



Impact of prophylactic and 'rescue pack' antibiotics on the airway microbiome in chronic lung disease

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To cite: Rofael SAD, Brown J, Lipman MCI, *et al.* Impact of prophylactic and 'rescue pack' antibiotics on the airway microbiome in chronic lung disease. *BMJ Open Resp Res* 2023;**10**:e001335. doi:10.1136/bmjresp-2022-001335

► Additional supplemental material is published online only. To view, please visit the journal online (<http://dx.doi.org/10.1136/bmjresp-2022-001335>).

Received 14 June 2022
Accepted 24 March 2023



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ABSTRACT

The management of many chronic lung diseases involves multiple antibiotic prescriptions either to treat acute exacerbations or as prophylactic therapy to reduce the frequency of exacerbations and improve patients' quality of life.

Aim To investigate the effects of antibiotics on the homeostasis of bacterial communities in the airways, and how this may contribute to antimicrobial resistance (AMR) among respiratory pathogens and microbiota.

Methods Within an observational cohort study, sputum was collected from 84 patients with chronic obstructive pulmonary disease and/or bronchiectasis at stable state: 47 were receiving antibiotic prophylaxis therapy. V3-V4 16S-rRNA sequencing on Illumina MiSeq, quantitative PCR for typical respiratory pathogens, bacteriology cultures and antimicrobial susceptibility testing of sputum isolates, resistome analysis on a subset of 17 sputum samples using MinION metagenomics sequencing were performed.

Finding The phylogenetic α -diversity and the total bacterial density in sputum were significantly lower in patients receiving prophylactic antibiotics ($p=0.014$ and 0.029 , respectively). Antibiotic prophylaxis was associated with significantly lower relative abundance of respiratory pathogens such as *Pseudomonas aeruginosa*, *Moraxella catarrhalis* and members of family Enterobacteriaceae in the airway microbiome, but not *Haemophilus influenzae* and *Streptococcus pneumoniae*. No major definite directional shifts in the microbiota composition were identified with prophylactic antibiotic use at the cohort level. Surveillance of AMR and resistome analysis revealed a high frequency of resistance to macrolide and tetracycline in the cohort. AMR expressed by pathogenic bacterial isolates was associated with antibiotics prescribed as 'rescue packs' for prompt initiation of self-treatment of exacerbations (Spearman's $\rho=0.408$, $p=0.02$).

Conclusions Antibiotic prophylactic therapy suppresses recognised pathogenic bacteria in the sputum of patients with chronic lung disease. The use of antibiotic rescue packs may be driving AMR in this cohort rather than prophylactic antibiotics.

INTRODUCTION

Chronic lung diseases such as bronchiectasis and chronic obstructive pulmonary disease (COPD) are associated with both a

WHAT IS ALREADY KNOWN ON THIS TOPIC

- ⇒ In the management of chronic lung diseases, substantial amounts of antibiotics are prescribed either as prophylaxis or treatment of acute exacerbations.
- ⇒ Substantial scientific evidence supports the clinical usefulness of prophylactic antibiotic use, especially macrolides which can reduce the rate of exacerbations.
- ⇒ A key question is which of these strategies leads to a greater risk of altering the microbiome and/or the development of antimicrobial resistance.

WHAT THIS STUDY ADDS

- ⇒ We found that antibiotic prophylaxis suppressed common pathogenic bacteria such as *Pseudomonas aeruginosa* in sputum from patients with chronic lung diseases; however, use of antibiotic rescue packs to self-treat exacerbations may be associated with antimicrobial resistance in our cohort.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- ⇒ The potential impact of prescribed antibiotic rescue packs is concerning and requires review, especially in patients with frequent exacerbations who might benefit more from antibiotic prophylaxis.

considerable socioeconomic burden and impacts on patients' lives.¹ Many people with chronic lung conditions also periodically experience intermittent acute deteriorations in respiratory health (exacerbations) which cause significant morbidity, impact significantly on quality of life and necessitate a change in regular medication.^{2 3} Frequent exacerbations have been associated with progressive lung damage, faster decline in lung function and worse quality of life.^{4 5}

Guidelines for bronchiectasis and COPD recommend that patients have a self-management plan (SMP) in place for acute exacerbations. Within these SMPs, antibiotics are often prescribed and kept at home by patients as a 'rescue pack' to allow for prompt start of antibiotic treatment.^{2 3}

Reducing the rate of exacerbations has been a major aim of patient management.⁶ Evidence from systematic reviews and meta-analyses of randomised placebo-controlled clinical trials has shown the clinical usefulness of prophylactic antibiotics in reducing the frequency of exacerbations in COPD^{7,8} and bronchiectasis.^{9,10} Azithromycin specifically has very good penetration into respiratory tissues, and a long serum half-life (up to 60 hours), permitting once-daily and even thrice-weekly dosing, with a characteristic postantibiotic effect¹¹—making it the macrolide of choice.

Nevertheless, the adverse effects of long-term use of antimicrobials in chronic respiratory disease has not been fully investigated. Concern exists that the selection pressure on microbiota from a prolonged antibiotic regimen may alter host microbial homeostasis. In addition, there is the risk of AMR emergence in human microbiota, which may act as a reservoir for antimicrobial resistance dissemination into the wider population.¹² Given that one aim of prophylactic antibiotics is to reduce the need and use of acute ‘rescue’ antibiotics in people with chronic lung diseases, a key question is which of these strategies leads to a greater risk of altering the microbiome and/or the development of antimicrobial resistance?

Here, we investigated the impact of prolonged antibiotic prophylactic therapy on the airway microbiome in patients with chronic lung disease, and the influence of prophylactic antibiotics and acute ‘rescue packs’ on the resistome and AMR in sputum bacterial isolates.

METHODS

Study design and population

Participants with diagnosis of bronchiectasis (on CT) or COPD (on spirometry) were recruited between February 2017 and February 2018, participants were followed-up for 12 months. This was a convenience sample. The inclusion criteria were: age ≥ 20 years, confirmed diagnosis of COPD and/or bronchiectasis, history of frequent exacerbations (≥ 2 /year), ability to spontaneously expectorate sputum and consent to participate. The exclusion criteria were diagnosis of cystic fibrosis or lung cancer, history of lung transplantation, known tuberculosis or HIV infections at the time of recruitment and clinical instability (sputum collected within 1 week before and 2 weeks after antibiotic treatment of an exacerbation or any other acute infection was excluded in the presented work). Clinical data collection included interviewing participants, and reviewing medical records and daily diary cards filled by participants reporting changes in the symptoms and treatment.

Patient and public involvement

Our research proposal was discussed with academics, physicians and students at University College London and with a sample of patients and their families/carers in pulmonary rehabilitation programme held in Peckwater Centre, London prior to submitting the ethics

application. We adjusted the design in the light of their comments. Feedback on the study’s procedure was obtained from participants during clinic visits. Any concerning findings in sputum microbiology results were communicated with the treating physician.

Sputum processing and bacteriology cultures

Details on the methods are provided in online supplemental data. Sputum samples were processed with Sputasol (Oxoid, UK), comprehensive bacteriology cultures were obtained by plating sputum on Columbia agar with chocolate horse blood, Columbia colistin-nalidixic acid agar and cystine-lactose-electrolyte-deficient agar. All morphologically distinct colonies on the three cultures were isolated, purified and identified using matrix-assisted laser desorption/ionisation-time-of-flight (MALDI-TOF) mass spectrometry. Antimicrobial susceptibility of bacterial isolates was determined using the Kirby-Bauer agar diffusion technique according to the standards of the European Committee on Antimicrobial Susceptibility Testing (EUCAST).¹³

DNA extraction

DNA was then extracted from 1 mL sputum samples that had been centrifuged, pellet heated at 95°C for 30 min, then mechanically disrupted by bead-beating step, on the automated LIAISON Ixt extraction platform using DiaSorin Arrow-DNA extraction kit.

The total bacterial density in sputum indicated by the number of copies of the 16S rRNA gene per μL ; in addition, the bacterial loads of *Pseudomonas aeruginosa*,¹⁴ and *Haemophilus influenzae*, *Moraxella catarrhalis* and *Streptococcus pneumoniae*¹⁵ were quantified using two multiplex quantitative PCR (qPCR) Taq-Man assays on a Qiagen Rotor-gene 6000 machine (online supplemental table S1).¹⁶ A mean of technical triplicates was taken for each sample. An internal amplification control, SPUD A, was used to test for PCR inhibition.

16S rRNA profiling

A sequence library was created by amplification of V3-V4 regions of the bacterial 16S rRNA gene using 341 forward-primer and 805 reverse-primer. The PCR products (584 bp amplicons) were cleaned using Agencourt AMPure XP beads (Beckman Coulter, UK). The samples were pooled in an equimolar ratio at 8 nM into one library which was checked by TapeStation (Agilent, USA). Sequencing was performed using Illumina MiSeq Platform using costume sequencing primers, and MiSeq Reagent Kit v2 (500 cycles).¹⁷

An extraction negative control and a no-template PCR control (water) were run alongside each batch of samples throughout the process as negative controls. A laboratory-prepared mock community was run as a positive control.

We adopted the workflow established by Microbiome helper¹⁸ using QIIME pipeline V.1.9.1.¹⁹ The appropriate

statistical significance tests were calculated using QIIME wrapper scripts. STAMP (V.2.1.3)²⁰ was used to visualise the results and testing the differential abundances of taxa between the two study groups using White's non-parametric t-test.²¹ All p values were corrected using Benjamini-Hochberg false discovery rate method for multiple comparisons. The statistical analysis was performed using IBM SPSS V.25.0.²²

Resistome analysis

Metagenomic sequencing was carried out on a subset of 17 sputum samples: 8 were from patients with bronchiectasis on prophylactic antibiotic therapy and 9 from the comparator group, using Oxford Nanopore MinION system. Human DNA was depleted as per the published method by Charalampous *et al.*²³ Metagenomics libraries of six multiplexed samples were prepared using the Rapid Barcoding Kit (Ref: SQK-RLB004, ONT). Sequencing was performed on MinION flow cell (R.9.4.1) for 48 hours. The genomes were assembled using the miniasm/minimap pipeline.²⁴ A Basic Local Alignment Search Tool (BLAST) search against the ResFinder database performed²⁵ to detect AMR genes. The alignments with accuracy <90% have been excluded. The prevalence of AMR genes within the samples was measured in parts per million reads (ppm), that is, the number of sequence reads identified as AMR genes relative to the total number of reads representing the sample.

RESULTS

The clinical and demographic characteristics of patients who were and were not on prescribed antibiotic prophylaxis therapy are shown in table 1. Forty-seven of 84 participants were prescribed antibiotic prophylaxis therapy for over a year, in the context of their routine medical care; in 66% these were macrolides, specifically, 29 patients were on azithromycin 250 mg thrice weekly and 8 on 500 mg thrice weekly; 2 were on clarithromycin (250 mg twice daily). Other prescribed antibiotics were co-trimoxazole (960 mg once daily or 1920 mg, divided into two doses, thrice weekly), ciprofloxacin (250 mg twice daily), doxycycline (100 mg once daily), amoxicillin (250 mg twice daily), cephalexin (500 mg twice daily) and phenoxymethyl penicillin (250 mg twice daily).

There were no significant differences in the proportions of patients with COPD and bronchiectasis in the two groups ($p=0.157$). All participants were regularly receiving the influenza vaccine and had pneumococcal vaccine. Lung function as indicated by the spirometry results was comparable between the two study groups (table 1).

A significantly higher proportion of patients with common variable immunodeficiency (CVID) were on antibiotic prophylaxis therapy (66%), compared with seven in the comparator group (19%) ($p<0.0001$); 90% of the patients with CVID were on immunoglobulin replacement therapy.

Seventy-nine per cent of the participants were prescribed a rescue pack of antibiotics to keep at home as an SMP; 47% of the rescue packs contained amoxicillin/clavulanic acid, 20% doxycycline, 14% quinolones (either ciprofloxacin, levofloxacin or moxifloxacin), 15% amoxicillin and 7.5% had macrolides either azithromycin or clarithromycin.

For comparing the microbiome of patients who were and were not receiving antibiotic prophylaxis therapy, one sputum sample per participant was selected to represent the microbiome profile at stable state. To avoid biases due to exacerbations and antibiotic treatment, sputum samples collected within 1 week before and 2 weeks after exacerbation and/or breakthrough antibiotic treatment were excluded.

The total bacteria density and the phylogenetic α -diversity were significantly lower in the sputum collected from patients on prophylactic antibiotics in comparison with those who were not (figure 1). The median 16S rRNA gene copies (IQR) was 6.20 (5.54–6.73) \log_{10} copies/ μ L in the antibiotic prophylaxis group vs 6.60 (6.17–6.92) \log_{10} copies/ μ L in the comparator group ($p=0.029$) (figure 1A). The difference of means of the phylogenetic α -diversity index PD whole tree in the two populations (SD) was 1.4 (0.56), $p=0.014$. This was despite the greater proportion of patients with bronchiectasis in the prophylactic antibiotic group (table 1) and the fact that the total bacterial density was significantly higher in the patients with bronchiectasis compared with those with COPD, where the median 16S rRNA gene copies (IQR) were 6.63 (6.18–6.96) vs 6.18 (5.41–6.88) \log_{10} copies/ μ L ($p=0.042$), respectively (figure 1B).

Antibiotic prophylaxis was the only significant covariate in weighted-UniFrac β -diversity index ($p=0.038$ by permutational multivariate analysis of variance (PERMANOVA)) and unweighted-UniFrac β -diversity index ($p=0.012$ by PERMANOVA) (online supplemental data, online supplemental figure S1). Chronic lung disease (whether bronchiectasis or COPD) was a significant covariate in unweighted-UniFrac β -diversity index ($p=0.01$ by PERMANOVA) but not in weighted-UniFrac β -diversity index ($p=0.152$ by PERMANOVA). Primary immunodeficiency status (CVID) was not a significant covariate in both weighted and unweighted-UniFrac β -diversity indices ($p>0.05$ by PERMANOVA) (online supplemental data, online supplemental figure S2).

The bacterial community structure was shifted towards more *Proteobacteria* ($p=0.041$) in patients with bronchiectasis while in patients with COPD it was shifted towards more *Firmicutes* ($p=0.048$). *Bacteroidetes* was slightly lower in patients with COPD and was significantly associated with airflow obstruction ($p=0.049$) (online supplemental figures S3 and S4).

The microbiome profiles demonstrated that the microbiota composition in sputum was similar in patients who did or did not use prophylactic antibiotics at the phylum level. Only the phylum *Synergistetes*, which is represented by a minor taxon present at relative abundance (RA) of

**Table 1** Demographic and clinical characteristics of participants

		On prophylactic antibiotic treatment (n=47)	Comparator group (n=37)	P value
Prophylactic antibiotics*				
Macrolides	Azithromycin	29 (62%)		
	Clarithromycin	2 (4%)		
Quinolones	Ciprofloxacin	2 (4%)		
Tetracyclines	Doxycycline	4 (9%)		
β-Lactams		3 (6%)		
Sulfonamides	Co-trimoxazole	7 (15%)		
Age† (years)		67 (12)	62 (19)	0.39‡
Sex*	Males	22 (47%)	18 (49%)	0.87§
BMI (kg/m ²)†		27.79 (4.40)	27.48 (9.14)	0.30‡
Chronic lung disease*				
	Bronchiectasis	37 (79%)	24 (65%)	0.16§
	COPD	10 (21%)	13 (35%)	
CVID*		31 (66%)	7 (19%)	<0.001§
Smoking*	Smokers	1 (2%)	5 (14%)	0.14¶
	Ex-smokers	11 (23%)	9 (24%)	
	Never-smokers	35 (74%)	23 (62%)	
Passive smoking*		10 (63%)	12 (60%)	0.88§
Number of exacerbation events/year**		2 (2–3)	3 (2–5)	0.18‡
Frequency of rescue pack consumption/year**		2 (2–3)	2.5 (2–4)	0.20‡
Rescue pack*		42 (89%)	24 (65%)	0.007§
Rescue pack antibiotics*				
β-Lactams	Co-amoxiclav	23 (49%)	8 (22%)	
	Amoxicillin	3 (6%)	7 (19%)	
FQ	Ciprofloxacin, levofloxacin, moxifloxacin	7 (15%)	2 (5%)	
Tetracyclines	Doxycycline	6 (13%)	7 (19%)	
Macrolides	Azithromycin, clarithromycin	3 (6%)	2 (5%)	
Rescue pack corticosteroids*		9 (19%)	5 (14%)	0.025§
Prescribed CIP course for <i>Pseudomonas aeruginosa</i> infection*		11 (23%)	7 (19%)	0.62§
Oral corticosteroids (<10 mg/day)*		10 (21%)	5 (14%)	0.36§
Carbocisteine*		10 (21%)	7 (19%)	0.79§
Oxygen therapy*		4 (9%)	3 (8%)	1.00¶
Inhaled respiratory medication*				
SABA		16 (34%)	16 (43%)	0.39§
LABA		19 (40%)	16 (43%)	0.80§
LAMA		9 (19%)	13 (35%)	0.10§
ICS*		21 (45%)	17 (46%)	0.91§
FEV1 (L)**		1.96 (1.06–2.91)	1.52 (0.94–2.72)	0.37‡
FEV1 % predicted**		80.5% (43.8%–109%)	75% (46%–92%)	0.56‡
FVC (L)**		2.99 (2.31–3.59)	2.29 (1.72–3.72)	0.22‡
FVC % predicted**		105.9% (70.8%–122%)	91% (73%–122%)	0.45‡

Continued

Table 1 Continued

	On prophylactic antibiotic treatment (n=47)	Comparator group (n=37)	P value
FEV1/FVC**	0.73 (0.50–0.80)	0.67 (0.51–0.74)	0.58‡

*N (%).

‡Mean (SD).

‡P value by Mann-Whitney U test.

§P value by χ^2 test.

¶P value by Fisher's exact test.

**Median (IQR).

BMI, body mass index; CIP, Ciprofloxacin; COPD, chronic obstructive pulmonary disease; CVID, common variable immunodeficiency; FEV1, forced expiratory volume in 1 s; FQ, fluoroquinolones; FVC, forced vital capacity; ICS, Inhaled Corticosteroids; LABA, Long-Acting Beta-2 Agonists; LAMA, Long-Acting Muscarinic receptor Antagonists; SABA, Short-Acting Beta-2 Agonists.

<0.5% in all samples, was significantly less abundant in the patients on prophylactic antibiotic therapy ($p=0.002$) (figure 2). At the genus level, the major taxa constituting the microbiome profiles and representing the core respiratory microbiota were similar in the patients who did or did not use prophylactic antibiotics (figure 3). Nevertheless, potentially pathogenic taxa were significantly less abundant in patients on prophylactic antibiotic therapy including: *Pseudomonas* ($p=0.027$), Enterobacteriaceae ($p=0.021$), *Klebsiella* ($p=0.046$), *Pasteurella* ($p=0.012$) and *Morganella* ($p<0.0001$). The genus *Moraxella* tended to be less abundant in patients on prophylactic antibiotic therapy; however, the observed difference did not reach

statistical significance ($p=0.087$) (figure 4). Some minor taxa with RA <1% such as *Bacteroidetes* ($p=0.02$), *Schwartzia* ($p=0.001$) and *Sphaerochaeta* ($p=0.01$) were also significantly lower in the antibiotic prophylaxis group.

qPCR confirmed that *P. aeruginosa* load was significantly suppressed in the sputum of patients on antibiotic prophylaxis; the median load (IQR) was 2.89 (2.62–3.64) \log_{10} CFU/mL vs 7.23 (3.44–7.95) \log_{10} CFU/mL in those who were not ($p=0.001$), even though *P. aeruginosa* was more frequently detected in the antibiotic prophylaxis group; 61% vs 44% in the comparator group (figure 5D). *M. catarrhalis* was significantly less prevalent in the sputum of patients on antibiotic prophylaxis (4%) compared with 20% in those who were not ($p=0.039$). However, when detected, the load of *M. catarrhalis* was no different between the two groups (figure 5A,C). The prevalence and loads of both *H. influenzae* and *S. pneumoniae* were similar in both groups.

Co-existence of two respiratory pathogens was detected in 39% of the examined sputum samples (figure 5B).

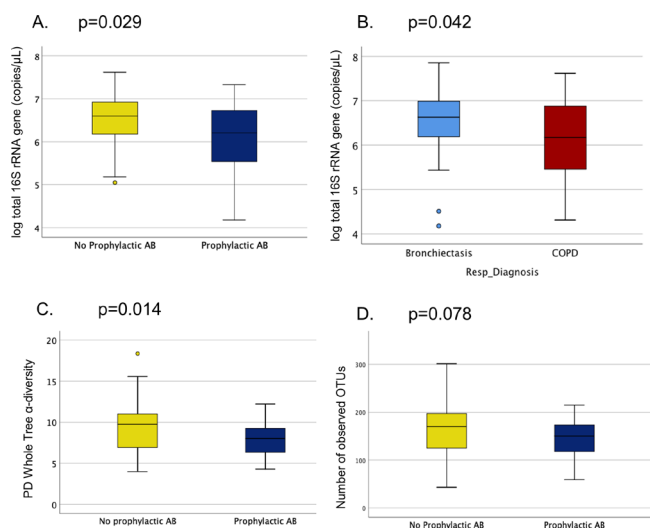


Figure 1 The total bacterial density, α -diversity and richness of airway bacterial community in patients with prophylactic antibiotics (AB) (n=47, navy blue) and those who were not (n=37, yellow). (A) Total number of 16S rRNA copies (a proxy to total bacterial density in sputum) was significantly lower in the prophylactic antibiotic group ($p=0.029$); however, (B) it was significantly higher in the patients with bronchiectasis (sky blue, n=50) compared with patients with chronic obstructive pulmonary disease (COPD) (red, n=23) ($p=0.042$). (C) α -Diversity expressed by phylogenetic diversity index: PD whole tree ($p=0.014$) and (D) richness expressed by total number of observed Operational Taxonomic Units (OTUs) ($p=0.078$).

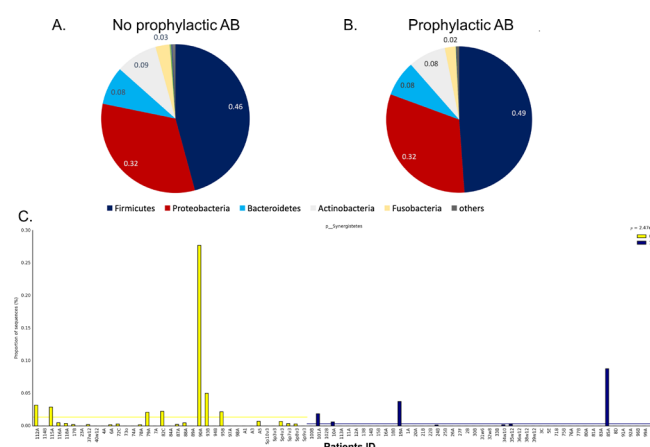


Figure 2 Comparison between the average airway microbiome profile of patients not receiving antibiotic (AB) prophylaxis (comparator group) (A) and those on prophylactic AB therapy (B) at the phylum level ($p>0.05$); (C) the relative abundance of phylum *Synergistetes* was significantly lower in patients receiving antibiotic prophylaxis therapy ($p=0.00247$); yellow: comparator group, navy blue: AB prophylactic group.

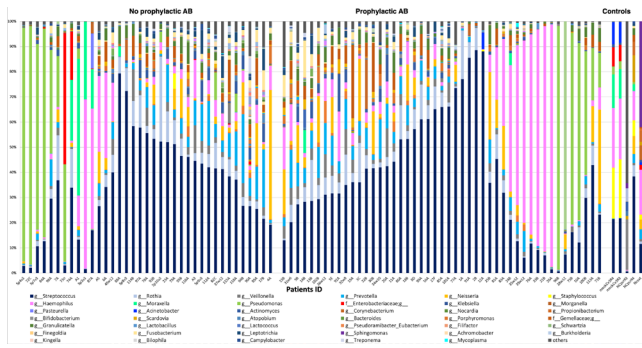


Figure 3 Airway microbiome profiles of patients with chronic lung disease who were receiving antibiotic (AB) prophylaxis therapy in comparison with patients in the comparator group; not receiving antibiotic prophylaxis; profiles of the Mock community resulted from two sequencing runs R3 and R4. f, family; g, genus; NC, no template negative control; NCext, extraction negative control.

The most common combinations were *P. aeruginosa*+*H. influenzae* (12%) and *H. influenzae*+*S. pneumoniae* (9%). The co-existence of these three pathogens was detected in 7% of samples. Four pathogens were detected in one patient with bronchiectasis who was not receiving antibiotic prophylaxis. However, there were no significant differences in the co-existence of these respiratory pathogens in the sputum of patients who were or were not on antibiotic prophylaxis ($p=0.203$).

In bacterial cultures, a total of 187 distinct bacterial isolates were isolated from sputum cultures and identified by MALDI-TOF. The prevalence of the 68 sputum isolates; 36 from patients on prophylactic antibiotics and 32 from the comparator group are shown in figure 6. The variety and frequency of bacterial pathogens isolated from the

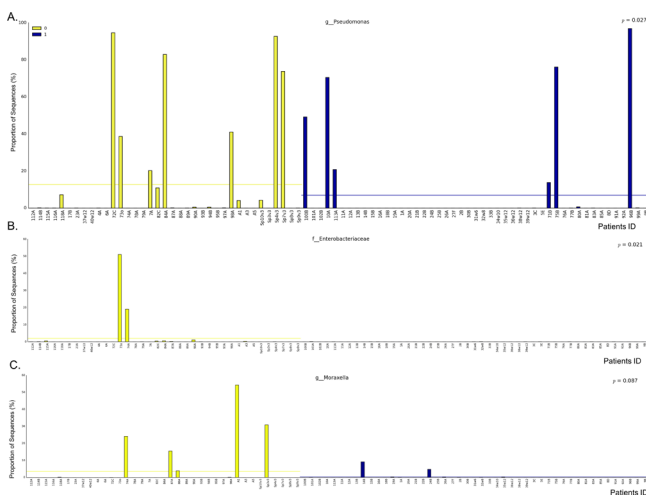


Figure 4 Suppression of potential pathogenic taxa in the sputum of patients on prophylactic antibiotic therapy (navy blue) compared with others in the comparator group (yellow): (A) genus *Pseudomonas* ($p=0.027$), (B) family Enterobacteriaceae ($p=0.021$), (C) genus *Moraxella* ($p=0.087$).

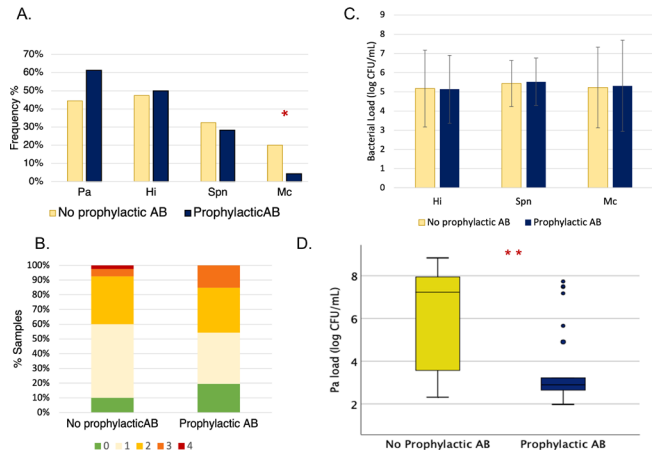


Figure 5 Prevalence, co-existence and bacterial loads of pathogenic airway bacteria in patients with chronic lung disease who were receiving antibiotic (AB) prophylaxis therapy (navy blue) and those not (comparator group, yellow) as determined by the multiplex quantitative PCRs. (A) Percentage of samples tested positive for the tested pathogens, (B) number of organisms tested positive within the same sputum samples ($p=0.203$), (C) mean bacterial loads of *Haemophilus influenzae* (Hi), *Moraxella catarrhalis* (Mc), *Streptococcus pneumoniae* (Spn) were similar in both groups ($p=0.189$, multivariate analysis of variance). (D) *Pseudomonas aeruginosa* (Pa) load was significantly lower in the prophylactic AB group ($p=0.001$). Bacterial load was measured in CFU/mL of original sputum sample for each bacteria. Samples which gave negative results for a given bacteria were excluded from the analysis. Error bars show ± 1 SD. * $P<0.05$, ** $p\leq 0.01$.

sputum of patients using prophylactic antibiotics were significantly less than those not receiving prophylactic antibiotics in the comparator group. Twenty-six per cent of the Gram-negative isolates were isolated from sputum cultures of patients on prophylactic antibiotics compared with 48% from the rest of the cohort ($p=0.011$).

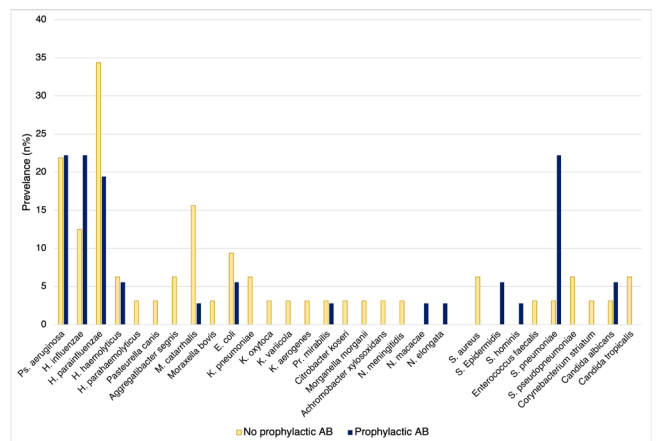


Figure 6 The taxa isolated from the sputum of patients with chronic lung disease and their prevalence among the patients who were receiving antibiotic (AB) prophylaxis therapy ($n=36$, navy blue) and those not (comparator group, yellow, $n=32$).

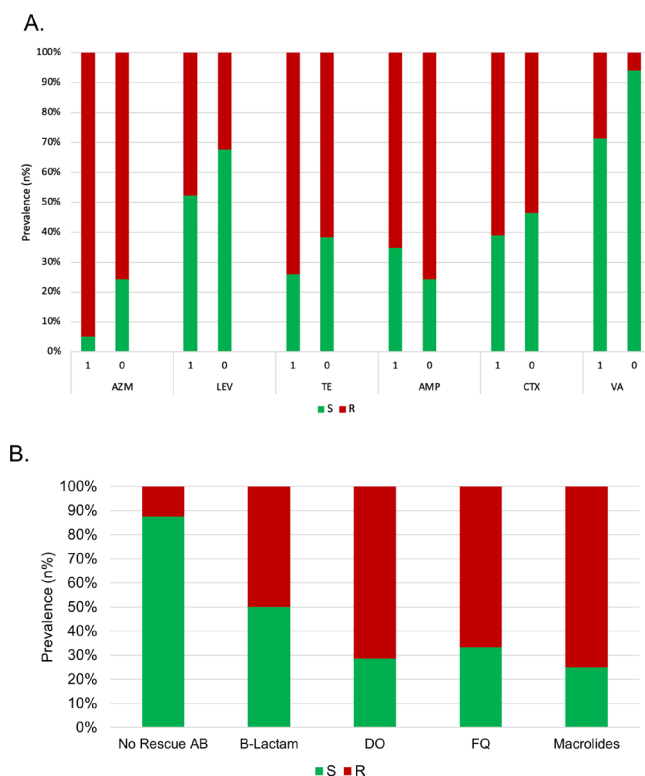


Figure 7 (A) Comparison between the antimicrobial resistance (AMR) in the commensal viridans streptococci group isolated from the sputum of patients receiving antibiotic (AB) prophylaxis therapy (group 1) and those not (comparator (group 0) ($p < 0.0001$), (B) association between the AMR in pathogenic sputum isolates and the prescribed ABs in the rescue packs for the self-treatment of acute exacerbations ($p = 0.04$) prevalence: percentage of the resistant isolates. AZM, azithromycin; AMP, ampicillin; CTX, cefotaxime; DO, doxycycline, FQ, fluoroquinolone; LEV, levofloxacin; R, resistant (red); S, sensitive (green); TE, tetracycline; VA, vancomycin.

Antibiotic susceptibility testing revealed high proportions of resistance to macrolide (64% and 29%), tetracycline (45% and 34%) and ampicillin (10% and 67%) of the tested Gram-positive and Gram-negative isolates, respectively in the whole cohort. Ciprofloxacin and cefotaxime resistance was detected in 21% and 17% of the Gram-negative isolates, respectively (online supplemental figures S5 and S6). The observed AMR in the bacterial isolates was associated with the corresponding prescribed antibiotics in the rescue packs for self-treatment of acute exacerbations (Spearman's correlation $\rho = 0.408$, $p = 0.02$) but not with the prophylactic antibiotics used (Spearman's correlation $\rho = -0.419$, $p = 0.136$) (figure 7B).

Among 119 tested viridans streptococci isolates, resistance to multiple antibiotics was common in both groups such as: azithromycin (95% in the antibiotic prophylaxis group vs 76% in the comparator group), tetracycline (74% vs 62%, respectively), ampicillin (65% vs 76%, respectively), cefotaxime (61% vs 54%, respectively), levofloxacin (48% vs 32%, respectively) and vancomycin (29% vs 6%, respectively). Nevertheless, resistance was

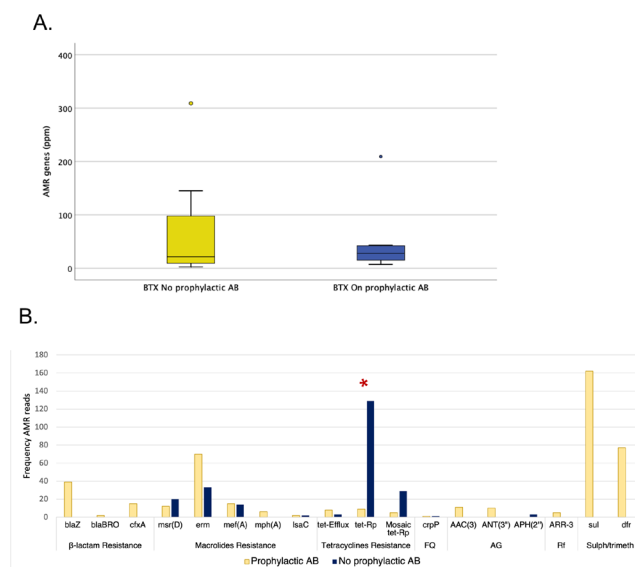


Figure 8 (A) Comparison between the relative abundance of the total antimicrobial resistance (AMR) genes (measured in ppm sequence reads) in sputum samples from patients with bronchiectasis (BTX) on antibiotic (AB) prophylaxis (blue, $n = 8$) and other patients with BTX having frequent exacerbations treated with AB rescue packs but not receiving AB prophylaxis therapy (yellow, $n = 9$) ($p = 0.48$ by Mann–Whitney). (B) The prevalence of AMR genes in the patients on antibiotic prophylaxis and those not ($*p < 0.05$ by MW). AG, aminoglycosides resistance; FQ, fluoroquinolones resistance; Rf, rifamycin resistance; Rp, ribosomal protection proteins; Sulph/trimeth, sulfonamides/trimethoprim.

significantly more frequently detected in the antibiotic prophylaxis group compared with the comparator group ($p < 0.0001$) (figure 7A).

In the resistome analysis, no significant differences in the prevalence of AMR genes were found in the sputum samples of patients with bronchiectasis who were on prophylactic antibiotics (seven were on 250 mg azithromycin thrice weekly and one on ciprofloxacin 250 mg twice daily) compared with those who were not ($n = 9$) ($p = 0.48$). Although not statistically significant due to the small sample sizes, a trend was observed in which the patients who were not receiving antibiotic prophylaxis therapy but had frequent exacerbations, median frequency of exacerbations/year 4 (IQR 2–5), exhibited a broader range of AMR gene prevalence: the median (IQR) was 21 ppm (6–121 ppm, $n = 9$) compared with those on prophylactic antibiotics which was 28 ppm (IQR 14–43 ppm, $n = 8$) (figure 8A). Also, a greater diversity of AMR genes conferring resistance to multiple antibiotic classes was observed in the frequent exacerbator patients using rescue packs and not on prophylactic antibiotics (figure 8B).

DISCUSSION

Antibiotic prophylaxis is a common approach in the management of chronic lung diseases including COPD



and bronchiectasis, especially in advanced cases with frequent and/or severe exacerbations. Here, we report the impact of prolonged antibiotic prophylaxis therapy on the airway microbiome within a cohort of 84 patients with chronic respiratory conditions: COPD and/or bronchiectasis, 47 received prophylactic antibiotics prescribed for at least 1 year prior to joining the study as part of their routine clinical care. We also relate AMR in sputum-resistome and sputum-derived bacterial isolates to antibiotics: prophylactic and acute 'rescue' prescription.

Antibiotic prophylaxis was associated with lower α -diversity of the airway bacterial community, as indicated by the PD whole tree (a quantitative measure of the phylogenetic diversity within an ecosystem)²⁶ and decline in the total bacterial burden in sputum. Previous studies have also reported a decline in richness and α -diversity in bronchoalveolar lavage (BAL) samples from patients with COPD and moderate-to-severe asthma who were receiving azithromycin prophylaxis therapy.^{27 28}

The microbiome profiles at the phylum level were similar between those prescribed and not prescribed prophylactic antibiotics. *Synergistetes* (a minor phylum) was the only phylum significantly less abundant in the antibiotic prophylaxis group. *Synergistetes* is a recently recognised bacterial phylum which has been detected in various body microbiomes such as the oral cavity, gut, umbilicus and vagina. Since its presence has been associated with disease in sites such as periodontitis, abscesses and cysts, *Synergistetes* are best considered opportunistic pathogens.²⁹

There were no significant differences in the relative abundances of any of the genera that would normally be defined as components of the healthy microbiota, between the patients receiving antibiotic prophylaxis and those who did not. Similarly, Rogers *et al* reported that there were no significant differences in microbiota composition between erythromycin treatment and placebo arms in the bronchiectasis and low-dose erythromycin study (BLESS).³⁰

Some pathogenic genera such as *Pseudomonas*, Enterobacteriaceae, *Klebsiella*, *Pasteurella* and *Morganella* were significantly less abundant in the patients receiving antibiotic prophylaxis compared with those who did not. Sequencing data suggested that *Moraxella* was also less abundant within the antibiotic prophylaxis group, although statistical significance was not achieved. Nevertheless, this observation was confirmed by specific qPCR ($p=0.039$).

qPCR is more sensitive and specific compared with 16S rRNA sequencing, data on the sensitivity and specificity of the methods used is presented in the online supplemental data, online supplemental figure S7, online supplemental tables S2 and S3. Apart from *S. pneumoniae* which could not be distinguished by V3-V4 16S rRNA sequencing from the viridans streptococci that are abundant in sputum, a strong significant correlation between the qPCR results of *H. influenzae* (Spearman's $\rho=0.798$ $p=8.4E-39$) and *M. catarrhalis* ($\rho=0.621$, $p=3.2E-15$) and

the corresponding taxa in the sequencing results of V3-V4 variable regions of 16S rRNA gene was found (online supplemental data, online supplemental figure S8). This correlation was less for *P. aeruginosa* ($\rho=0.238$ $p=2.9E-4$). Although *P. aeruginosa* was more frequently detected by qPCR in the antibiotic prophylaxis group, as a significantly higher proportion of CVID patients were in this study group and *P. aeruginosa* was significantly more prevalent in the patients with CVID (68%, $p=0.01$) compared with the rest of the cohort, the load of *P. aeruginosa* was in fact significantly lower in patients receiving prophylactic antibiotics ($p=0.001$). The same trend was observed after excluding the patients with CVID, although the sample size in this case was too small to assess true statistical significance (online supplemental figure S9).

The data from molecular methods were reflected in the bacterial culture results where pathogenic bacteria were isolated significantly less frequently from patients' sputum using prophylactic antibiotic therapy compared with the other participants. This is in line with previous clinical trials which evaluated the efficacy of antibiotic prophylaxis therapy in various chronic lung diseases, and reported that respiratory colonisation with typical respiratory bacterial pathogens was either eliminated or inhibited following introduction of the prophylactic antibiotic.³¹⁻³³ It is noteworthy that microbiology was a secondary outcome in these trials and the results in most cases were based on culture-dependent techniques only, which are less holistic compared with the microbiome approach and can be insensitive when detecting bacteria present at low loads.

This finding may explain how prophylactic antibiotics reduce the risk of exacerbations in chronic lung disease, where the mechanism would be long-term suppression of pathogenic bacteria within the airway microbiome rather than 'snapshot' elimination of pathogens at acute exacerbation events. This would support the use of antibiotic prophylaxis in the management of chronic lung disease.

Most participants (62%) in the antibiotic prophylaxis group in our cohort were on an intermittent azithromycin regimen (thrice weekly). Macrolides are reported to have additional anti-inflammatory and immunomodulatory properties³⁴; therefore, the mechanism of action may not be mediated solely through antimicrobial activity. For example, macrolides have poor antibacterial activity against *P. aeruginosa*, but azithromycin was found to inhibit its biofilm formation by interfering with essential quorum sensing pathways.^{35 36} Segal *et al* have demonstrated that azithromycin could modulate the metabolome of the microbiome which in turn mediated the anti-inflammatory and immunomodulatory activity in the BAL samples of patients with COPD receiving azithromycin prophylaxis therapy.²⁷

Surveillance of AMR revealed a high frequency of resistance across the whole cohort; however, it was significantly greater in the antibiotic prophylaxis group. For example, azithromycin resistance was detected in 95% of the viridans streptococci isolates in the antibiotic prophylaxis

group vs 75% in the comparator group. Previous studies confirmed that the oropharyngeal carriage of macrolide resistant viridans streptococci is common in general populations; it was estimated as 71% in a Belgian cohort of healthy adults and 94% in another Spanish cohort. In the Belgian study, co-resistance to tetracycline was identified in 73% of the isolates.^{37 38}

The resistome analysis revealed similar trends in which AMR genes were slightly richer (in terms of frequency of detection) in the samples from patients with bronchiectasis on prophylactic antibiotics, however, the broadest diversity of AMR genes was observed in those patients who had frequent exacerbations events treated with antibiotic rescue packs but were not receiving antibiotic prophylaxis. Nevertheless, due to the small sample sizes, the study lacked the power to demonstrate that any of the observed trends were statistically significant. The acquired and/or inherent resistance of the microbiota might explain the resilience of the airway microbial community in response to antimicrobial treatment.

The relationship between the development of AMR and use of antibiotic prophylaxis is unclear within the current literature. Many studies report a significant rise in the acquisition of AMR, or a significant elevation in the minimum inhibitory concentrations of clinical isolates, in response to antibiotic prophylaxis.^{31 39 40} The viridans streptococci develop resistance rapidly, especially to macrolides and tetracyclines.^{41 42} Whole genome sequencing of commensal streptococci in one study revealed that macrolide transmissible resistance genes were carried with tetracycline-resistance determinants on transposable elements.⁴² Commensal streptococci were also reported to carry the same macrolide resistance genes as pathogenic streptococci.⁴³ Therefore, microbiota may be regarded as a reservoir for AMR in the microbial ecosystem.

On the other hand, some studies reported no significant differences in the detection rates of the antibiotic resistance between patients receiving antibiotic prophylaxis therapy and those who did not.^{32 44} Other studies have found that the resistance acquired during the antibiotic prophylaxis therapy was temporary, this suggested that acquired resistance is at the cost of fitness and does not persist.^{45 46}

In current clinical practice, courses of antibiotics are often prescribed in 'rescue packs' kept by patients to initiate treatment of acute exacerbations promptly. The decision on antibiotic choice is based on clinician preference and may consider the individual's clinical condition, drug tolerance and allergies, previous sputum bacteriology culture results and the antimicrobial susceptibility profile (antibiogram) of previous sputum pathogenic bacterial isolates. Nevertheless, the benefit of antibiotics in the treatment of acute exacerbation, especially in COPD, remains controversial, and the evidence supporting the universal treatment of exacerbation with antibiotics is limited.² In our results, AMR detected in sputum isolates was more closely related to antibiotic rescue packs rather

than to prophylactic antibiotics. Therefore, the practice of prescribing antibiotic rescue packs might be more concerning in this sector rather than antibiotic prophylaxis therapy, especially in patients who suffer frequent exacerbations. This may justify the benefit of antibiotic prophylaxis in these patients. Patients' education on the rational use of antibiotics during exacerbations is crucial (such as having clear criteria when to start and stop them). Also, rotating prescriptions between different classes of antibiotics may theoretically help to mitigate AMR in this sector, although the available options may be limited and our data cannot inform on this.

There are several limitations to our results. As this was an observational study, participants were not randomly assigned to receive prophylactic antibiotics, therefore, our results demonstrate association but cannot prove causation. The decision to place a patient on antibiotic prophylaxis therapy was a clinical decision taken by the treating clinicians prior to joining the study. Antimicrobial prescription behaviour can be subjective and clinicians have different thresholds for prescribing antibiotic prophylaxis, although in most cases this decision is reserved for severe cases. This may explain why the frequency of exacerbations was comparable in both study groups despite the evidence that antibiotic prophylaxis therapy reduces the rate of exacerbations. Our cohort included patients with COPD, bronchiectasis and 45% of participants had an underlying diagnosis of CVID (the majority of whom were receiving immunoglobulin replacement). A relatively higher proportion of participants had bronchiectasis compared with COPD in the whole cohort; nevertheless, similar proportions of patients with bronchiectasis and COPD were present in both study groups. Apart from azithromycin, the small number of participants in the other six antibiotic prophylaxis regimes did not allow for comparisons between different antibiotics. The residual antimicrobial activity in the samples could have biased the bacteriology cultures in the antibiotic prophylaxis group but the molecular methods confirmed the findings of the culture-based approach. All these sources of variability might have masked some significant trends in the data.

In conclusion, antibiotic prophylaxis therapy was associated with reduced phylogenetic α -diversity of the bacterial communities and lower bacterial density in sputum. It selectively suppressed specific taxa that represent bacterial respiratory pathogens without disrupting the homeostasis of the respiratory microbiota. It did not induce a definitive compositional shift in the airway microbiota composition at the cohort level. In general, macrolide resistance was high in the whole cohort, nevertheless, it was significantly greater in patients receiving antibiotic prophylaxis. The practice of antibiotic rescue packs may be driving AMR in this cohort since the detected AMR expressed by sputum bacterial isolates was associated with prescribed antibiotics in the rescue packs kept by patients for self-treatment of exacerbations. Therefore, the clinical decision of antibiotic prophylaxis in the

management of chronic lung disease should be carefully considered on an individual basis by weighing the benefits of suppressing pathogenic bacteria and reducing the rate of exacerbations, and hence need for acute antibiotic treatment courses, against the risk of enriching resistance to the prescribed antibiotic among bacterial populations and the considerable adverse effects that can result from the long-term use of these antimicrobial chemotherapeutic agents.

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Acknowledgements We would like to thank Sary Workman and Fernando Moreira in the Department of Clinical Immunology Royal Free London NHS Foundation Trust for their help and support in the recruitment and samples' collection. The first author would like to thank the Missions sector in the Egyptian Ministry of Higher Education and the British Council Egypt for funding her PhD as part of the Newton-Mosharafa scheme. The funder had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Contributors SR, JRH, TMCh, ML, DS and JB designed the study. SR, SQ, ML, DML and JB recruited participants, conducted the study and collected clinical data and samples. SR processed the samples, did the laboratory work, performed the bioinformatics and data analyses. SR, JRH, TMCh, ML, DML and DS interpreted the data. TMCh is the principal investigator and acts a guarantor of the study. SR wrote the first draft of the manuscript with contributions from all other authors.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Disclaimer The funder had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Competing interests Newton Mosharafa funds by British Council Egypt and Egyptian Ministry of Higher Education sponsored the PhD of SR.

Patient and public involvement Patients and/or the public were involved in the design, or conduct, or reporting, or dissemination plans of this research. Refer to the 'Methods' section for further details.

Patient consent for publication Not applicable.

Ethics approval The prospective observational cohort study conducted in the Royal Free London NHS Foundation Trust was approved by Harrow Research Ethics Committee (16/LO/1490). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available on reasonable request.

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Impact of Prophylactic and 'Recue Pack' Antibiotics on Airway Microbiome in Chronic Lung Disease

(Data Supplement)

1. DNA extraction

In a microbiological safety cabinet in a Containment Level 3 laboratory, 1 millilitre of each sample was allowed to thaw at room temperature, then centrifuged at 13,000xg for 10 min; the supernatant was discarded and the pellets were heated at 95°C for 30 min, then they were disrupted with silica beads (FastPrep® lysis matrix B) on FastPrep®-24 Instrument (MP Biomedicals™, Fisher Scientific, UK) speed 6.5 m/s for 45 sec. DNA was then extracted on the automated LIAISON® Ixt extraction platform using DiaSorin® Arrow DNA extraction kit.

2. Multiplex qPCR for respiratory pathogens

This method was previously described by Garcha et al. [1]. The master-mix was prepared using Platinum® quantitative PCR Supermix-UDG (Thermo-Fisher Scientific, UK) and additional magnesium chloride at final concentration of 3 mM. The thermocycles of 95°C for 3 min followed by 40 cycles of 95°C for 10 sec and 60°C for 45 sec were carried out on Qiagen Rotor-gene® 6000 real-time PCR machine (Corbett Research UK, Cambridgeshire, UK). An internal amplification control, Spud A: 101 bp of the PhyB gene of *Solanum tuberosum*, was used at final concentration of 0.04 pM to test for PCR inhibition.[2]

3. *P. aeruginosa* and total bacterial load Diplex qPCR

The total bacterial load (copies/μL) and load of *P. aeruginosa* (CFU/mL) were determined in sputum samples using another Taq-Man® assay diplex qPCR targeting a 466 bp fragment of 16S rRNA gene (V3-V4 hypervariable regions) using the universal bacterial primers Bact340F and Bact806R and a 65 bp fragment of the *regA* gene in *P. aeruginosa*. [3] The IAC was also employed in this method to test for PCR inhibition. The master-mix was prepared using Platinum® quantitative PCR Supermix-

UDG (Thermo-Fisher Scientific, UK). The thermo-cycling conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec as carried out on Qiagen Rotor– gene® 6000 real-time PCR machine (Corbett Research UK, Cambridgeshire, UK). The signal of *P. aeruginosa* was captured on the green channel, 16S rRNA on the orange channel and IAC on the red channel. Load of *P. aeruginosa* was calculated in colony forming unit (CFU) per mL of sputum while the load of 16S rRNA was calculated as copies/ μ L.

Table S1: The primers and probes for the bacterial targets and the internal amplification control in qPCR for typical respiratory pathogens

Targets	5'–3' Sequence
<i>P. aeruginosa</i>	
Ps Forward Primer	TGCTGGTGGCACAGGACAT
Ps Reverse Primer	TTGTTGGTGCAGTTCCTCATTG
Ps probe	[FAM] CAGATGCTTTGCCTCAA [TAM]
<i>S. pneumoniae</i>	
Spn Forward primer	AGTCGTTCCAAGGTAACAAGTCT
Spn Reverse primer	ACCAACTCGACCACCTCTTT
Spn Probe	ROX-TACATGTAGGAACTATTTTCCTCACAAA-BHQ2
<i>H. influenzae</i>	
Hi Forward primer	CCGGGTGCGGTAGAATTTAATAA
Hi Reverse primer	CTGATTTTTCAGTGCTGTCTTTGC
Hi Probe	6FAM-ACAGCCACAACGGTAAAGTGTCTACGT-DB
<i>M. catarrhalis</i>	
Mc Forward primer	GTGAGTGCCGCTTTTACAACC
Mc Reverse primer	TGTATCGCCTGCCAAGACAA
Mc Probe	6JOE-TGCTTTTGCAGCTGTTAGCCAGCCTAA-BHQ1
16S rRNA qPCR	
q16S rRNA Forward Primer (Bact340F)	TCCTACGGGAGGCAGCAGT
q16S rRNA Reverse Primer (Bact806R)	GGACTACCAGGGTATCTAATCTT
q16S rRNA probe	[ROX] CGTATTACCGCGGCTGCTGGCAC [BHQ2]
IAC	
Spud Forward primer	AACTTGGCTTTAATGGACCTCCA
Spud Reverse primer	ACATTCATCCTTACATGGCACCA
Spud Probe	Cy5-TGCACAAGCTATGGAACACCACGT-BBQ
SpudA	AACTTGGCTTTAATGGACCTCCAATTTTGAGTGTG CACAAGCTATGGAACACCACGTAAGACATAAAAC GGCCACATATGGTGCCATGTAAGGATGAATGT

4. 16S rRNA gene sequencing

A sequence library was created by amplification of V3-V4 regions of the bacterial 16S *rrna* gene through conventional PCR on the extracted metagenomic DNA using 341 forward primer (CCTACGGGNGGCWGCAG) and 805 reverse primer (GACTACHVGGGTATCTAATCC).[4, 5] Each sample was assigned a unique pair combination of standard Illumina® dual indexed primers (with adaptors attached: P5 and P7 in the forward and reverse primers respectively). The PCR master-mix per reaction was composed of; 0.2 µM for each of the forward and reverse primers, 10.8 µL Mol Taq 16S basic Master-mix (Molzym, VH Bio Limited, UK). The amount of DNA template added was adjusted such that the final DNA input per reaction was 300 ng. The thermo-cycling conditions were 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 55°C for 40 sec and 72°C for 1 min, in addition to a final extension phase at 72°C for 10 min. The PCR products (577bp amplicons) were cleaned up using Agencourt AMPure XP beads (Beckman Coulter, UK) with a binding buffer of 2.5 M sodium chloride and 20 g% PEG-8000, 80% ethanol and EB Buffer® (Qiagen, UK) to remove nonspecific amplicons <200bp and primer dimers. DNA in the cleaned products was then quantified using Qubit™ dsDNA HS kit and Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, UK). The samples were pooled in an equimolar ratio at 5 nM into one library. The library was checked on bioanalyzer. Sequencing was performed using Illumina MiSeq Platform using costume sequencing primers for read 1: GCGAGTCAGTCAGCGGACTACHVGGGTATCTAATCC, read 2: GCGAGTCAGTCAGCGGACTACHVGGGTATCTAATCC and index i7: GGATTAGATACCCBDGTAGTCCGCTGACTGACTCGC, MiSeq® Reagent Kit v2 (500 cycles) (cat no. MS-102-2003) and PhiX control V3 KIT (cat no. FC-110-3001) as internal control for the sequencing run (Illumina Cambridge, Ltd, UK). The extraction negative control and a no-template PCR control (water) were run throughout the amplification and sequencing process as negative controls to allow for the evaluation of potential contamination.

A mock community composed of equal proportions of DNA concentrations from *Streptococcus pneumoniae* ATCC 49619, *Haemophilus influenzae* ATCC 8468, *Streptococcus pyogenes* ATCC 19615, *Escherichia coli* ATCC 27853, *Klebsiella*

pneumoniae NCTC 13438, *Staphylococcus aureus* ATCC 29213, *Acinetobacter baumannii* clinical isolate, *Pseudomonas aeruginosa* clinical isolate, *Moraxella catarrhalis* clinical isolate was run as a positive control.

5. Bioinformatic and Statistical Analyses

In bioinformatic analysis we adopted the workflow established by Microbiome helper.[6] Briefly, the paired end reads were stitched together using PEAR.[7] The low-quality reads with quality score <30 over 10% of its bases and length less than 400 bp were filtered out using FASTX-toolkit (v.0.0.14).[8] The reads were then screened for possible chimeras that may have resulted from PCR using VSEARCH (v1.11.1). [9] The subsequent steps were through QIIME pipeline v1.9.1 [10] where the sequences were clustered based on 97% similarity into Operational Taxonomic Units (OTU) and taxonomic classification was assigned to OTUs using open reference OTU picking against Greengenes database version 13_8. The OTU table was then rarefied per sample to 5000. Alpha and beta diversity indices were calculated on the rarefied OTU table using QIIME. PERMANOVA and ANOSIM were used to test the associations between the metadata and the microbiome β -diversity. PERMANOVA is a non-parametric multivariate analysis test that partitions a distance matrix among sources of variation in order to describe the strength and significance that a categorical covariate has in determining variation of distances; while, ANOSIM is a non-parametric hypothesis test that assesses whether two or more groups of samples are significantly different based on a categorical covariate in the metadata. [11]

The appropriate statistical significance tests were calculated using SPSS v. 23 or QIIME wrapper scripts. STAMP (v2.1.3) [12] was used to visualize the results and explore the OTUs showing significant differences across the two study groups using White's non-parametric t-test.[13] Whenever applicable the p-values were corrected using Benjamini-Hochberd False Discovery Rate (FDR) method for multiple comparisons.

6. Statistical Analysis

The primary outcome was defining the associations between the microbiome parameters (α and β diversity indices, and differential abundances of OTUs/taxa) and treatment regimes. Since there was no prior work published on which formal power calculations could be based on; therefore, the sample size was established on a pragmatic recruitment rate from clinics in each study within the time frame of the doctoral degree. One sputum sample per patient at baseline that comply with the pre-specified inclusion criteria was selected to be included in the presented cross-sectional study. Missing data was dealt with by removing the related data.

For continuous data, the significance of the observed differences in the results was tested using the parametric t-test as appropriate when the normal distribution and other assumptions are satisfied, otherwise the alternative non-parametric Mann-Whitney test was used. The distribution of each continuous variable had been checked prior to the selection of the most appropriate significance test. Normal distribution was confirmed when Shapiro-Wilk test was insignificant ($p > 0.05$), and skewness and kurtosis z-scores were within ± 1.96 while equal variance assumption was confirmed when the Levene's test was insignificant ($p > 0.05$).

Chi squared tests or Fisher exact test (when the expected frequency is < 5 in 25% of the contingency table) were used to test significance in comparisons involving categorical data as appropriate. Other tests are specified in the text. Stratification analysis was used whenever a source of bias is identified using PERMANOVA test.

The statistical analysis was performed using IBM SPSS Statistics, Version 25.0. [14]. Significance was set at $p < 0.05$. [15]

7. Resistome analysis

In a pilot study metagenomic sequencing was carried out on 17 sputum samples using MinION system (Oxford Nanopore Technologies, ONT, UK). Aliquots of sputum samples homogenised with Sputasol which had not been heat killed, were treated with saponin 2.5% and HL-SAN endonuclease (Arcticzymes®, Norway) to deplete human DNA as per the published method [16]. Then, metagenomic DNA was extracted on the automated LIAISON® lxt extraction platform

The extracted DNA was purified using magnetic AMPure XP beads (Beckman Coulter, UK) and washed twice with ethanol 80%. The sequencing library was prepared by multiplexing six samples in addition to the negative control per run using the Rapid Barcoding Kit (Ref: SQK-RLB004) which involved a tagmentation step followed by a non-specific PCR amplification step of 25 cycles of: 15 sec denaturation at 95°C, 15 sec annealing at 56 °C and 6 min extension at 65 °C, and a final 6 min extension step at 65 °C which enriches and tags all DNA fragments from each sample with unique barcodes. The DNA from differently tagged samples was pooled at equal concentrations at a maximum of 500 ng each; then the pooled library was purified using magnetic AMPure XP beads (Beckman Coulter, UK) and double ethanol 80% washes. The sizes of the DNA fragments were checked on Tape Station Automated Electrophoresis (Agilent, UK) and they were around 3500 bp (a sample report is in Appendix 3.9). The library was adjusted to 10 μ L of 200 fmol final DNA concentration (20 nM) prior it to adding the rapid adaptors and other reagents from the kit (as per manufacturer's directions). The sequencing was run for 48 hrs on Oxford Nanopore MinION flow cell (R.9.4.1).

ONT Albacore Sequencing Pipeline Software (version 2.3.4) was used to carry out base-calling, de-multiplex the samples per run. The genomes were assembled using the miniasm/minimap pipeline [17] and a BLAST search against the ResFinder database performed [18] to detect the antimicrobial resistance (AMR) genes. The alignments with accuracy less than 90% have been excluded. The prevalence of AMR genes within the samples was measured in part per million reads (ppm) i.e. the number of sequence reads identified as AMR genes relative to the total number of reads representing the sample.

Results

Recruitment

The respiratory clinic lists were screened for potential participants with a confirmed diagnosis of bronchiectasis (CT) or COPD (spirometry), in the period between February 2017 and May 2018, in total 147 patients were approached; and 90 patients were recruited from three outpatient respiratory clinics at the Royal Free hospital. In the period between February 2017 and July 2019, a total of 163 sputum samples were collected in the context of the longitudinal cohort study from participants with a median of two samples per patient. 84 patients provided sputum samples and completed the study.

Resistome

The most frequently detected AMR gene families were *tet* genes (32% of the total detected AMR genes) particularly 25% were *tet* genes which encode ribosomal protection proteins and confer resistance to tetracyclines by target protection such as *tet(O)* and *tet(M)* and 5% were mosaic *tet* genes such as *tet(O/32/O)*, *tet(S/M)*, *tet(W/32/O)*, *tet(O/W/32/O)*, *tet(O/W/32/O/W/O)*. Whereas, the *tet* genes which confer resistance to tetracyclines through efflux pumps such as *tet(A)*, *tet(B)* represented 2% of detected AMR genes. The prevalence of *tet* genes was significantly higher in the antibiotic prophylaxis group ($p=0.041$).

Macrolide resistance genes were the second most frequent, 14% of the detected AMR genes were *erm*; particularly *erm(B)* and *erm(F)* which encodes 23S ribosomal methyltransferase and confers Macrolide–Lincosamide–StreptograminB (MLSb) resistant phenotype. Both the major facilitator efflux pump encoding gene *mef(A)* and the MDR ATP binding cassette (ABC-F) encoding gene *msr(D)* which confer resistance to macrolides and multiple antimicrobials that target the protein synthesis process such

as tetracyclines, phenicols, lincosamides, and oxazolidinones, each of these gene families represented 5% of the detected AMR genes. Although there were no statistically significant differences in the prevalence of the above described AMR genes between the two study groups, it is noteworthy to highlight that the prevalence of macrolide resistance genes was also slightly higher in the group of bronchiectasis patients not receiving antibiotic prophylaxis therapy (**Figure 8B**).

Both the MDR ABC-F ribosomal protection protein gene family *Isa(C)* and ciprofloxacin phosphotransferase encoding gene *crpP* were equally detected in both groups. The detected *crpP* gene which confer resistance to fluoroquinolones through antibiotic inactivation was not detected in the patient who was on ciprofloxacin prophylaxis therapy but in a patient on azithromycin prophylaxis having chronic *P. aeruginosa* colonization and was prescribed ciprofloxacin in the rescue pack for the treatment of exacerbations.

The following AMR genes were detected only in the group of bronchiectasis patients not receiving antibiotic prophylaxis. First AMR genes which encode antibiotic inactivating enzymes such as class A β -lactamase enzymes encoding genes: *blaBRO*, *blaZ* and *cfxA*, macrolide phosphotransferase encoding genes *mphA*, aminoglycosides inactivating enzymes encoding genes: *aac(3)* and *ant(3'')*, rifampin ADP-ribosyltransferase encoding gene *arr-3*. Second, the AMR genes which confer resistance through antibiotic target modification: sulfonamide resistant dihydropteroate synthase encoding genes *sul1* and trimethoprim resistant dihydrofolate reductase encoding gene *dfrA17* (**Figure 8B**).

Forty-three percent of the AMR genes detected such as *msr(D)*, *mef(A)*, *tet(O)*, *crpP*, *ACC (3)*, and *ANT(3")* have been known to be linked to mobile genetic elements such as plasmids, transposons and integrons. (Alcock et al., 2020)

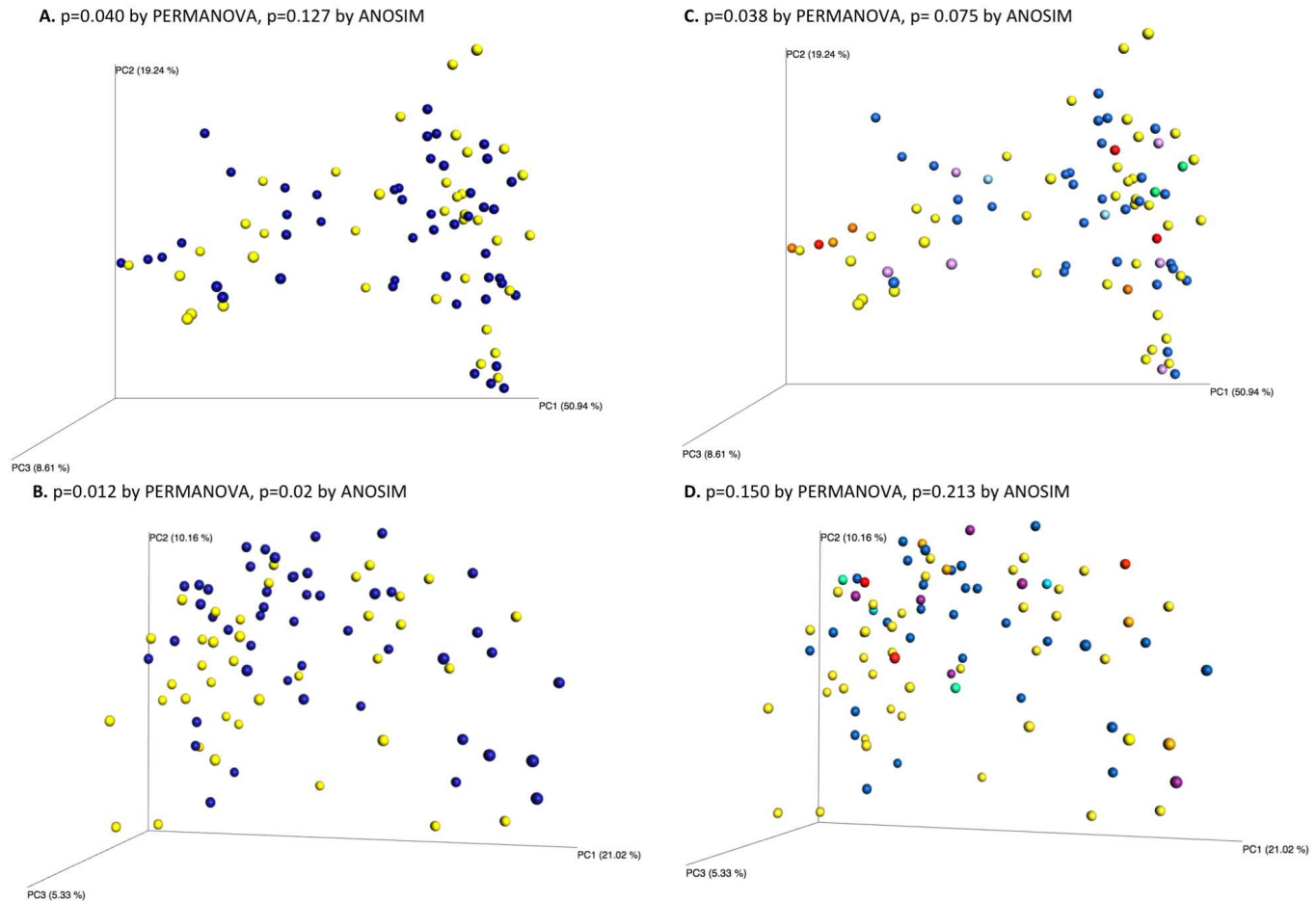


Figure S1: PCoA plot of weighted UniFrac (**A and C**) and unweighted UniFrac (**B and D**), samples are coloured with respect to: (**A and B**) being on prophylactic antibiotic therapy (navy blue, $n=47$) or not (comparator group, yellow, $n=37$) (**C and D**) the prophylactic antibiotic regime: azithromycin (blue, $n=29$), clarithromycin (light blue, $n=2$), doxycycline (orange, $n=4$), ciprofloxacin (green, $n=2$), β -lactam (red, $n=3$) co-trimoxazole (purple, $n=7$).

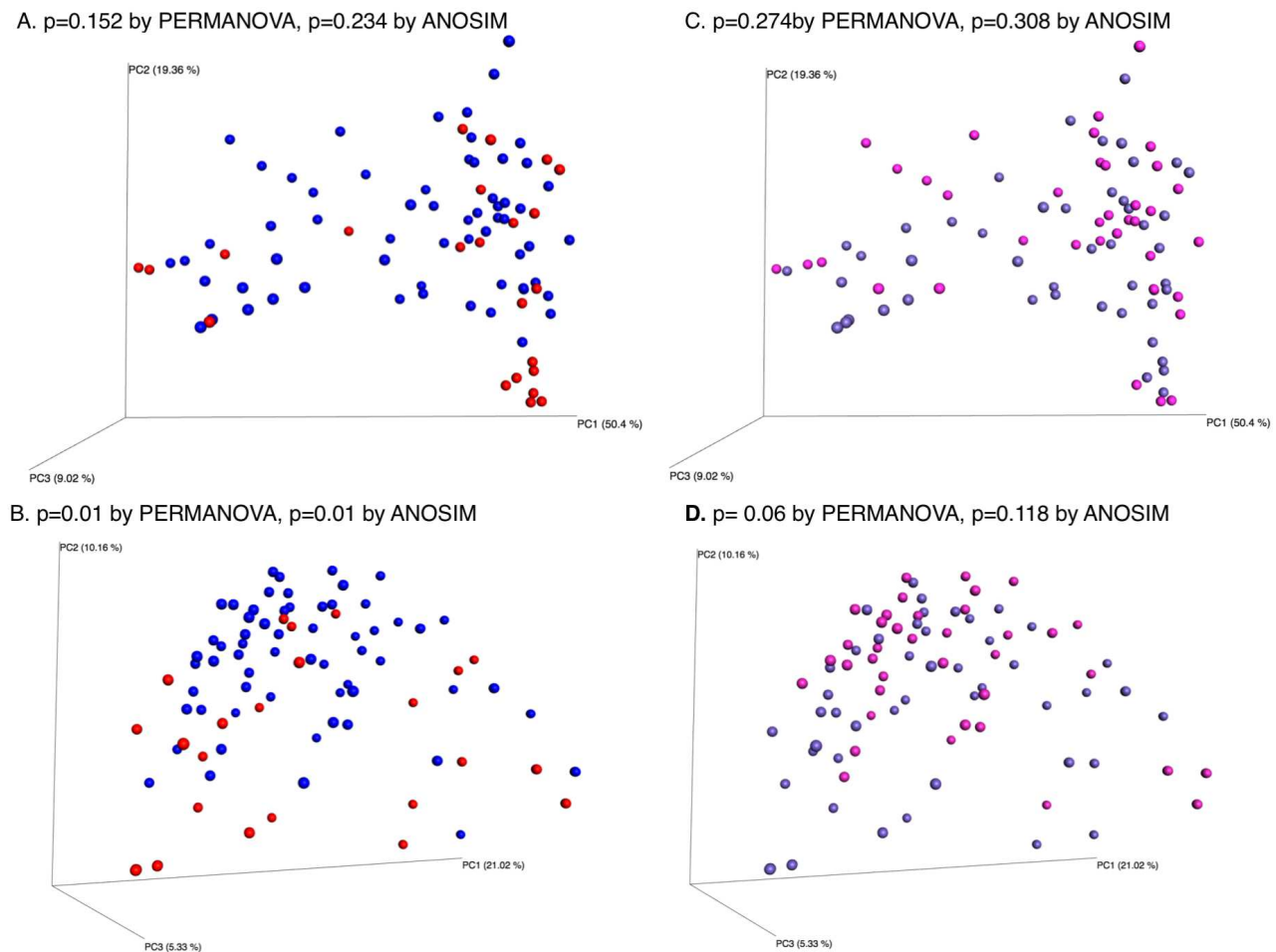


Figure S2: PCoA plot of weighted UniFrac (A and C) and unweighted UniFrac (B and D), samples are coloured with respect to: (A and B) **chronic respiratory condition (blue: bronchiectasis, $n=61$), (red: COPD, $n=23$)** (C and D) **CVID status pink: CVID, $n=38$ (purple: immunocompetent, $n=46$).**

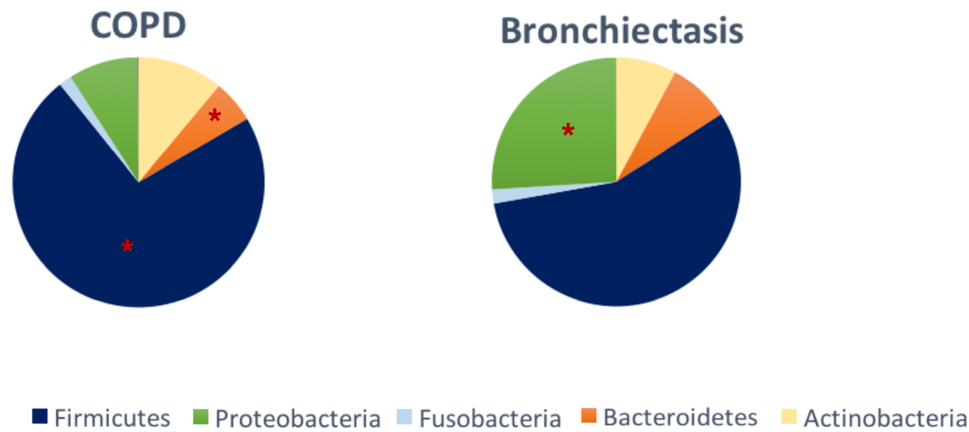
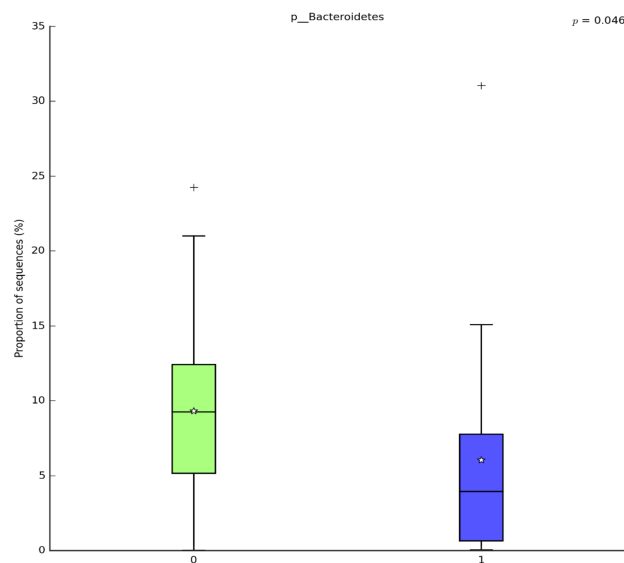
**B.**

Figure S3: Comparison between the airway microbiome profiles of COPD and bronchiectasis patients at phylum level (A) In COPD, *Firmicutes* ($p=0.048$) was significantly higher while *Bacteroidetes* was lower ($p=0.049$). On the other hand *Proteobacteria* ($p=0.041$) was significantly higher in bronchiectasis **(B)** *Bacteroidetes* was significantly lower in patients with airflow obstruction ($p=0.046$) (purple (group 1): airflow obstruction, $n=22$), (green (group 0): no airflow obstruction, $n=23$).

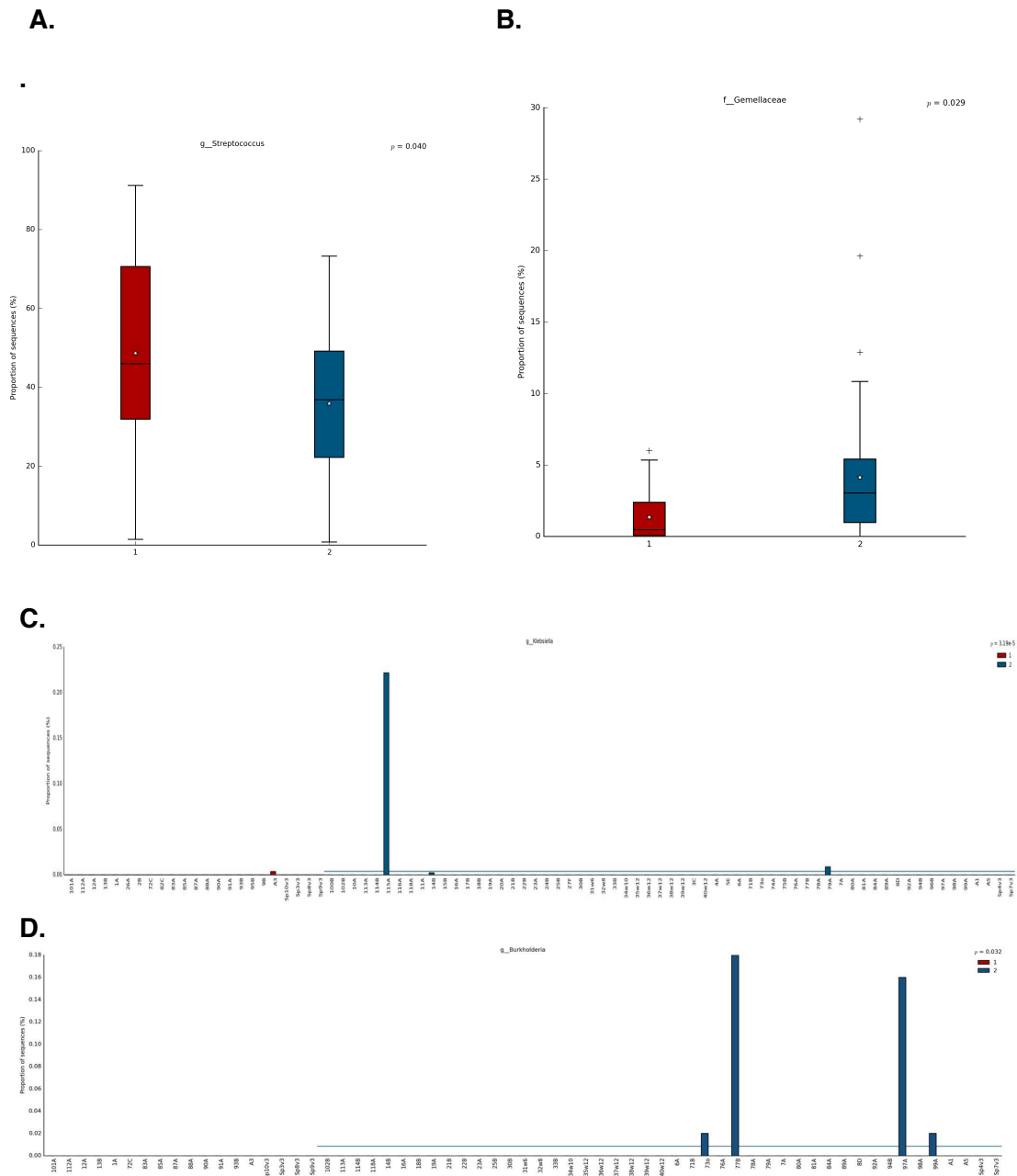


Figure S4: Differential abundance of the following taxa in the airways of COPD (group 1: red, n=23) and bronchiectasis patients (group 2: blue, n=61) **(A)** *Streptococcus* ($p=0.04$) more abundant in COPD; whereas, in bronchiectasis, **(B)** Family *Gemellaceae* ($p=0.029$), **(C)** *Klebsiella* ($p=0.003$) **(D)** *Burkholderia* ($p=0.032$) were more abundant and/or frequent.

A.

AB	<i>S. pneumoniae</i>									<i>S. aureus</i>		<i>E. faecalis</i>		
	AZM	R	R	R	R	R	R	R	R	S	S	S	S	S
E										S	S	I		
CIP	S										S	S	S	
TE	R	S	S	R	S	R	R	S	S	S	S	R		
AMP	S	S	S	S	S	S	S	S	S	R		S		
CTX	S	S	S	S	S	S	S	S	S					
C	S	S	S	S	S	S	S	S	S		S	I		
RIF	S	S	S	S	S	S	S	S	S	S		I		
VA	S	S	S	S	S	S	S	S	S			S		
OX	R	S	R	S	S	S	R	R			R			
DA	R											S		

B.

AB	<i>P. aeruginosa</i>							<i>P. aeruginosa</i>						
	CIP	S	R	R	R	S	S	S	S	S	S	S	S	S
LEV	S	R	S	R	S	S	S	S	S	S	S	S	S	S
TZP	S	S	S	S	S	S	S	S	S	S	S	S	S	S
AK	S	S	S	S	S	S	S	S	S	S	S	S	S	S
CAZ	S	S	S	S	S	S	S	S	S	S	S	S	S	S
CTX	R	R	S	S	R	S	S	S	S	S	S	S	S	S
MEM	S	S	S	S	S	S	S	I	S	S	S	S	S	S

Figure S5: Comparison between the AMR prevalence in the Gram positive and *P. aeruginosa* sputum isolates from patients receiving antibiotic prophylaxis therapy and those not (A) Antibiogram of Gram positive bacteria: *S. pneumoniae* (n=8), *S. aureus* (n=2) and *E. faecalis* (n=1) (p=0.014 by Fisher exact test) and (B) Antibiogram of *P. aeruginosa* isolates (n=14) (p=0.03 by Fisher exact test); antibiotic prophylaxis group (Blue) and the comparator group (orange). R: resistant (red), I: intermediate sensitivity (peach), S: sensitive (green), AB: antibiotic, AZM: azithromycin, E: erythromycin, CIP: ciprofloxacin, LEV: levofloxacin, TE: tetracycline, AMP: ampicillin, TZP: piperacillin/tazobactam, CTX: cefotaxime, CAZ: ceftazidime, MEM: meropenem, C: chloramphenicol, RIF: rifampicin, VA: vancomycin, OX: oxacillin, DA: clindamycin, AK: amikacin, grey shade: data not available.

AB	H. influenzae												H. influenzae			H. para		H. para	Pc	Mc	M. catarrhalis					E. coli			Pr.	Pr	Ck	K. pn	Kv	Ko	K. aer	Mm			
CIP	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	R	R	R	I	S	S	S	S	R	S	S	S	S	S	S	S	S
TE	S	R	S	S	S	S	S	S	S	S	I	S	S	S	R	R	S	S	S	S	R	S	R	R	R	R	R	R	S	S	S	S	S	S	S	S	S	R	
AZM	R	S	S	S	S	S	S	S	R	S	S	S	S	R	S	S	S	S	S	R	R	R	S	R	R	R	R	S	S	R	S	S	S	S	S	S	S	S	
AMP	S	R	R	R	R	R	S	S	S	S	R	R	S	R	R	R	S	S	S	S	R			R	R	R			R	R	R	R	R	R	R	R	R		
AMC	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R	S	R	R	S	S	S	S	R	S	S	R	S	S	R			
CAZ																				S	R				S	S											S		
CTX	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	R	S	R	S	S	S	I	R	S	S	S	S	S	S		S	S	S	S	S	S			
FEB									S								S			S	S	I				S	S							S	S				
MEM		S	S	S	S	S	S	S	S	S	S	S					S			S	S	S	S	S		S	S							S	S				
C	S	S	S	S	S	S	S	S	S	S	S	S	S	S	I				S	S	R	S	S	S	S	S		S			S	R	S	S	S				
SXT	S								S	S			S	S	S	S	S	S			R	R	S	R			S	S	R	S	S								
CN																					S		R		S	S									S	S			

Figure S6: Comparison between the AMR prevalence in the Gram negative sputum isolates from patients receiving antimicrobial prophylaxis and those not. Each column represents antibiogram of a Gram negative isolate: *H. influenzae* (Hi, n=12), *H. parainfluenzae* (H. para, n=3), *Pasteurella canis* (Pc, n=1), *M. catarrhalis* (Mc, n=5) *Proteus mirabilis* (Pr, n=2), *Citrobacter koseri* (Ck, n=1,) *K. pneumoniae* (K.pn, n=2), *K. variicola* (Kv, n=1), *K. oxytoca* (Ko, n=1), *K. aerogenes* (K.aer, n=1), *M. morganii* (M. mor, n=1):isolated from the sputum of chronic lung disease patients who were receiving antimicrobial prophylaxis (Blue) and those not (orange) (p=0.390 by Chi square test)

R: resistant (red), I: intermediate sensitivity (peach), S: sensitive (green), grey shade: data not available.

AB: Antibiotics, CIP: ciprofloxacin, TE: tetracycline, AZM: azithromycin, AMP: ampicillin, AMC: amoxicillin/clavulanic acid, CAZ: ceftazidime, CTX: cefotaxime, FEB: cefepime, MEM: Meropenem, C: chloramphenicol, SXT: Sulfamethoxazole/ Trimethoprim, CN: gentamycin, Hi: *H. influenzae*, H. para: *H. parainfluenzae*, Pc: *Pasteurella canis*, Mc: *M. catarrhalis*, Pr: *Proteus mirabilis*, Ck: *Citrobacter koseri*, K.pn: *K. pneumoniae*, Kv: *K. variicola*, Ko: *K. oxytoca*, K.aer: *K. aerogenes*, m: *M. morganii*.

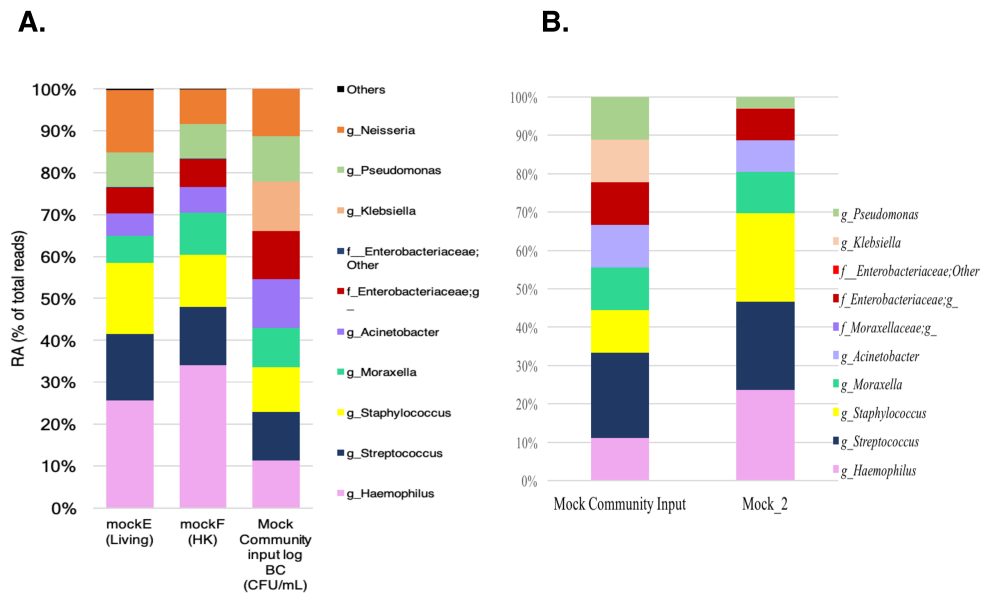


Figure S7: A. Mock community E and F used to assess the efficiency of the DNA extraction procedure and the effect of the heat killing step on the bacterial community composition representation **B.** The microbiome profile of laboratory prepared mock community; Mock_2 is composed of equal proportions of DNA concentrations from each of the constituting bacteria measured by Qubit™ HS Kit.

Table S2: The limit of quantification (LOQ) and limit of detection (LOD) of the qPCR for respiratory pathogens

	<i>P. aeruginosa</i>	<i>S. pneumoniae</i>	<i>H. influenzae</i>	<i>M. Catarrhalis</i>
LOQ	700 CFU/mL 2.9 log ₁₀ CFU/mL	25,000 CFU/mL 4.4 log ₁₀ CFU/mL	20,000 CFU/mL 4.3 log ₁₀ CFU/mL	2500 CFU/mL 3.4 log ₁₀ CFU/mL
LOD	350 CFU/mL 2.5 log ₁₀ CFU/mL	3700 CFU/mL 3.7 log ₁₀ CFU/mL	1000 CFU/mL 3 log ₁₀ CFU/mL	500 CFU/mL 2.7 log ₁₀ CFU/mL

Table S3: Sensitivity, Specificity and Accuracy of the 16S rRNA sequencing relative to the qPCR

taxa	Sensitivity	Specificity	Accuracy
<i>Pseudomonas</i>	90.5%	77.2%	80.7
<i>Moraxella</i>	82.5%	84.1%	83.9
<i>Haemophilus</i>	100%	0%	41.2%
<i>H. influenzae</i>	92.7%	81.1%	85.8%

Sensitivity is the true positive rate

Specificity is the true negative rate [19]

Accuracy is the true positives true negatives rate [20]

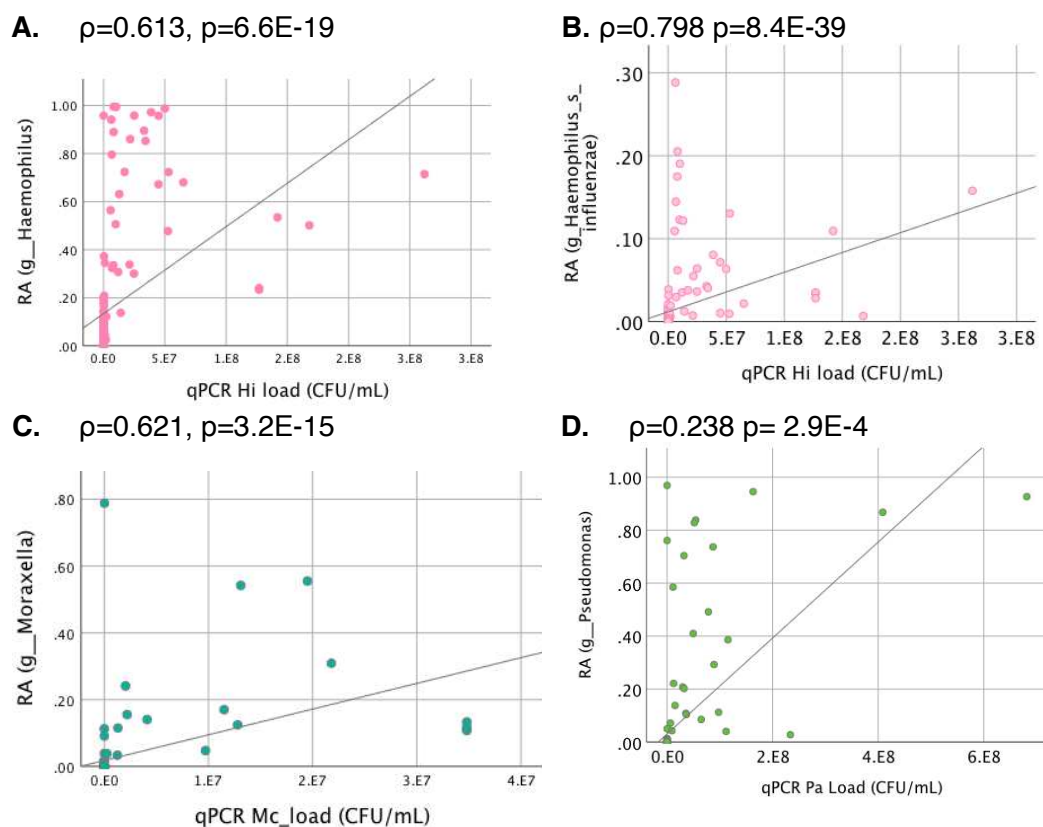


Figure S8. Correlation between the Relative Abundance (RA) results of V3-V4 16S rRNA sequencing and the quantitative loads of qPCR results (CFU/mL) in 182 tested sputum samples (A) Correlation between relative abundance of genus *Haemophilus* and load of *H. influenzae* (Hi) (B) Correlation between relative abundance of *H. influenzae* OTUs and load of *H. influenzae* (C) Correlation between relative abundance of genus *Moraxella* and load of *M. catarrhalis* (Mc) (D) Correlation between relative abundance of genus *Pseudomonas* and load of *P. aeruginosa* (Pa). ρ : Spearman's rho Correlation.

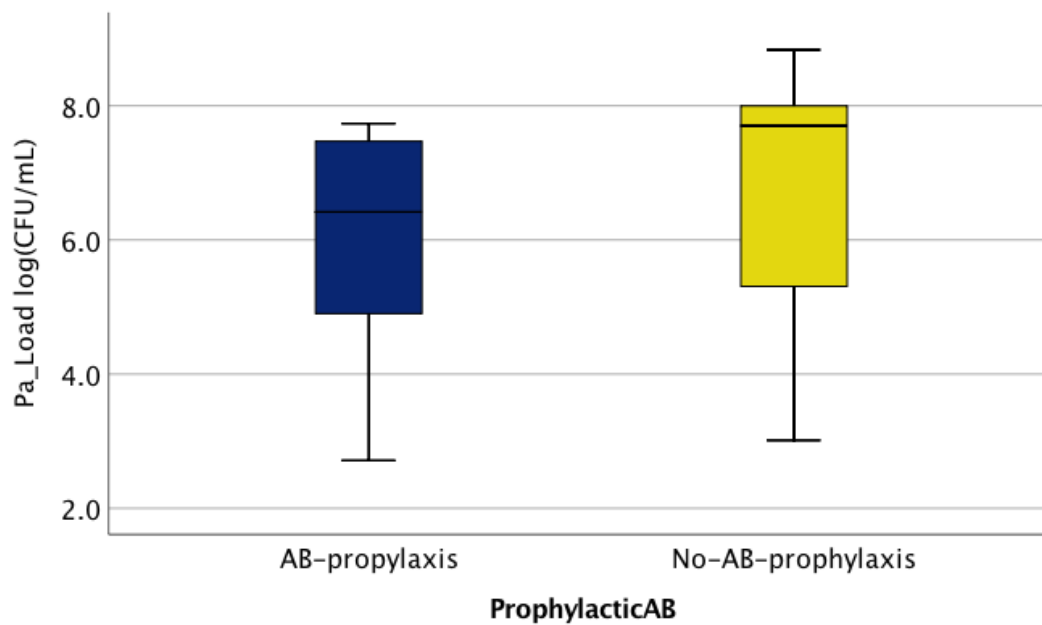


Figure S9: Comparison between the *P. aeruginosa* load in the sputum of patients in the antibiotic (AB) prophylaxis group (navy blue, n= 6) and the comparator group not receiving antibiotic prophylaxis (yellow, n=12) after excluding the COVID patients in both study groups (p=0.223 by MW)

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