



Anaerobic bacteria cultured from cystic fibrosis airways correlate to milder disease: a multisite study

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Anaerobic bacteria are cultured across all ages, occur as communities and correlate with milder CF disease in adults <http://ow.ly/7wQ430khMmE>

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ABSTRACT Anaerobic and aerobic bacteria were quantitated in respiratory samples across three cystic fibrosis (CF) centres using extended culture methods. Subjects aged 1–69 years who were clinically stable provided sputum (n=200) or bronchoalveolar lavage (n=55). 18 anaerobic and 39 aerobic genera were cultured from 59% and 95% of samples, respectively; 16 out of 57 genera had a $\geq 5\%$ prevalence across centres.

Analyses of microbial communities using co-occurrence networks in sputum samples showed groupings of oral, including anaerobic, bacteria, whereas typical CF pathogens formed distinct entities. *Pseudomonas* was associated with worse nutrition and F508del genotype, whereas anaerobe prevalence was positively associated with pancreatic sufficiency, better nutrition and better lung function. A higher total anaerobe/total aerobe CFU ratio was associated with pancreatic sufficiency and better nutrition. Subjects grouped by factor analysis who had relative dominance of anaerobes over aerobes had milder disease compared with a *Pseudomonas*-dominated group with similar proportions of subjects that were homozygous for F508del.

In summary, anaerobic bacteria occurred at an early age. In sputum-producing subjects anaerobic bacteria were associated with milder disease, suggesting that targeted eradication of anaerobes may not be warranted in sputum-producing CF subjects.

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Introduction

Antibiotic therapies for cystic fibrosis (CF) respiratory infections are targeted at specific bacteria, typically *Staphylococcus aureus*, *Pseudomonas aeruginosa* and other Gram-negative species. However, both culture-dependent and culture-independent molecular methods have redefined CF respiratory infections as polymicrobial, with a diverse lower airways bacterial community [1]. Anaerobic conditions have been described in CF lower airways [2, 3]. Importantly, obligate and facultative anaerobic bacteria, typically associated with the oral cavity, have been cultured in abundance from CF respiratory samples [4, 5]. Indeed, extended anaerobic culture methods identified anaerobic bacteria in high numbers in >60% of CF sputum samples from adults [6].

The role of anaerobic bacteria in CF lower airways disease is not clearly understood. Studies have identified anaerobic bacteria from direct lung samples, suggesting their presence does not simply reflect salivary contamination during sampling [7]. *In vitro* studies have demonstrated that anaerobes release virulence factors, including proteases and pro-inflammatory short-chain fatty acids [8, 9], suggesting that anaerobes may be pathogenic in the CF lung. In contrast, *in vivo* molecular studies indicate that greater anaerobe diversity is associated with milder disease [10, 11]. Molecular methods highlight the breadth of bacteria, and novel preparation methods and metagenomics address a shortcoming of conventional next-generation sequencing, *i.e.* the amplification of sequences of nonviable bacteria [12–14]. Bacterial abundance can be measured by quantitative PCR; however, 16S rRNA copy numbers per genome vary between genera and quantitation of different genera in multispecies infections remains imprecise [15, 16]. Culture allows enumeration of the different bacterial genera in a polymicrobial infection, demonstrates viability and growth in lower airways, and enables subsequent *in vitro* studies [17, 18]. Culturing anaerobic and bacteria not typically considered as pathogenic is also relevant to clinical care, *i.e.* would knowledge about abundance of these bacteria be useful for treatment?

Here, we assessed the association of strict anaerobes with disease severity in CF across all age ranges at three CF centres with different genetic and geographic backgrounds and medications. Although “CF pathogens” can grow under anaerobic conditions, this likely reflects adaptation to the microenvironment and not distinct genera/infections. We used extended culture methods attempting to investigate the role of anaerobes in CF lung disease. We focused on strict anaerobic bacteria to match genera reported in molecular studies, and to ascertain the load and viability of these anaerobes in lower airways in relation to typical CF pathogens. Three key questions were investigated. 1) Does the prevalence of anaerobes *versus* aerobes in subjects with CF change as a function of age and site/climate? 2) Are there associations between prevalence or quantity of anaerobes and genotype, disease severity and medications? 3) Are community structures established by anaerobes and do they differ across ages, disease severity or associations with aerobes?

Methods

Subjects and sample collection

People diagnosed with CF attending CF centres in Belfast in the UK (Adult and Paediatric CF Clinics, Belfast Health and Social Care Trust), University of North Carolina (UNC) at Chapel Hill, NC in the USA (UNC Hospital Pediatric and Adult Clinics) and Dublin in Ireland (Beaumont Hospital and Our Lady's Children's Hospital, Crumlin) were prospectively enrolled into this study between July 2010 and November 2013. Subjects who had undergone transplant, were taking CF transmembrane conductance regulator potentiators/modulators or enrolled in interventional therapeutic trials were excluded. Ethical

approval for the study was obtained at each institution, and informed consent/assent was obtained from all adults, parents and paediatric subjects.

Either bronchoalveolar lavage (BAL) or sputum (if able to expectorate) and concomitant clinical information were collected when subjects were clinically stable, defined as not having received antibiotics beyond chronic maintenance therapy and ≥ 6 weeks post-completion of intravenous antibiotic therapy. Bronchoscopy was performed at UNC if a child had a procedure requiring anaesthesia and for routine surveillance at Dublin. At both sites, bronchoscopy was performed utilising a laryngeal mask airway or endotracheal tube.

Clinical data

Demographics, anthropometrics, lung function, medication history and disease complications were entered into a centralised database. Genotype was categorised by number of F508del alleles. Disease severity was assessed by nutritional status, pancreatic status and lung function (forced expiratory volume in 1 s (FEV₁) % pred; see supplementary material) if subjects were able to reliably perform spirometry as per American Thoracic Society/European Respiratory Society criteria [19]. For comparisons of body mass index (BMI) across paediatric and adult age ranges, BMI was stratified into poor, acceptable and well-nourished categories based on percentiles in children (≤ 5 th, 6–50th and > 50 th) and absolute BMI in adults [20].

Bacterial isolation and identification

Standardised culture protocols were used across the three sites as previously described [6], with further details provided in the supplementary material. Briefly, samples were treated for 15 min with dithiothreitol prior to serial dilution and plating on both selective and nonselective media (supplementary table S1). Plates were incubated under aerobic–micro-aerophilic and anaerobic conditions for between 2 and 7 days. All bacteria detected were quantified (CFU·g⁻¹ or CFU·mL⁻¹ in BAL) by total viable count. Each distinct colonial morphotype was identified by full-length 16S rRNA gene sequencing. Strict anaerobes were defined as bacteria known not to survive under atmospheric oxygen tension, whereas facultative anaerobic and aerobic bacteria that grew under anaerobic conditions were included as aerobes in all analyses.

Statistical analysis

Demographics, markers of disease severity and bacterial prevalence (presence of bacteria at any CFU > 0) were compared across clinical sites using the Chi-squared or Fisher's exact test for categorical variables and the Mantel–Haenszel mean score Chi-squared test for ordinal variables. Associations between presence of bacteria and markers of disease severity were assessed by the Mantel–Haenszel mean score Chi-squared test. Analyses were stratified by site as described.

Multivariate logistic regression was used to test for factors that predict prevalence of specific bacterial genera. Predictors were identified *via* a stepwise model selection (with significance level for entry or staying in the model of $p=0.10$ or $p=0.05$, respectively).

Analyses that included bacterial quantities/density were log-transformed to account for nonnormal distributions using $\log_{10}(\text{CFU}\cdot\text{g}^{-1}+1)$ to incorporate samples with no detectable bacteria. Bacterial diversity measures included richness (number of counted taxa), evenness and diversity (Shannon–Wiener index). Analyses of bacterial communities (network analyses and factor analysis) were conducted on sputum bacterial quantity for the most common genera. Co-occurrence (network analysis) between taxonomic groups was calculated as previously described [21]. Factor analysis as an unsupervised method to group genera was conducted using varimax rotations to achieve independent factor groupings. These factors were used to assign each subject to a group defined by their dominating factor based on similarity of bacterial quantities (see details in supplementary material).

Results

Subjects and samples

255 subjects were enrolled. Sputum samples ($n=200$) were obtained at all three sites, whereas BAL ($n=55$) samples were collected only at UNC ($n=24$) and Dublin ($n=31$). All samples from children aged < 6 years were BAL ($n=39$). Either sputum ($n=34$) or BAL ($n=12$) were collected from subjects aged 6– < 18 years; for subjects aged ≥ 18 years, 166 sputum and four BAL samples were collected.

Subject characteristics by site are shown in table 1. Site-specific differences in the study subjects included older age and fewer subjects positive for F508del mutations in Belfast compared with UNC or Dublin. Fewer subjects in Belfast were undernourished, and, despite being an older cohort, FEV₁ values in Belfast were similar to those in UNC and Dublin, consistent with milder disease in Belfast. Use of chronic medications was lower in those contributing BAL *versus* sputum (supplementary table S2a and b).

Prevalence and abundance of genera with age

18 anaerobic and 39 aerobic genera were cultured (supplementary table S3), representing 167 species (supplementary table S4). The prevalence of one or more strict anaerobic genus at any CFU >0 in a sample was 59% and was higher in sputum (67%) than BAL samples (31%) ($p < 0.001$). Aerobic bacteria were present at any CFU >0 in 197 out of 200 (99%) of sputum and in 51 out of 55 (93%) BAL samples. The mean \pm SEM total viable counts were higher for aerobic than anaerobic bacteria in sputum ($2.27 \times 10^8 \pm 3.80 \times 10^7$ versus $2.86 \times 10^7 \pm 2.07 \times 10^7$ CFU·g⁻¹; $p < 0.001$) and BAL ($3.97 \times 10^7 \pm 3.71 \times 10^7$ versus $2.27 \times 10^4 \pm 1.61 \times 10^4$ CFU·mL⁻¹; $p < 0.001$).

TABLE 1 Patient demographics by site

	Overall	Clinical site			p-value [#]
		Belfast	Chapel Hill	Dublin	
Patients	255	75	65	115	
Age years					
Mean \pm SD	21.9 \pm 12.7	29.1 \pm 12.2	16.1 \pm 10.3	20.5 \pm 12.1	
Median (range)	21.6 (1.0–68.2)	26.2 (8.3–68.2)	14.5 (1.0–50.0)	22.0 (1.0–61.2)	
Age group years	255	75	65	115	<0.0001
0–<6	39 (15)	0 (0)	14 (22)	25 (22)	
6–<13	21 (8)	4 (5)	13 (20)	4 (3)	
13–<18	25 (10)	6 (8)	9 (14)	10 (9)	
18–<25	77 (30)	22 (29)	18 (28)	37 (32)	
25–<30	44 (17)	19 (25)	5 (8)	20 (17)	
\geq 30	49 (19)	24 (32)	6 (9)	19 (17)	
Sex	255	75	65	115	0.13
Female	115 (45)	29 (39)	36 (55)	50 (43)	
Male	140 (55)	46 (61)	29 (45)	65 (57)	
F508del mutation	251	74	63	114	0.001
Homozygote	131 (52)	29 (39)	45 (71)	57 (50)	
Heterozygote	99 (39)	34 (46)	15 (24)	50 (44)	
None	21 (8)	11 (15)	3 (5)	7 (6)	
Pancreatic status	255	75	65	115	0.61
Pancreatic sufficiency	223 (87)	64 (85)	59 (91)	100 (87)	
Pancreatic insufficiency	32 (13)	11 (15)	6 (9)	15 (13)	
BMI	235	74	65	96	0.01
Undernourished	26 (11)	3 (4)	9 (14)	14 (15)	
Acceptable	118 (50)	34 (46)	37 (57)	47 (49)	
Well nourished	91 (39)	37 (50)	19 (29)	35 (36)	
FEV₁ % pred[¶]	212	74	51	87	0.02
<41	42 (20)	10 (14)	5 (10)	27 (31)	
41–80	115 (54)	48 (65)	28 (55)	39 (45)	
>80	55 (26)	16 (22)	18 (35)	21 (24)	
Sample type	255	75	65	115	<0.0001
BAL	55 (22)	0 (0)	24 (37)	31 (27)	
Sputum	200 (78)	75 (100)	41 (63)	84 (73)	
Chronic antibiotics	173 (68)	58 (77)	43 (66)	72 (63)	0.10
Flucloxacillin	18 (7)	8 (11)	1 (2)	9 (8)	0.09
Azithromycin	127 (50)	44 (59)	37 (57)	46 (40)	0.02
Inhaled antibiotic ⁺	130 (52)	42 (56)	30 (47)	58 (52)	0.58
Any mucolytic	205 (80)	59 (79)	54 (83)	92 (80)	0.79
DNase	164 (64)	54 (72)	43 (66)	67 (58)	0.15
Hypertonic saline	109 (43)	15 (20)	37 (57)	57 (50)	<0.0001
Inhaled corticosteroid	105 (41)	31 (41)	36 (55)	38 (33)	0.004
Antacid[§]	129 (51)	32 (43)	41 (63)	56 (49)	0.05
Insulin	34 (13)	7 (9)	8 (12)	19 (17)	0.35

Data are presented as n or n (%), unless otherwise stated. BMI: body mass index; FEV₁: forced expiratory volume in 1 s; BAL: bronchoalveolar lavage. Patients were continuously enrolled at each site, not necessarily reflecting the study site population. #: comparisons across sites using the Chi-squared, Mantel-Haenszel mean score Chi-squared or Fisher's exact test; ¶: based on Global Lung Initiative reference values [see supplementary material]; +: tobramycin, colistin and aztreonam; §: antacids, H₂-blockers and proton pump inhibitors.

The ratio of anaerobic to aerobic viable counts for BAL and sputum showed no differences across age groups (supplementary figure S1a). Bacterial richness was lower in BAL (3.03 ± 0.31) compared with sputum (5.18 ± 0.16) ($p < 0.001$), but similar within each sample type over age sextiles that approximate clinical progression (figure 1a). Shannon diversities showed similar trends (supplementary figure S1b). 16 genera that had a $\geq 5\%$ prevalence across BAL and sputum samples are shown by age sextiles in supplementary table S5. The most prevalent anaerobic and aerobic genera across all ages were *Prevotella*

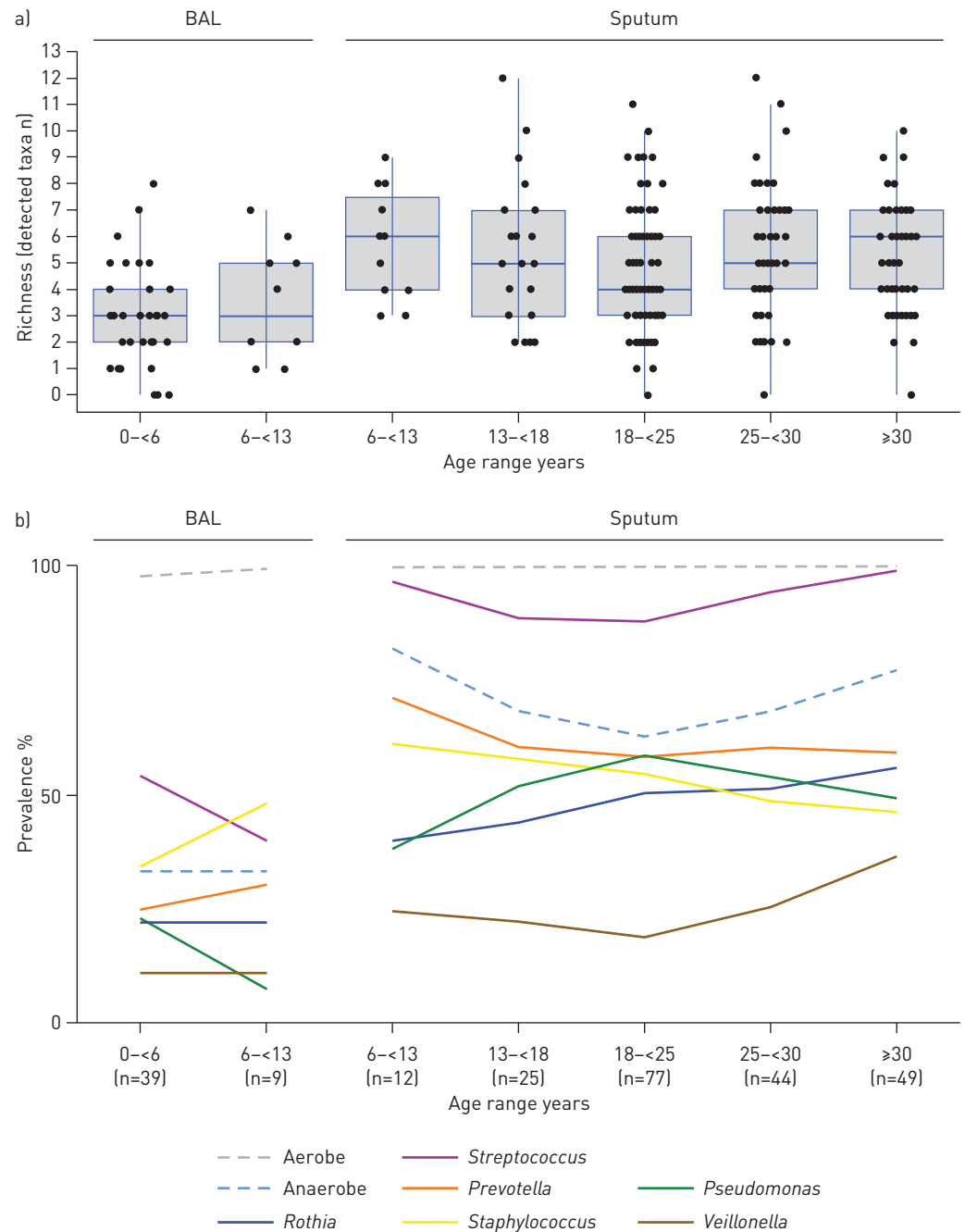


FIGURE 1 Changes in bacterial communities and prevalence with subject age: a) richness across age sextiles, and b) prevalence of anaerobes, aerobes and specific genera across age sextiles. BAL: bronchoalveolar lavage. a) Richness, defined as number of anaerobic and aerobic genera detected at any CFU >0 per sample, did not differ between age groups within sample types (BAL and sputum). Boxes indicate the 25th–75th quartiles with horizontal lines indicating the median; whiskers are 1.5 times the interquartile range. b) Prevalence for the most prevalent bacteria shown by age group. The number of samples for each group is given in parentheses. Bacterial density and prevalence were lower in BAL than sputum. Only BAL samples are included in subjects aged <6 years.

(51%) and *Streptococcus* (82%), respectively. More prevalent bacteria were more abundant with the exception of *Burkholderia* with a 5% prevalence, but being most abundant when present, and *Prevotella* with high prevalence, but low abundance. Changes in prevalence of the most frequently cultured anaerobes and aerobes with age showed decreasing prevalence from childhood to mid-adulthood, with subsequent increases for anaerobic genera and *Streptococcus*. Inverse trends were seen for *Pseudomonas* (figure 1b).

Analyses of samples by sites revealed differences in bacterial richness across sites, but similar diversity in sputum samples (supplementary figure S2a). There were no differences in richness or diversity in BAL samples from Dublin versus UNC (supplementary figure S2b). The prevalence of *Pseudomonas* was highest at UNC, with *Burkholderia*, *Haemophilus*, *Actinomyces*, *Rothia* and *Gemella* being more prevalent in Belfast. *Neisseria* prevalence was lowest in Belfast. Dublin had the lowest prevalence of *Prevotella* and *Veillonella*. Further details are shown by site and sample type in supplementary table S3 and supplementary figure S3.

Relationship between bacterial prevalence, abundance and disease severity

In subjects who produced sputum (n=200), anaerobes were associated with phenotypically milder disease, e.g. better lung function and BMI, and with pancreatic sufficiency, absence of insulin and chronic

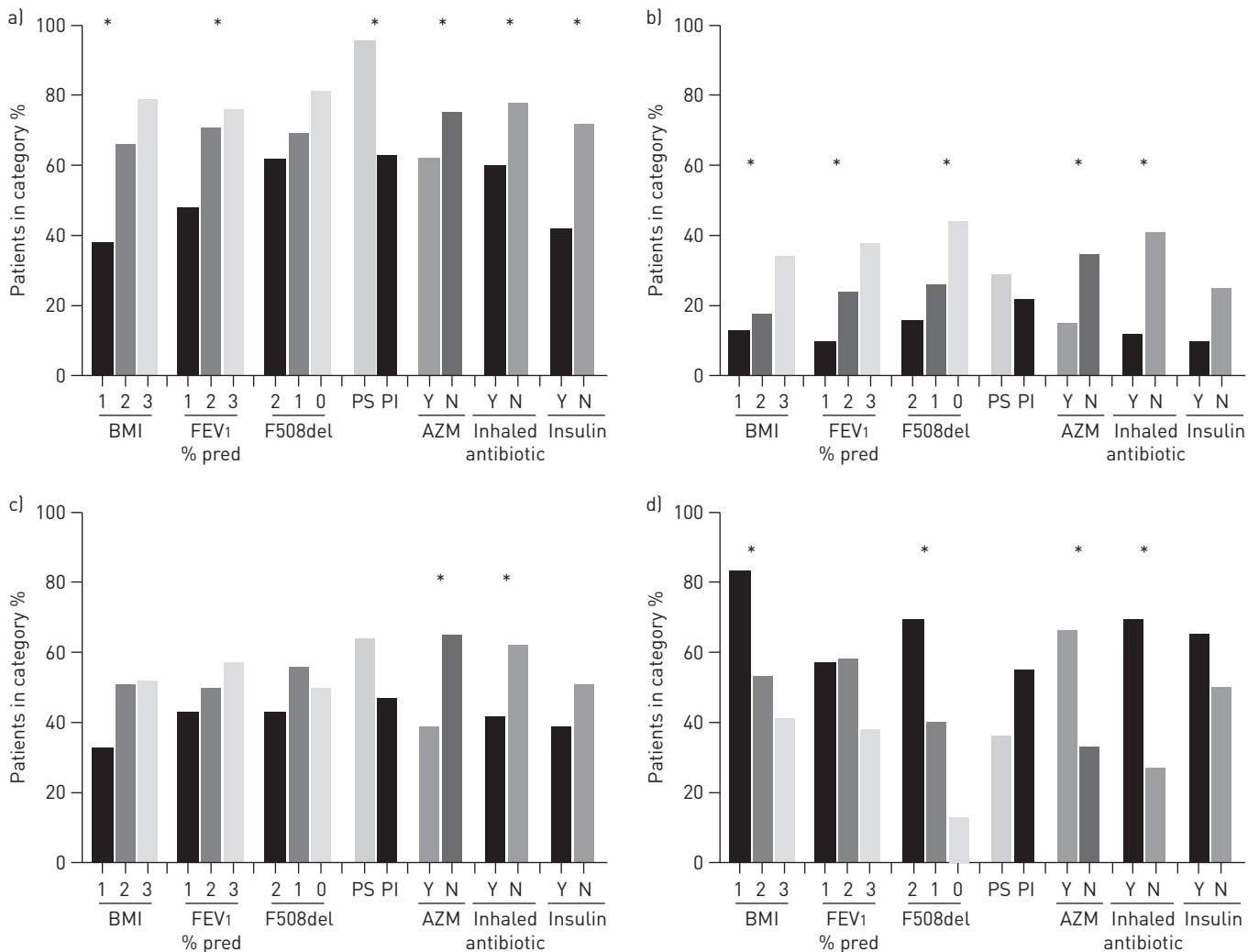


FIGURE 2 Prevalence as percentage of subjects within the category with any CFU >0 for a) anaerobes, b) *Haemophilus*, c) *Staphylococcus* and d) *Pseudomonas* in sputum and their association with clinical characteristics. BMI: body mass index; FEV1: forced expiratory volume in 1 s; PS: pancreatic sufficiency; PI: pancreatic insufficiency; AZM: azithromycin. 200 sputum samples included. Seven subjects had missing data for FEV1 and eight subjects had missing data for BMI. BMI categories are 1: poor nutritional status; 2: adequate; 3: well nourished. FEV1 % pred categories are 1: <40%; 2: 40–80%; 3: >80% (see supplementary material). Numbers per category are given as percentage of subjects positive for this characteristic, where each category (e.g. "BMI 1") can reach 100%. Inhaled antibiotics included tobramycin, colistin and aztreonam. *: significant difference between groups by the Mantel-Haenszel mean score Chi-squared test stratified by site.

TABLE 2 Odds ratios from logistic regression models of bacterial prevalence in sputum

	Anaerobes	<i>Haemophilus</i>	<i>Staphylococcus</i>	<i>Pseudomonas</i>
Patients n	197	197	200	195
F508del 1 versus 0				8.24 [1.00–68.20]
F508del 2 versus 0				17.63 [2.13–146.04]
Pancreatic insufficiency	0.12 [0.03–0.53]			
Azithromycin		0.41 [0.18–0.92]	0.35 [0.19–0.62]	2.26 [1.10–4.67]
Inhaled antibiotic		0.26 [0.12–0.57]		3.97 [1.96–8.05]
Centre C versus B	0.58 [0.22–1.52] [#]	0.41 [0.15–1.08] [#]		
Centre D versus B	0.17 [0.08–0.38]	0.17 [0.07–0.41]		

Data are presented as OR (95% CI), unless otherwise stated. Variables evaluated for model inclusion were: sex, age, centre (B: Belfast; C: Chapel Hill; D: Dublin), forced expiratory volume in 1 s and body mass index as categories (as in figure 2), F508del, pancreatic status, chronic use of flucloxacillin, azithromycin, inhaled antibiotics, DNase, hypertonic saline, corticosteroids, and antacids. Blank cells indicate these variables were not selected for the final model because they were not significant predictors as determined by stepwise model selection with $p=0.10$ for entry and $p=0.05$ for staying in the model. [#]: difference is not significant but comparison included in model for comparison to centre D versus B.

antibiotic use, but not genotype (figure 2a). *Haemophilus influenzae*, an aerobe observed in early CF disease, followed a similar pattern (figure 2b). *Staphylococcus* exhibited an intermediate pattern with negative associations, *i.e.* was less prevalent, with use of chronic antibiotics (figure 2c). The pattern for *Pseudomonas*, however, was distinctly different from anaerobes. *Pseudomonas* prevalence was positively associated with worse BMI, number of F508del alleles and chronic antibiotics (figure 2d).

Logistic regression adjusting for covariates with $p<0.05$ identified through stepwise modelling revealed a lower prevalence of anaerobes in pancreatic-insufficient subjects (table 2). With respect to aerobes, a lower prevalence of *Haemophilus* and *Staphylococcus* was associated with use of azithromycin. *Pseudomonas* prevalence again was associated with F508del alleles, and both azithromycin and inhaled antibiotic use. For BAL, clinical or site variables were not associated with bacterial prevalence.

No associations were observed for absolute anaerobic or aerobic viable counts and clinical variables or study site in sputum or BAL. However, calculation of the anaerobe/aerobe CFU ratio showed that subjects with a relatively higher load of anaerobes had milder disease (pancreatic sufficiency and higher BMI) than aerobe-dominated subjects (supplementary figure S4).

Community structure in sputum and association with subject characteristics

Bacterial communities reflecting the 16 genera with a $\geq 5\%$ prevalence were explored in sputum samples ($n=200$) using microbial co-occurrence network analysis. Bacteria typical of the oropharynx, *e.g.* *Streptococcus*, *Rothia* and *Gemella*, associated with each other. In contrast, *Staphylococcus*, *Pseudomonas* and *Burkholderia* each formed distinct entities (figure 3).

Next, factor analysis was used to reduce the complexity of the microbiota per sample into a set of variables that best defined sample community composition. The bacterial community compositions were generally consistent with the co-occurrence networks. Five components/factors explained 49% of the variation (figure 4). Factors 1 and 2 were dominated by oral-associated bacteria, including *Streptococcus*, *Gemella* and *Rothia*. Factor 3 included *Haemophilus* and *Neisseria*. Factor 4 was dominated by obligate anaerobes. *Pseudomonas* defined Factor 5, which was also negatively associated with *Staphylococcus* and *Burkholderia*.

To define the clinical relevance of these microbial communities, patient sputum samples were defined by their dominating factor (table 3). Thus, each sample was assigned to Groups 1–5. There were no differences in pancreatic status, use of azithromycin, mucolytics and anaerobic or aerobic bacterial load/density between groups. Groups 4 and 5 had the highest percentage of F508del homozygous subjects, yet differed significantly with regard to both anaerobes and *Pseudomonas*. The group with higher anaerobic density/diversity (Group 4) tended to have a higher FEV₁ and a lower incidence of poor nutritional status compared with the *Pseudomonas*-dominated Group 5. Notably, Group 3 included the fewest subjects with poor nutritional status and the most with a high FEV₁, compared with other groups, but had the highest proportion of subjects aged <20 years.

Discussion

Anaerobic and aerobic bacteria were cultured from respiratory samples across all age ranges to assess bacterial prevalence and quantify density in CF respiratory secretions. The prevalence and quantities of both

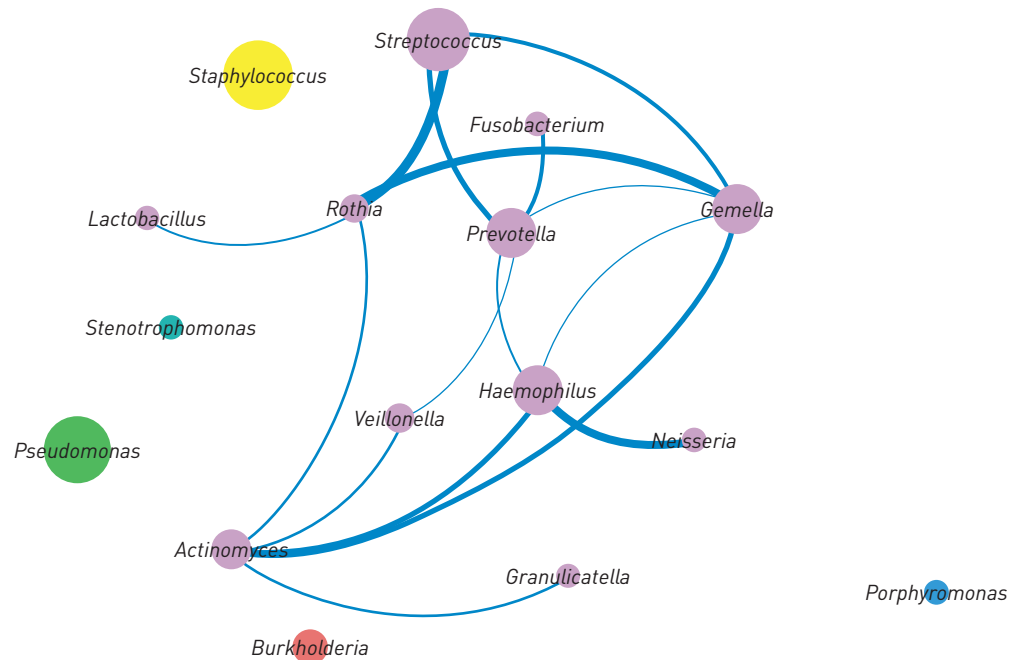


FIGURE 3 Bacterial co-occurrence network between genera detected by extended microbial culture. The 16 genera detected at a $\geq 5\%$ prevalence from sputum samples are contained within the network. Co-occurring microbial taxa are shown with nodes (circles) denoting a particular genus within the network and each edge (blue lines) denoting a significant co-occurrence relationship (Spearman's rank correlation coefficient) for positive correlations (lines); adjusted p-value < 0.05 [Benjamini-Hochberg-Yekutieli false discovery rate correction; see supplementary material]. The size of the corresponding nodes demonstrates the relative proportion of each genus within the current study and the thickness of the edges represents the strength of the corresponding associations.

anaerobic and aerobic bacteria were lower in BAL compared with sputum. Interestingly, the anaerobe/aerobe ratios in BAL, *i.e.* incorporating quantity, were similar to sputum. Facultative anaerobes and micro-aerophilic bacteria were also cultured in both sample types (supplementary table S3) and were included with aerobes, potentially underestimating the true anaerobic burden in the CF lung. *Pseudomonas* and *Staphylococcus* also grow anaerobically, a growth mode associated with persistence in chronic infection, but the focus in this study was on strict anaerobes as determined in molecular studies. The prevalence of anaerobes and *Streptococcus* declined from early childhood to early adulthood, *i.e.* a time when many patients experience worsening disease. In older age groups, anaerobes were more prevalent again, possibly related to less severe disease (figure 1b) as reflected by the higher anaerobe/aerobe ratio in subjects with better nutritional status (supplementary figure S4). Anaerobic and streptococcal species are highly abundant in oral secretions, and are detected in the oral cavity in the first months of life [22]. Although oral secretions are a source of contamination of sputum and BAL, the high abundance of anaerobic genera makes sample contamination an unlikely explanation for our findings. Although BAL samples were obtained *via* a laryngeal mask airway or endotracheal tube, which decreases the risk of contamination, the lower anaerobe prevalence in BAL compared with sputum likely reflects the younger BAL subject age with lower bacterial load and sample dilution. Aerobe prevalence and abundance were also lower in BAL than sputum. Typically, BAL is assumed to represent a 30- to 80-fold dilution of epithelial lining fluid [23, 24].

The source of CF lower airways oral bacteria likely reflects micro-aspiration as reported in healthy subjects, in whom oral bacteria are found in proximal airways but continuous mucociliary clearance prevents growth to high densities [25, 26]. We suggest that starting in infancy, abnormal mucociliary clearance in CF enhances retention of aspirated flora in an anaerobic airways environment, allowing anaerobic bacteria to proliferate. Such a scenario is consistent with recent CF infant BAL microbiome analyses [27–29].

The contribution of anaerobic bacteria to lung disease is not clearly understood. This large study, which analysed samples from 255 CF subjects across three sites and a wide age range, characterised the anaerobic and aerobic microbial community composition in the CF airways using extended culture methods. A prior molecular study had shown geographical differences in the CF microbiome [30]. In our study, differences occurred by study site but not by continent, making climate a less relevant factor than genotype (*e.g.* F508del) and associated disease severity. We observed that anaerobic bacteria were more prevalent in

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
<i>Streptococcus</i>	0.672	-0.056	0.166	-0.032	-0.076
<i>Gemella</i>	0.574	0.274	0.087	0.109	-0.098
<i>Rothia</i>	0.550	0.434	-0.162	0.121	0.004
<i>Stenotrophomonas</i>	0.496	-0.329	-0.123	-0.138	0.300
<i>Granulicatella</i>	0.035	0.608	0.219	-0.095	0.150
<i>Lactobacillus</i>	-0.085	0.597	-0.282	0.122	-0.081
<i>Actinomyces</i>	0.320	0.562	0.135	-0.094	-0.235
<i>Neisseria</i>	0.028	-0.075	0.736	-0.057	0.106
<i>Haemophilus</i>	0.162	0.263	0.654	0.123	-0.166
<i>Fusobacterium</i>	-0.182	0.101	0.049	0.654	0.209
<i>Porphyromonas</i>	0.014	-0.201	-0.162	0.630	0.022
<i>Prevotella</i>	0.378	-0.027	0.352	0.557	-0.092
<i>Veillonella</i>	0.165	0.275	0.108	0.454	-0.236
<i>Pseudomonas</i>	-0.034	-0.097	-0.051	0.051	0.735
<i>Staphylococcus</i>	-0.245	-0.265	0.325	0.160	-0.491
<i>Burkholderia</i>	0.179	0.039	-0.318	-0.097	-0.533
Eigenvalue	2.42	1.62	1.37	1.31	1.16
Variance explained %	11.41	9.94	9.80	9.31	8.90

FIGURE 4 Loadings from factor analysis showing groupings of bacteria with each other based on quantity in sputum (n=200 samples). Five factors explain 49% of the total variation. Blue indicates positive associations and red indicates negative associations, with darker colours indicating the robustness of associations.

sputum of subjects with milder disease. Higher lung function, increased BMI, pancreatic sufficiency and absence of requirement for insulin were associated with higher prevalence of anaerobes in univariate analyses (figure 2). Lower prevalence of anaerobes and *Haemophilus* in subjects on azithromycin could be due to the antibacterial activity of azithromycin. Azithromycin exhibits good activity against those organisms in non-CF disease; however, high rates of resistance are seen in CF that may mitigate its activity [31, 32]. Notably, associations with pancreatic sufficiency remained significant when adjusting for multiple medications and clinical parameters (table 2). A sensitivity analyses with $p=0.1$ for staying in the model additionally included FEV₁ for *Pseudomonas*, azithromycin and insulin for anaerobes, and hypertonic saline for *Haemophilus* (supplementary table S6).

Our culture-based findings are consistent with observations from molecular microbiome studies describing higher diversity with less severe CF lung disease [5, 33] and decreasing diversity in patients with more rapid lung function decline [5, 11]. Our quantitative cultures allowed these relationships to be extended to anaerobic and aerobic bacterial density, anaerobe/aerobe ratio and diversity. A higher anaerobe/aerobe ratio was associated with pancreatic sufficiency and better nutrition, with trends for higher lung function in those with a higher anaerobe/aerobe ratio (supplementary figure S4). However, associations between absolute aerobic or anaerobic bacterial concentrations/density and FEV₁ were not detected. We hypothesise that the absence of such an association partly reflects the spatial heterogeneity of CF lung disease, with bacterial density reflecting disease severity in focal bronchiectatic areas, whereas FEV₁ reflects the overall extent of diseased lung. The hypothesis that FEV₁ is not sufficiently sensitive to detect regional, bronchiectatic disease is supported by a study that reported associations between anaerobic density in CF patients and lung clearance index, but not FEV₁ [34]. Moreover, it should be noted that age-based CF-specific lung function parameters developed for children [35] are not available for adults and effects of genetic mutations on lung function were not included. Thus, although we used Global Lung Initiative reference equations (see supplementary material) for comparison across body stature and centre, analyses of relationships of bacterial density with FEV₁ may have been more sensitive if CF- and age-specific references were available.

Analyses of anaerobe pathogenicity *in vitro* may also yield insight into the relationships between anaerobes and CF disease severity. *Prevotella* was the most abundant and prevalent anaerobic genus in this study,

TABLE 3 Patient characteristics within the groups characterised by bacterial communities identified by factor analysis

	Group 1	Group 2	Group 3	Group 4	Group 5	p-value [#]
Patients n	48	35	46	27	44	
Age years	27.56±1.49	28.62±1.75	22.99±1.53	24.34±1.99	26.82±1.56	0.089
Age <20 years	27	17	43	37	20	0.048
Homozygote F508del	40	54	37	64	61	0.046
Null F508del	15	11	6	4	2	0.2
FEV₁ % pred	59.47±3.17 ^f	55.79±3.78 ⁺	75.64±3.28 ^{+,s,f}	61.58±4.26	57.08±3.35 [§]	0.0002
BMI kg·m⁻²	21.9±0.52	22.9±0.7	21.8±0.5	20.8±0.7	20.8±0.5	0.1
BMI category poor nutritional status	10	6	2	15	29	0.0033
Inhaled antibiotic use	67	66	35	56	78	0.0007
<i>Pseudomonas</i>-positive	38	37	39	59	91	<0.0001
<i>Staphylococcus</i>-positive	31	49	78	70	27	<0.0001
Any anaerobe	81	60	72	100	32	<0.0001
Aerobe richness	6.83±0.43 ⁺	6.69±0.50 [§]	6.07±0.44	5.7±0.57 [¶]	4.57±0.45 ^{+,§}	0.0038
Anaerobe richness	1.75±1.19 ⁺	1.20±0.23	1.59±0.20 [§]	3.22±0.26 [¶]	0.68±0.21 ^{+,§}	<0.0001

Data are presented as mean±SD or %, unless otherwise stated. BMI: body mass index; FEV₁: forced expiratory volume in 1 s. Groups 1 and 2 dominated by oral bacteria; Group 3 *Haemophilus* and *Neisseria*; Group 4 anaerobes; Group 5 *Pseudomonas*-dominated. Inhaled antibiotics included tobramycin, colistin and aztreonam. [#]: p-values for comparison of categorical (Pearson) or continuous variables (ANOVA) across groups (p<0.05 considered significant). Adjusted difference (Tukey's): [¶]: differs from all other groups; ^{+,§,f}: differences to corresponding symbol by row.

and has been reported consistently in other studies [5, 6, 10, 36–38]. *In vitro* studies of laboratory strains of *Prevotella* demonstrated that they produce short-chain fatty acids and proteases that may be pro-inflammatory [9]. However, *Prevotella* elicits diminished pro-inflammatory responses in respiratory cell lines and murine models compared with classic aerobic pathogens [39]. Thus, conceivably the relatively lower virulence of anaerobes contributes to their association with a milder CF phenotype.

Co-occurrence networks to visualise potential bacterial interactions showed groupings of orally derived anaerobic bacteria, which occupied niches distinct from the known pathogens (e.g. *Pseudomonas* and *Staphylococcus*) that were themselves highly dissimilar (figure 3). Both *Burkholderia* and *Pseudomonas* release products that are toxic to other bacteria, which may contribute to their distinct niches as well as their dominance [40]. CF registry data also demonstrate decreasing prevalence of *Staphylococcus* in older patients as *Pseudomonas* prevalence increases, suggesting a similar community competition for these two bacteria [41, 42].

Factor analyses (figure 4) revealed bacterial groupings similar to the co-occurrence networks. These factors enabled testing of associations with clinical parameters based on samples defined by the highest factor loadings (table 3). Group 4 with the highest prevalence and diversity of anaerobes had milder disease than the *Pseudomonas*-dominated group, but not the mildest disease, possibly reflecting that Group 4 exhibited the highest proportion of F508del homozygote subjects and moderate/high rates of *Pseudomonas*.

A challenge to the present study is the inclusion of paediatric and adult age groups, necessitating comparisons between BAL and sputum. For age-wide comparisons, prevalence was used as a surrogate in addition to the ratio of anaerobic to aerobic bacterial load. Similar to prior studies, this study is limited by its observational design and thus cannot determine the association *versus* causality with respect to anaerobe-induced disease severity. This difference has an impact when considering targeted anaerobe antibiotic therapy in subjects with CF. A recent study in infants with CF demonstrated more airway inflammation in children whose airway microbiome was dominated by anaerobes compared with “sterile” airways [29]. Anaerobes may also condition the lower CF airways to promote infection with more classic pathogens [43]. Such data may support treatment of anaerobes in early CF disease. In contrast, bacterial culture and molecular studies in older, sputum-producing subjects suggest that a diverse lung microbiota with relatively abundant anaerobes is associated with milder disease. Conceivably, higher anaerobic diversity in adults with milder disease reflects lower antibiotic use in such subjects [44] and/or lower intrinsic virulence of anaerobes [39]. Therefore, targeted “eradication” of anaerobes in older patients, when clinically at baseline, might result in little clinical benefit and more resistant organisms. Anaerobes may increase at onset of exacerbations and therefore their role may fluctuate with short-term changes in disease state [6, 14]. Anaerobes are not routinely cultured for in the clinical setting. This approach may be justified in older subjects if antimicrobial therapy is not being considered.

In summary, anaerobic bacteria were detected in CF respiratory secretions at all ages. In the age groups investigated in this study, aerobes dominated over anaerobes with respect to bacterial numbers. The prevalence of anaerobic genera was associated with milder disease and lower use of antibiotics, suggesting that a diverse anaerobic microbiota may be a marker of better health in older subjects with CF. Sputum anaerobic/aerobic bacterial genera clustered into communities, which were associated with clinical outcomes. Cross-sectional studies do not permit the distinction of anaerobes as markers *versus* causative factors in the severity of lung disease. Collectively, our data suggest that targeted eradication of anaerobes in the context of aerobic pathogenic bacteria may not be warranted until further data are available.

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