In addition, longitudinal sample sets that span both clinically and microbiologically important time periods will allow us to better understand the *in vivo* mechanisms that lead to predominance of *H. influenzae* or *P. aeruginosa* and its associated worse clinical outcomes. However, obtaining informative longitudinal sample sets in adult bronchiectasis has significant challenges. Intensive, long-term sample collection would be required to span rare and unpredictable events, such as the acquisition of *P. aeruginosa* in a condition that has relatively slow progression (Martínez-García *et al*., 2007) and is commonly idiopathic (Anwar *et al*., 2013). Given their potential to provide mechanistic insight into the relationship between recognised airway pathogens, the wider airway microbiota, host immunity, and clinical outcome, such long-term frequent sample collection represents an important next step.

In conclusion, we present evidence supporting the contribution of both interspecific competition, and direct and/or indirect interaction between predominant infective taxa and the wider bacterial community, to determining whether *H. influenzae* or *P. aeruginosa* dominates the chronic lung infections associate with bronchiectasis. Given the prognostic implications of *P. aeruginosa* dominance, these findings provide a basis for identifying the mechanisms that underpin this airway microbial ecology, and perhaps offering novel therapeutic opportunities.

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Conflicts of interest

The authors declare no conflict of interest.

Supplementary information is available at ISMEJ's website

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443 **Figure legends**

444 **Fig. 1.** Relative percentage abundances of *P. aeruginosa* (green circles) and *H. influenzae* (blue) in
445 samples from within *P. aeruginosa*- and *H. influenzae*-dominated groups (P1-P26 and H1-H34,
446 respectively).

447

448 **Fig. 2.** Canonical correspondence biplots for microbiota (a) with and (b) without *Pseudomonas* and
449 *Haemophilus* included. Dots represent microbiota samples from the *Pseudomonas* (denoted with
450 green filled circles) and *Haemophilus* (blue filled squares) groups. In each instance, the 95 %
451 concentration ellipses are given for the *Pseudomonas* (green) and *Haemophilus* (blue) groups. Biplot
452 lines for the clinical variables included in the analyses show the direction of increase for each variable,
453 and the length of each line indicates the degree of correlation with the ordination axes. CCA field
454 labels: "AH" – antihypertensive, "LABA/ICS" – long acting β-agonist, "SABA" – short acting β-agonist,
455 "prior IE" – number of pulmonary exacerbations in the prior twelve months. Percentage of community
456 variation explained by each axis is given in parentheses.

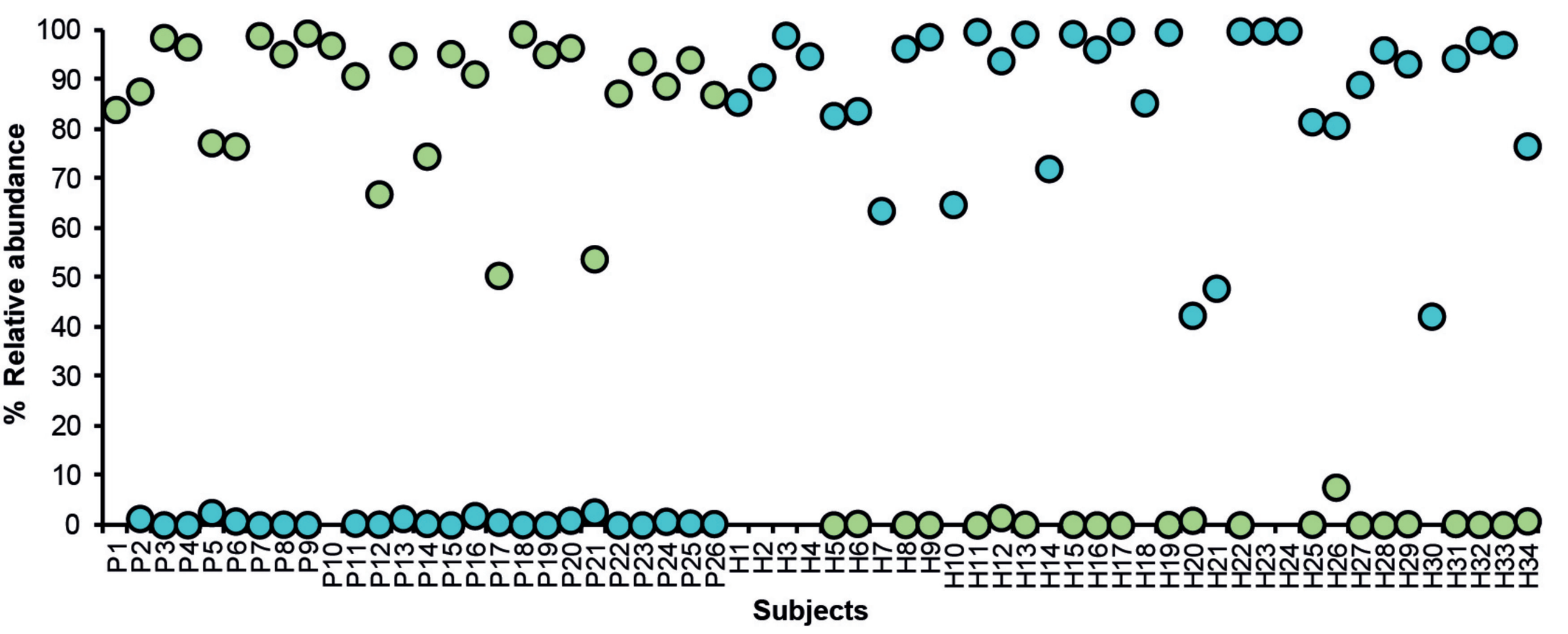
457

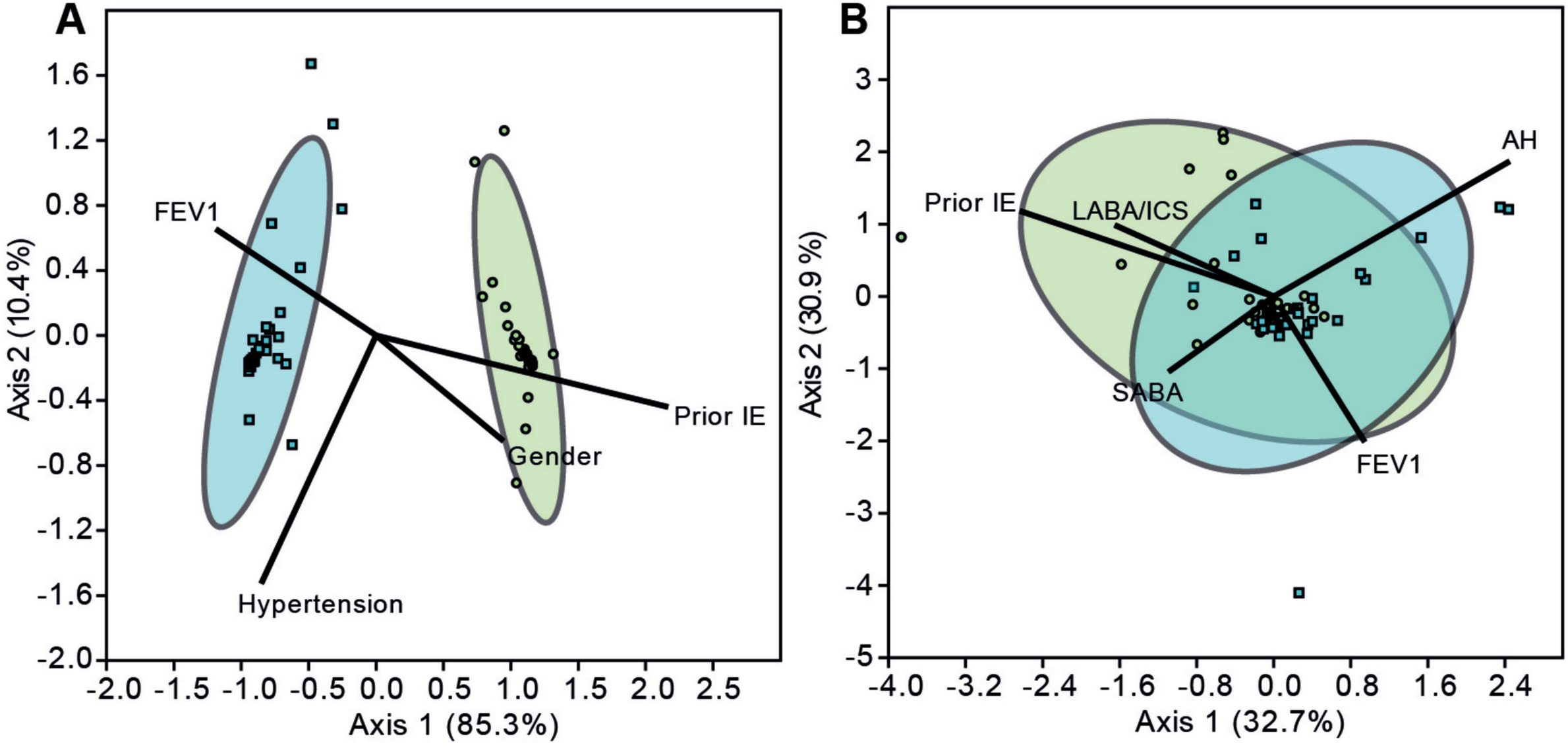
458 **Fig. 3.** Distribution and dispersal of bacterial taxa among *Haemophilus*- and *Pseudomonas*-dominated
459 microbiota samples. (a & b) The number of samples for which each detected bacterial taxon (open
460 circles) was observed, plotted against the abundance ($\log_{10}$ scale) of that species among all samples
461 within each group ((a) *Haemophilus* group, $r^2 = 0.27$, $F_{1,92} = 33.2$, $P < 0.0001$; and (b) *Pseudomonas*
462 group, $r^2 = 0.33$, $F_{1,68} = 33.5$, $P < 0.0001$). Also given are dispersal plots to identify which bacterial taxa
463 are randomly distributed within the (c) *Haemophilus* and (d) *Pseudomonas* groups; a measure used to
464 assign core versus satellite status. Index of dispersion was calculated as the ratio of variance to mean
465 of abundance for each taxon within each group and plotted for each sample. The line depicts the 2.5
466 % confidence limit for the χ^2 distribution. Taxa that fall below this line are randomly distributed and
467 were considered satellite taxa, whereas those that are above the line are non-randomly distributed
468 and were considered core taxa. The 97.5 % confidence limit was not plotted, as no taxon fell below
469 that line.

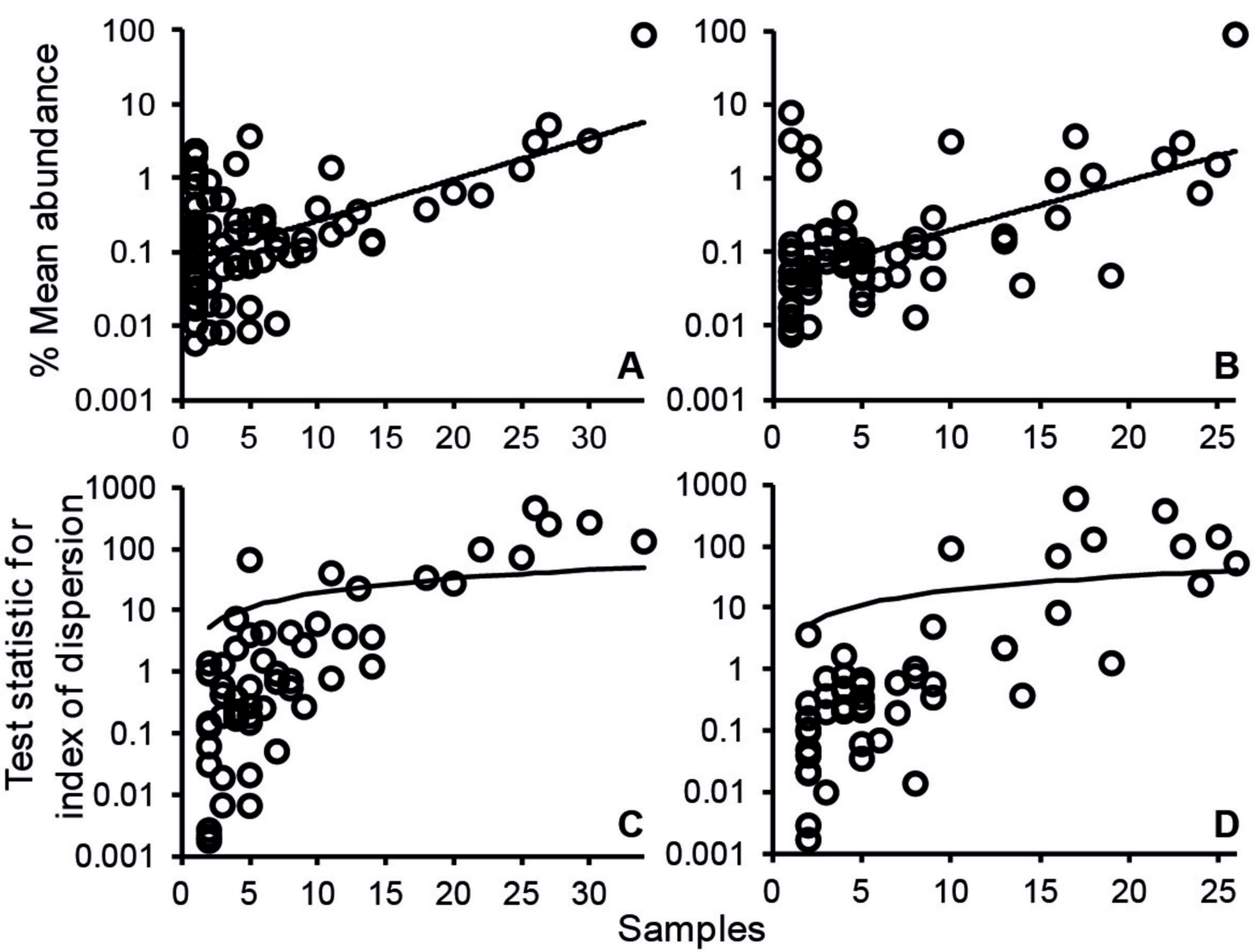
470

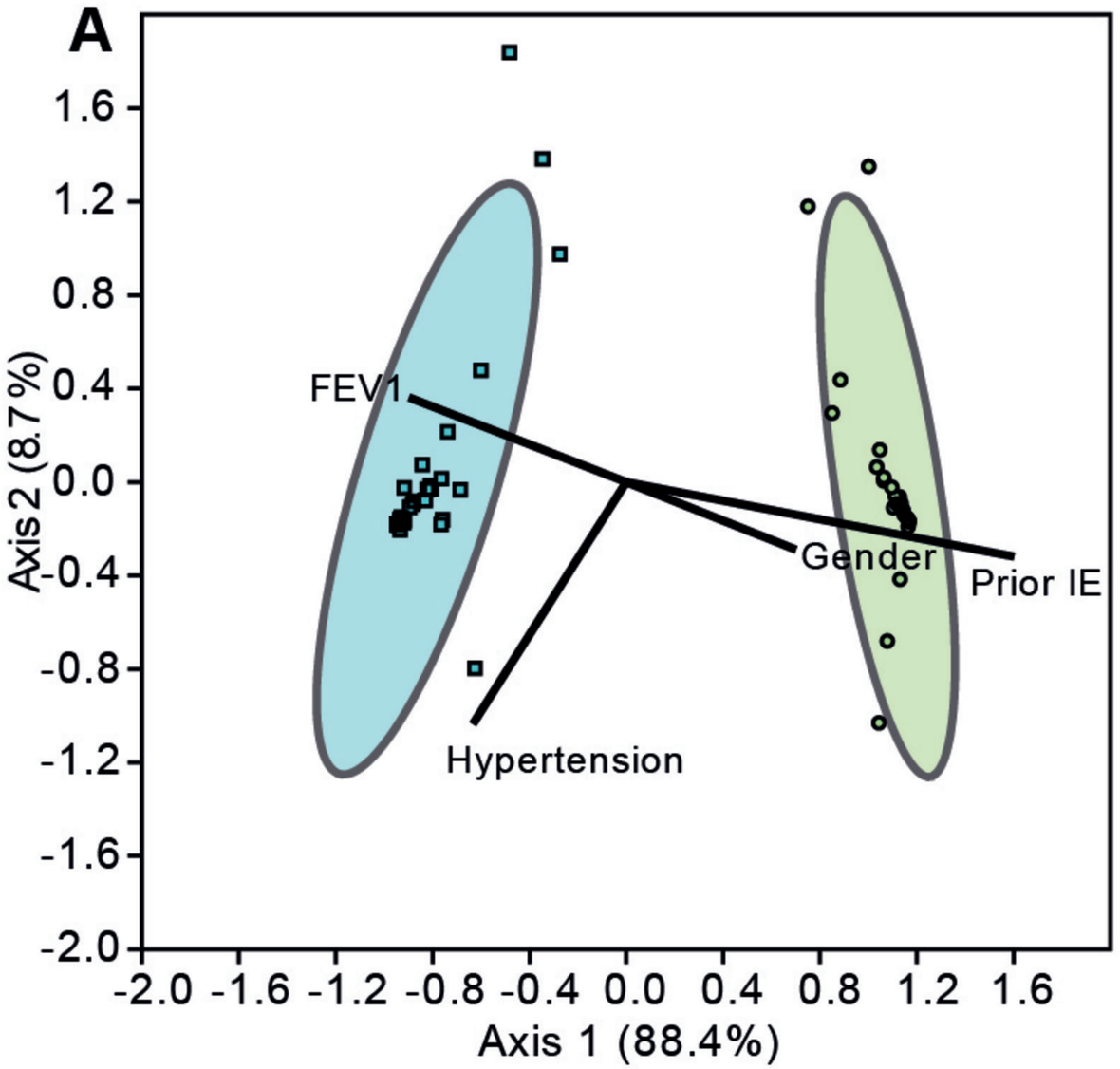
471 **Fig. 4.** Canonical correspondence biplots for core microbiota (a) with and (b) without *Pseudomonas*
472 and *Haemophilus* included. Dots represent core microbiota from the *Pseudomonas* (denoted with

473 green filled circles) and *Haemophilus* (blue filled squares) groups. In each instance, the 95 %
474 concentration ellipses are given for the *Pseudomonas* (green) and *Haemophilus* (blue) groups. Biplot
475 lines for the clinical variables included in the analyses show the direction of increase for each variable,
476 and the length of each line indicates the degree of correlation with the ordination axes. CCA field
477 labels: “AH” - antihypertensive, “LCS” – Leicester cough score, “prior IE” – number of pulmonary
478 exacerbations in the prior twelve months. Percentage of community variation explained by each axis is
479 given in parentheses.


% Relative abundance
100
90
80
70
60
50
40
30
20
10
0
P1 P2 P3 P4 P5 P6 P7 P8 P9 P10 P11 P12 P13 P14 P15 P16 P17 P18 P19 P20 P21 P22 P23 P24 P25 P26
H1 H2 H3 H4 H5 H6 H7 H8 H9 H10 H11 H12 H13 H14 H15 H16 H17 H18 H19 H20 H21 H22 H23 H24 H25 H26 H27 H28 H29 H30 H31 H32 H33 H34
Subjects


A
FEV1
Prior IE
Gender
Hypertension
Axis 2 (10.4%)
Axis 1 (85.3%)
1.6
1.2
0.8
0.4
0.0
-0.4
-0.8
-1.2
-1.6
-2.0
-2.0 -1.5 -1.0 -0.5 0.0 0.5 1.0 1.5 2.0 2.5
B
AH
Prior IE
LABA/ICS
SABA
FEV1
Axis 2 (30.9 %)
Axis 1 (32.7%)
3
2
1
0
-1
-2
-3
-4
-5
-4.0 -3.2 -2.4 -1.6 -0.8 0.0 0.8 1.6 2.4


% Mean abundance
Test statistic for index of dispersion
Samples
A
B
C
D
100
10
1
0.1
0.01
0.001
1000
0
5
10
15
20
25
30


A
1.6
1.2
0.8
0.4
0.0
-0.4
-0.8
-1.2
-1.6
-2.0
Axis 2 (8.7%)
FEV1
Gender
Prior IE
Hypertension
-2.0 -1.6 -1.2 -0.8 -0.4 0.0 0.4 0.8 1.2 1.6
Axis 1 (88.4%)

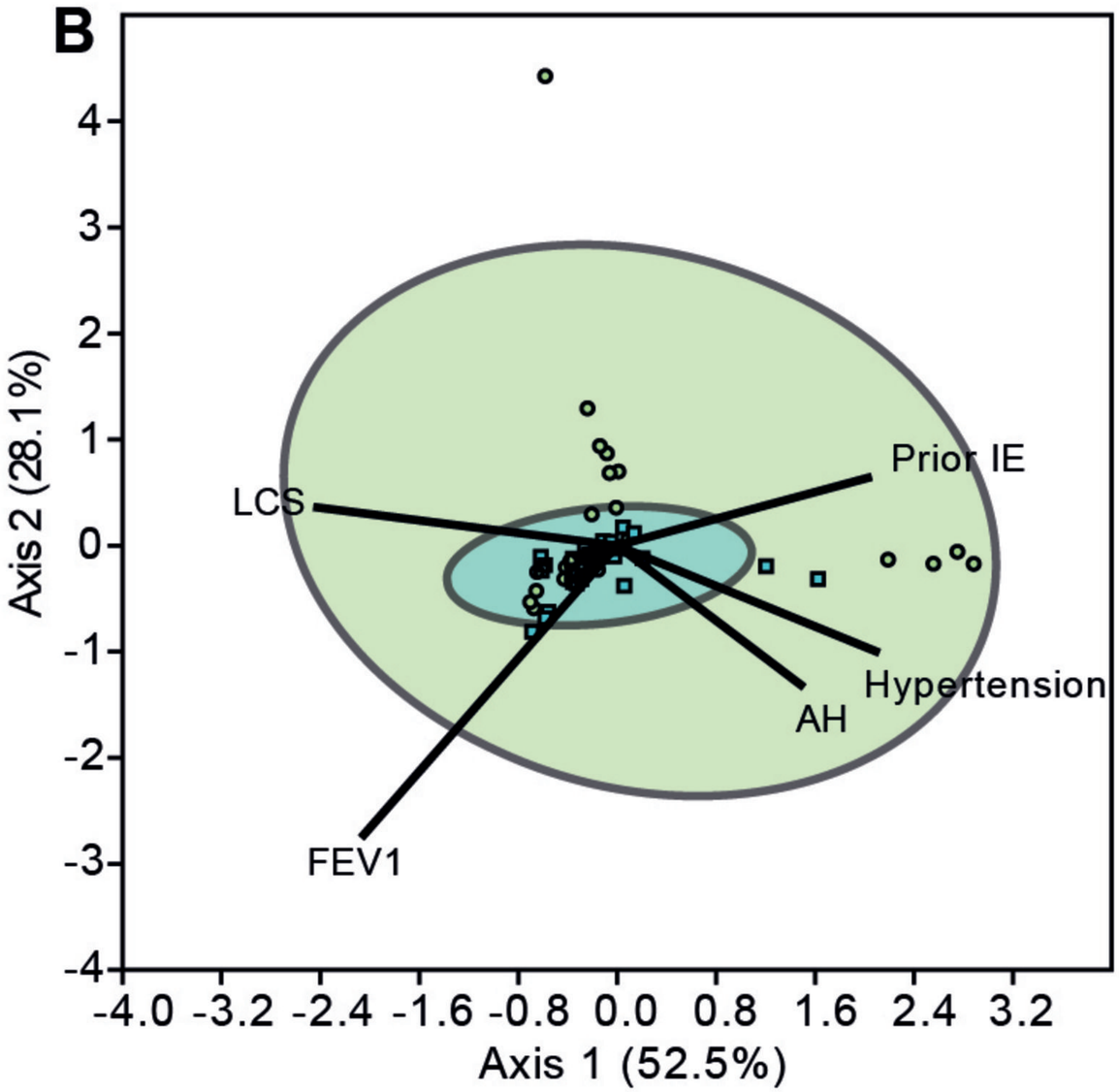

B
4
3
2
1
0
-1
-2
-3
-4
Axis 2 (28.1%)
LCS
Prior IE
Hypertension
AH
FEV1
-4.0 -3.2 -2.4 -1.6 -0.8 0.0 0.8 1.6 2.4 3.2
Axis 1 (52.5%)

Table 1. Clinical, treatment and comorbidity data for patient population. FEV_1%* – forced expiratory volume in one second, expressed as a percentage of predicted and measured following administration of a bronchodilator; SGRQ, St George's Respiratory Questionnaire – range 1-100, lower scores indicate better quality of life. Leicester cough score – lower scores indicate worse cough symptoms.

	P. aeruginosa		***H. influenzae***	
Gender (male:female)	6:20		16:18	
Clinical measures of disease	**Range**	**Mean (± SD)**	**Range**	**Mean (± SD)**
FEV1%*	30.7-94.2	60.5 (± 18.1)	37.2-114.7	71.2 (± 15.1)
Duration of bronchiectasis (years)	1-70	45.4 (± 19.6)	10-65	45.4 (± 17.5)
Pulmonary exacerbations in prior 12 months	2-12	6.3 (± 3.0)	2-7	3.4 (± 1.3)
Leicester cough score	5.5-20.2	14.8 (± 3.5)	7.7-19.9	15.6 (± 2.6)
SGRQ total	8.6-79.6	39.9 (± 16.6)	14.9-58.9	36.7 (± 14.0)
Six minute walk test	291-650	488.7 (± 85.1)	275-710	519.6 (± 101.6)
C-reactive protein (mg/litre)	0-21	6.8 (± 6.0)	0-19	7.1 (± 6.1)
Induced sputum IL-8 (ng/ml)	27.4-1053.8	337.6 (± 340.3)	28.8-1326.7	275.2 (± 295.7)
Induced sputum IL-1β (ng/ml)	0.25-36.2	9.8 (± 10.7)	0.6-115.8	11.4 (± 22.3)
Treatment				
Short acting β-agonist	14		10	
Inhaled corticosteroid	16		17	
Inhaled corticosteroid + long acting β-agonist	13		10	
Anti-cholinergics	6		2	
Aspirin	3		9	
Beta blocker	0		3	
Nasal steroids	2		4	
Prednisolone	3		1	
Antihypertensive	9		16	
Comorbidity				
Cerebrovascular disease	1		4	
Heart disease	2		4	
Hypertension	6		14	
Diabetes	1		0	

Table 2. Similarity of percentages (SIMPER) analysis of bacterial community dissimilarity (Bray-Curtis) between core taxa groups without *Pseudomonas* and *Haemophilus*. Given is mean % abundance of sequences for each taxon across the samples they were observed to occupy. Analysis was based on an average of 10647 sequences per sample (SD 5070, range 2395-28916). Also given is the average dissimilarity between samples (overall mean = 61.9%). Percentage contribution is the mean contribution divided by mean dissimilarity across samples. SIMPER analysis with *Pseudomonas* and *Haemophilus* is presented in Table S1.

Taxon	% Mean abundance		Samples detected in		Average dissimilarity	% Contribution	Cumulative %
	Pseudomonas group	*Haemophilus* group	*Pseudomonas* group	*Haemophilus* group			
Prevotella	31.9	31	23	27	14.96	24.17	24.17
Veillonella	20.8	26.7	25	30	11.85	19.14	43.31
Streptococcus	13.2	18.3	22	26	10.51	16.99	60.30
Moraxella	14	4.9	10	5	8.69	14.04	74.34
Neisseria	7.8	13.6	18	25	7.59	12.26	86.60
Flavobacterium	8.7	0	17	0	4.35	7.02	93.62
Leptotrichia	3.6	2.0	16	18	2.22	3.59	97.21
Fusobacterium	0	3.5	0	11	1.72	2.79	100

Table 3. Canonical correspondence analyses for determination of percent variation in lung microbiota from bronchiectasis subjects by clinical variables. LABA, long acting β-agonist; ICS, inhaled corticosteroid; SABA, short acting β-agonist.

	Whole microbiota		Whole microbiota without P & H		Core microbiota		Core microbiota without P & H	
Variable	% of variance	Probability	% of variance	Probability	% of variance	Probability	% of variance	Probability
Prior Exacerbations	11.35	0.01	2.35	0.02	13.8	0.01	2.7	0.01
FEV_1 % predicted	4.60	0.01	2.41	0.01	5.5	0.01	4.9	0.01
Hypertension	3.08	0.01	-	-	3.5	0.01	2.7	0.01
Gender	3.01	0.01	-	-	3.4	0.01	-	-
Anti-Hypertensive	-	-	2.20	0.01	-	-	2.3	0.01
LABA + ICS	-	-	1.81	0.02	-	-	-	-
SABA	-	-	1.54	0.02	-	-	-	-
Leicester cough score	-	-	-	-	-	-	3.1	0.02

Table S1. Similarity of percentages (SIMPER) analysis of bacterial community dissimilarity (Bray-Curtis) between core taxa groups with *Pseudomonas* and *Haemophilus*. Given is mean % abundance of sequences for each taxon across the samples they were observed to occupy. Analysis was based on an average of 10647 sequences per sample (SD 5070, range 2395-28916). Also given is the average dissimilarity between samples (overall mean = 61.9%). Percentage contribution is the mean contribution divided by mean dissimilarity across samples.

Taxon	% Mean abundance		Samples detected in				
	Pseudomonas group	*Haemophilus* group	*Pseudomonas* group	*Haemophilus* group	Average dissimilarity	% Contribution	Cumulative %
Pseudomonas	87.3	0.37	26	22	44.44	45.53	45.53
Haemophilus	0.56	86	24	34	43.93	45.01	90.54
Prevotella	2.61	4.14	23	27	2.51	2.57	93.11
Streptococcus	1.51	2.35	22	26	1.69	1.73	94.84
Veillonella	1.44	2.8	25	30	1.67	1.71	96.55
Flavobacterium	2.38	0	17	0	1.21	1.24	97.79
Moraxella	1.18	0.54	10	5	0.83	0.85	98.65
Neisseria	0.74	0.97	18	25	0.74	0.75	99.40
Leptotrichia	0.57	0.20	16	18	0.35	0.36	99.77
Fusobacterium	0	0.45	0	11	0.23	0.23	100

Supplementary Methods

Samples were collected from participants in the Bronchiectasis and Low-dosen Erythromycin Study (BLESS) trial (Serisier *et al*., 2013). Adult patients aged 20 to 85 years were eligible if they had bronchiectasis documented by high-resolution computed tomographic scan, at least 2 separate pulmonary exacerbations requiring supplemental systemic antibiotic therapy in the preceding 12 months, and daily sputum production. Participants were required to have been clinically stable for at least 4 weeks prior to enrolment (defined as no symptoms of exacerbation, no requirement for supplemental antibiotic therapy, and forced expiratory volume in the firs second of expiration [FEV_1] within 10% of best recently recorded value where available). Exclusion criteria included CF, current mycobacterial disease or bronchopulmonary aspergillosis, any reversible cause for exacerbations, maintenance oral antibiotic prophylaxis, prior macrolide use except short-term, changes to medications in the preceding 4 weeks, cigarette smoking within 6 months, and medications or comorbidities with the potential for important interactions with erythromycin. All participants required negative results from sputum mycobacterial cultures prior to randomization. The study was approved by the Mater Health Service human research ethics committee, and all participants provided written, informed consent.

Sample collection

Subjects were instructed to perform their usual chest physiotherapy regime on the morning of the sputum induction procedure. Prior to commencement of hypertonic saline inhalation, any spontaneous sputum expectorated was collected for standard culture. Sputum induction (SI) was performed after inhalation of 400 ug of albuterol, using 4.5% hypertonic saline nebulised from an ultrasonic nebuliser (output >1 mL/ min) for 20 minutes in 4 periods of 5 minutes each, according to the standardised protocol recommended by the European Respiratory Society taskforce [Paggiaro *et al*., 2002] Following mouth-rinsing and expectoration, sputum was collected following each nebulisation period, on each occasion preceded immediately by spirometry. The first sputum sample was refrigerated immediately following collection and frozen at -80 °C within an hour. A cold chain was maintained up until the point of DNA extraction.

DNA extraction protocol

Nucleic acid extractions were performed on 500 µL cell suspensions. Guanidinium thiocyanate–EDTA–sarkosyl (500 µL) and PBS (500 µL), pH 8.0, were added to samples. Cell disruption was achieved using a Fastprep-24 Instrument (MP Biomedicals Europe, Illkirch, France) 6.5 m/s, 60 s, followed by incubation at 90 °C for 1 min and −20 °C for 5 min. Cell debris was pelleted by centrifugation at 12 000 × g for 2 min at 4 °C. Supernatant was transferred to a fresh microfuge tube. NaCl (to a final concentration of 0.5 mol/L and polyethylene glycol (to a final concentration of 15%) were added and DNA precipitated at 4 °C for 30 min. DNA was pelleted by centrifugation at 12 000 × g for 2min at 4 °C and resuspended in 300 µL of sterile distilled water. Samples were heated at 90 °C for 30 s and vortexed. Phenol/chloroform (1:1) (300 µL) was added, and samples were vortexed for 20 s before centrifugation at 12 000 × g at 4 °C for 3min. The upper phase was then transferred to a fresh microfuge tube. Total DNA was then precipitated by the addition of an equal volume of isopropanol, a 0.1-volume 10 mol/L ammonium acetate, and 1 µL of GenElute linear polyacrylamide (Sigma-Aldrich, Gillingham, UK) and incubated at −20 °C for 25 min. DNA was pelleted by centrifugation at 12 000 × g at 4 °C for 5 min. Pelleted DNA was then washed 3 times in 70% ethanol, dried, and resuspended in 50 µL of sterile distilled water. DNA extracts were quantified using the Picodrop Microlitre Spectrophotometer (GRI, Braintree, UK). Negative controls, consisting of sterile water, were included in the PMA treatment, DNA extraction, and PCR amplification steps.

Pyrosequencing

Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) was performed as described previously using Gray28F 5'-TTTGATCNTGGCTCAG-3' and Gray519r 5'-GTNTTACNGCGGCKGCTG-3'). Initial generation of the sequencing library involved a one-step PCR of 30 cycles, using a mixture of Hot Start and HotStar high fidelity Taq DNA polymerase, as described previously (4). Tag-encoded FLX amplicon pyrosequencing analyses utilized Roche 454 FLX instrument with Titanium reagents, titanium procedures performed at the Research and Testing Laboratory (Lubbock, TX) using RTL protocols (www.researchandtesting.com).

Following sequencing, all failed sequence reads, low quality sequence ends and tags and primers were removed. Sequences with ambiguous base calls, sequences with homopolymers > 6bp were removed. Further, any non-bacterial ribosomal sequences and chimeras using B2C2 (Dowd *et al.*, 2008) as described previously (Dowd *et al.*, 2008). To determine the identity of bacterial species in the

remaining sequences, sequences were de-noised, assembled into OUT clusters at 97% identity, and queried using a distributed .NEt algorithm that utilizes Blastn+ (KrakenBLAST www.krakenblast.com) against a database of high quality 16S rRNA gene bacterial sequences. Using a .NET and C# analysis pipeline the resulting BLASTn+ outputs were compiled, data reduction analysis performed, and sequence identity classification carried out, as described previously (Dowd *et al*., 2008).

Quantitative PCR

P. aeruginosa density was determined using a Taqman assay, in which a 117 bp region between positions 330 to 447 of the *P. aeruginosa* OprL gene was amplified, as described previously (Feizabadi *et al*., 2010). Primers (PsF: 5'-CGAGTACAACATGGCTCTGG-3', EubR: 5'-ACCGGACGCTCTTTACCATA-3') were used at a concentration of 500 nM each, and the probe (EubPr: 5'-FAM- CCTGCAGCACCAGGTAGCGC -TAMRA-3') at a concentration of 250 nM. All reactions were carried out in a total volume of 20 µl containing primers at a concentration of 500 nM each, probe concentration of 250 nM, 1 µl of template and LightCycler 480 Probes Master (Roche Diagnostics GmbH, Mannheim, Germany) at 1x final concentration. Quantitative PCR assays were carried out using the Rotor-Gene Q (Qiagen, Crawley, UK) with a temperature profile of 95 °C for 5 min, followed by 45 cycles at 95 °C for 15 s and 58 °C for 45 s. Nutrient broth culture of *P. aeruginosa* (NCTC 12934/ATCC 27853) was incubated at 37 °C for 16 h, with cfu/ml estimated by incubation of dilutions (n=4) on Nutrient agar at 37° for 24 h, followed by colony counts. DNA was extracted from tenfold dilutions of the broth culture in the same way as for the sputum samples, and RT-PCR was carried out as above on the DNA extracts. The standard curve generated using *P. aeruginosa* (qPCR efficiency = 1.20; R^2 value = 0.996) was used as reference to allow direct comparisons to be made by the Rotor Gene Q-series Software (Qiagen, Crawley, UK).

H. influenzae density was determined using a Taqman assay, in which a 90-bp region between positions 518 to 608 of the *H. influenzae* Hel gene was amplified, using primers (HelSF: 5'-CCGGGTGCGGTAGAATTTAATAA-3', EubR: 5'-CTGATTTTTCAGTGCTGTCTTTGC-3') were used at a concentration of 100 nM each, and the probe (EubPr: 5'-FAM-ACAGCCACAACGGTAAAGTGTTCTACG-TAMRA-3') (Long, 2011). All PCR reactions were carried out in a total volume of 20 µl containing primers and probe at a concentration of 1000:500:200 nM (HelSF: HelSR; HelSPr), 1 µl of template and LightCycler 480 Probes Master (Roche Diagnostics

GmbH, Mannheim, Germany) at 1x final concentration. Quantitative PCR assays were carried out using the Rotor-Gene Q (Qiagen, Crawley, UK) with a temperature profile of 95 °C for 5 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 60 s. A standard curve was generated by qPCR settings as above using *H. influenzae* DNA extract obtained from the Health Protection Agency (*H. influenza* clinical isolate, chronic respiratory infection, 2012). *H. influenza* load (cfu/ml) was determined by direct comparison with the standard curve (qPCR efficiency = 1.05; R^2 value = 0.997) using the Rotor Gene Q-series Software (Qiagen, Crawley, UK).

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