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Original research

Lower airway microbiota in COPD and healthy controls

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ABSTRACT

Background The lower airway microbiota in patients with chronic obstructive pulmonary disease (COPD) are likely altered compared with the microbiota in healthy individuals. Information on how the microbiota is affected by smoking, use of inhaled corticosteroids (ICS) and COPD severity is still scarce.

Methods In the MicroCOPD Study, participant characteristics were obtained through standardised questionnaires and clinical measurements at a single centre from 2012 to 2015. Protected bronchoalveolar lavage samples from 97 patients with COPD and 97 controls were paired-end sequenced with the Illumina MiSeq System. Data were analysed in QIIME 2 and R.

Results Alpha-diversity was lower in patients with COPD than controls (Pielou evenness: COPD=0.76, control=0.80, $p=0.004$; Shannon entropy: COPD=3.98, control=4.34, $p=0.01$). Beta-diversity differed with smoking only in the COPD cohort (weighted UniFrac: permutational analysis of variance $R^2=0.04$, $p=0.03$). Nine genera were differentially abundant between COPD and controls. Genera enriched in COPD belonged to the *Firmicutes* phylum. Pack years were linked to differential abundance of taxa in controls only (ANCOM-BC (Analysis of Compositions of Microbiomes with Bias Correction) log-fold difference/q-values: *Haemophilus* −0.05/0.048; *Lachnoanaerobaculum* −0.04/0.03). *Oribacterium* was absent in smoking patients with COPD compared with non-smoking patients (ANCOM-BC log-fold difference/q-values: −1.46/0.03). We found no associations between the microbiota and COPD severity or ICS.

Conclusion The lower airway microbiota is equal in richness in patients with COPD to controls, but less even. Genera from the *Firmicutes* phylum thrive particularly in COPD airways. Smoking has different effects on diversity and taxonomic abundance in patients with COPD compared with controls. COPD severity and ICS use were not linked to the lower airway microbiota.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is an inflammatory disease of the lower airways. COPD often alternates between a stable state of chronic airflow obstruction and episodic exacerbation. Smoking is the main cause of COPD and a known irritant that affects innate and adaptive mucosal immunity in the lower airways. However, not all smokers develop COPD.^{1,2} Smoking has also been associated with an increased risk of pneumonia and with an altered lower airway bacterial community

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Many smokers do not develop chronic obstructive pulmonary disease (COPD), and other causative factors must exist. One potential factor is the lower airway microbiota. Inhaled corticosteroids (ICS) are both known to prevent COPD exacerbation and increase the risk of airway infections in patients with COPD. A dysbiosis of the lower airway microbiota may impact the risk of lower airway infections in COPD.

WHAT THIS STUDY ADDS

⇒ This study describes a loss of evenness in the airway microbiota in bronchoalveolar lavage (BAL) in patients with COPD, with genera belonging to the *Firmicutes* phylum proliferating compared with healthy controls. Additionally, this study suggests that ICS do not disrupt the microbiota in BAL in patients with stable COPD.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Future studies should explore the functionality of the microbiota, both in health and disease, especially in smokers and non-smokers. ICS may not be adversely affecting the microbiota, but this needs confirmation in longitudinal studies.

(microbiota).^{3,4} The lower airway microbiota itself has been suggested to contribute to the development and progression of several lung diseases, including COPD.^{5–7} Even before studies began characterising the lower airway microbiota using gene sequencing tools, it was acknowledged that acute exacerbation of COPD could be triggered by lower airway infections.⁸

Treatment for COPD may include inhaled corticosteroids (ICS) with anti-inflammatory properties. ICS is now recommended for patients with frequent and severe exacerbation, and those with elevated blood eosinophils and known concurrent asthma.⁹ Use of ICS in patients with COPD does however come with an increased risk of pneumonia, a risk of overgrowth of *Streptococcus* and *Haemophilus* spp, and possibly an altered lower airway microbiota.^{10–12} Thus, investigations of the lower airway microbiota in patients with COPD



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and healthy controls, across smoking habits, ICS use and exacerbation history, can be important to best tailor future therapeutic strategies.

The lower airway microbiota in COPD has been studied in different biological samples.^{13–15} Contamination confounds the use of sputum, as the samples pass unprotected through the high biomass oral cavity.^{16,17} Bronchoalveolar lavage (BAL) is less prone to contamination,¹⁷ and the sampling site is directly verified. Although a fairly safe procedure,¹⁸ bronchoscopy is uncomfortable and resource-intensive compared with sampling of sputum. Previous studies using BAL have tended to include small numbers of participants and controls.^{3,15,19–23} The largest study previously published is a substudy from the SPIROMICS cohort with 78 patients with COPD and 103 controls.¹³ The Bergen COPD Microbiome Study (MicroCOPD) is a single-centre study, where 100 patients with COPD and 100 controls were sampled with equipment designed for protected BAL sampling, reducing the risk of oropharyngeal contamination to a minimum.¹⁷

With this material at hand, we looked for differences in the bacterial composition between patients with COPD and healthy controls, and for patients with COPD, we investigated the effect of disease severity, use of ICS and exacerbation history.

METHODS

Study population

The MicroCOPD Study protocol has been published.²⁴ From the 249 participants enrolled at the Department of Thoracic Medicine, Haukeland University Hospital, Bergen, Norway in 2012–2015, the current study included all 97 patients with COPD and all 97 healthy controls with valid protected BAL samples (online supplemental figure 1). All bronchoscopies were performed without any other indication than study sampling. Patients with COPD were examined in a stable state defined as not having ongoing respiratory symptom exacerbation, and no use of antibiotics or oral corticosteroids within the last 2 weeks before inclusion. Subjects who met the eligibility criteria for the study but had recently used antibiotics or oral corticosteroids were requested to return to the outpatient clinic at a later date. Inclusion, collection of baseline data and the bronchoscopy procedure were scheduled within the same day.

The structured interviews performed included information on smoking habits, medication and exacerbation history. The COPD diagnosis was verified using clinical evaluation and standardised spirometry with a forced expiratory volume in 1 s (FEV₁)/forced vital capacity (FVC) cut-off at 0.7 according to international guidelines.⁹ The Global Initiative for Chronic Obstructive Lung Disease (GOLD) staging system was used to classify disease severity.⁹ COPD I/II had post-bronchodilator 80% > FEV₁ > 50% of expected, and COPD III/IV had FEV₁ < 50% of expected. Controls were enrolled if no known lung diseases and no significant airflow obstruction were present. To be classified as a non-smoker, participants had to be smoke-free > 1 year before inclusion. A total of 22 controls had FEV₁/FVC < 0.7 indicating obstructive airways, but with no other objective or subjective findings supporting a COPD diagnosis. After initial inclusion of all participants, three experienced pulmonologists at our department reviewed inclusion according to available spirometry, CT scans and medical history. One person included in the COPD cohort was recategorised as a healthy control, whereas five healthy participants were reclassified as patients with COPD.

Factors contradicting bronchoscopy and thus inclusion included oxygen saturation < 90% with oxygen supplementation, partial pressure of carbon dioxide in arterial blood > 6.65 kPa,

known increased bleeding risk, a known allergy towards the premedication, cardiac risk and use of antibiotics within 2 weeks prior to bronchoscopy. If measures of FEV₁ were both < 30% predicted and < 1 L, BAL sampling was not performed.²⁴

Sample collection

Bronchoscopy was performed through oral access with the patient in the supine position. Protected BAL was collected from all participants with FEV₁ > 30% predicted and five patients with FEV₁ < 30% predicted, but > 1 L. Sterile phosphate-buffered saline (PBS) was instilled in the right middle lobe through a sterile catheter (Combicath, Prodimed) with a sealed wax tip, followed by aspiration through the same catheter. PBS used for bronchoscopic sampling served as a negative control for the microbiota analysis. Further details on the bronchoscopic sampling procedure are published²⁵ and are available in the online supplemental file.

DNA extraction and target gene sequencing

A detailed protocol for laboratory processing has been published.²⁶ Bacterial DNA was extracted using both enzymatic and mechanical lysis methods. Samples were treated with the enzymes lysozyme, mutanolysin and lysostaphin (Sigma-Aldrich) as recommended.²⁷ Following DNA extraction, processing with the FastPrep-24 instrument with reagents from the FastDNA Spin Kit (MP Biomedicals, Solon, Ohio, USA) was performed. Amplicon PCR (45 cycles) and index PCR were run using primers from the Nextera XT Index Kit (Illumina, San Diego, California, USA). Finally, paired-end sequencing (2 × 300 base pairs) of the V3–V4 region of the 16S rRNA gene as instructed by the protocol for Metagenomic Sequencing Library Preparation for the Illumina MiSeq System was performed (Part #15044223 Rev. B, MiSeq Reagent Kit V3).

Statistical analyses

Bioinformatic terms and handling of the sequencing output are described in the online supplemental file. Briefly, the bioinformatic pipeline QIIME 2 V.2022.11²⁸ and the statistical computing environment R²⁹ were used. The sequencing output underwent quality control, sorting of sequences in amplicon sequence variants (ASVs) and removal of contaminants. Diversity analyses were preceded by normalising all samples at 2200 reads/sample, determined from rarefaction curves (online supplemental figure 2). To capture the complexity of the data, different indexes were calculated. For alpha-diversity: observed ASVs (richness), Pielou evenness, Shannon entropy and Faith's phylogenetic diversity (PD) (phylogenetic). For beta-diversity: Sorensen and Bray-Curtis (non-phylogenetic), weighted and unweighted UniFrac (phylogenetic) and Aitchison (compositional) distance matrices. To identify differentially abundant taxa, we used Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) in R.³⁰ Only ASVs found in > 10% of the samples were considered.³¹ The handling of zero counts is described in the online supplemental file.^{30,32}

Data generated by ST and TMLE for this manuscript including sequencing data, relevant QIIME 2 and R code, and the meta-data are available at the DRYAD data repository: <https://doi.org/10.5061/dryad.rfj6q57ff>.³³

RESULTS

Participant characteristics analysed for differences between current and non/ex-smokers are presented in table 1. Non-smoking patients with COPD were significantly older than

Table 1 Participant characteristics

	Current smoking COPD (n=35)	Non/ex-smoking COPD (n=62)	Current smoking controls (n=25)	Non/ex-smoking controls (n=72)	P value*
Sex					0.8
Female	48.6	41.9	48.0	40.3	
Male	51.4	58.1	52.0	59.7	
Age years, median (IQR)	68.5 (14.4)	68.9 (7.5)	62.7 (8.6)	67.7 (11.1)	0.04
Age					0.03
<60	25.7	14.5	36.0	19.4	
60–69.9	31.4	46.8	56.0	48.6	
70+	42.9	38.7	8.0	31.9	
Pack years	27.3 (21)	30.0 (21.2)	19.5 (15)	15.4 (20)	<0.0001
Lung function†					
FEV ₁ /FVC	0.49 (0.19)	0.47 (0.16)	0.70 (0.09)	0.74 (0.05)	<0.0001
FEV ₁ (% predicted)	59.7 (25.8)	61.8 (24.1)	100.4 (23.6)	106.1 (16.8)	<0.0001
FVC (% predicted)	94.7 (17.0)	98 (17.6)	110.5 (15.3)	113.2 (12.2)	<0.0001
COPD GOLD class					<0.0001
GOLD I/II (FEV ₁ >50%)	62.9	72.6			
GOLD III/IV (FEV ₁ <50%)	37.1	27.4			
Inhaled corticosteroids					<0.0001
Yes	54.3	66.1			
No	45.7	33.9			
Frequent exacerbation‡					<0.0001
Yes	22.9	11.3			
No	77.1	88.7			

Smoking defined as having smoked within the last year of inclusion. All others defined as non-smoking. Count values are percentages.

*Statistics: categorical data: Fisher's exact test; continuous data: Shapiro-Wilk test for distribution. FVC (mean/SD) only parametric variable. Others are median/IQR. Kruskal-Wallis rank-sum test for overall comparison.

†Post-bronchodilator FEV₁.

‡Frequent exacerbation was defined as >1 exacerbation/year.

COPD, chronic obstructive pulmonary disease; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; GOLD, Global Initiative for Chronic Obstructive Lung Disease.

smoking controls (Dunn's test $p=0.02$). Patients with COPD had smoked more pack years than controls. In the COPD cohort, the use of ICS was more common among non-smokers than smokers. Current smoking patients with COPD had more frequent exacerbation episodes (table 1).

In online supplemental table 1, the overall significant differences found using Kruskal-Wallis test were supplied with Dunn's test to show which pairs of participant groups from table 1 were significantly different.

Sequencing results

In BAL from patients with COPD, 1.79 million sequences were assigned to 399 ASVs. Sixty-two ASVs were unique to the COPD cohort. BAL from all 97 controls contained 1.58 million sequences assigned to 366 ASVs, of which 29 were not seen in patients with COPD (online supplemental figure 1).

Diversity

Alpha-diversity was lower in COPD compared with controls when considering Pielou evenness and Shannon entropy. Observed ASVs (richness) and Faith's PD were not associated with disease (figure 1).

Alpha-diversity was neither associated with FEV₁ in per cent predicted or GOLD stage as a dichotomous variable, nor the use of ICS in the COPD cohort. Lower richness and PD in BAL were found in those with frequent exacerbation (observed ASVs:

OR 0.95, CI 0.92 to 0.99, $p=0.02$; Faith's PD: OR 0.45, CI 0.23 to 0.88, $p=0.02$). No significant associations remained after adjusting for disease severity, sex, age and smoking (online supplemental tables 2–4).

Beta-diversity did not differ significantly between patients with COPD and controls, or across sex and age. Among patients with COPD, significant differences were seen between smokers and non-smokers, but only for the phylogenetic and abundance-aware weighted UniFrac matrix (permutational analysis of variance: effect size $R^2=4\%$, $p=0.01$).

Taxonomy

The relative abundances of phyla and genera in each sample, categorised by smoking status in participants with and without COPD, are presented in figure 2A,B. Taxa with an average relative abundance of less than 1% among all participants were categorised as low abundant and grouped as 'others'.

Online supplemental figure 3 shows differentially enriched ASVs in both groups, annotated with the highest resolution taxonomic information achieved from a self-trained Naive Bayes classifier and the Silva database V.138.1. Differential abundance was further tested with ANCOM-BC comparing patients with COPD with controls and comparing the two groups of participants separately. We investigated the effect of sex, age, smoking, and for the COPD cohort, COPD severity, ICS and exacerbation frequency. The results are summarised in table 2.

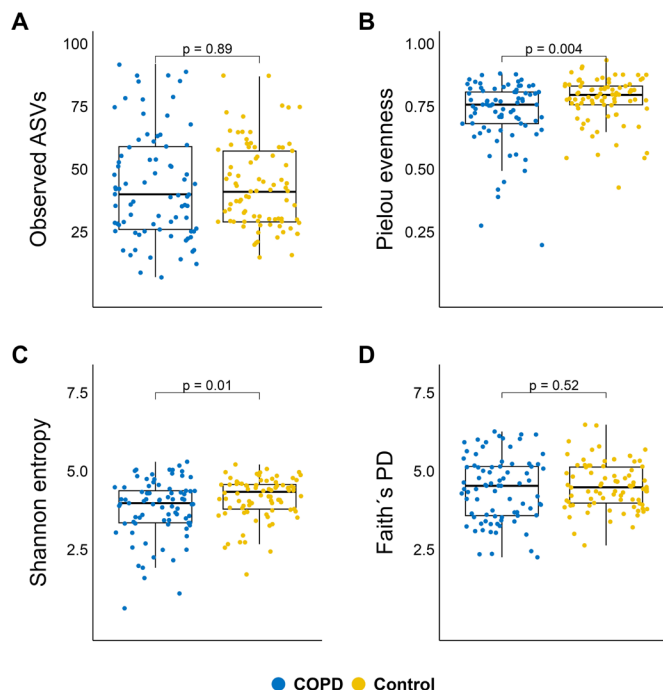


Figure 1 Alpha-diversity in bronchoalveolar lavage samples from participants with and without chronic obstructive pulmonary disease (COPD) showing significantly different evenness between groups after logistic regression analyses adjusting for sex, age and smoking (smoking vs non-smoking within the last year of inclusion). (A) Counts of different amplicon sequence variants (ASVs), that is, richness; (B) Pielou evenness; (C) Shannon entropy and (D) Faith's phylogenetic diversity (PD).

Comparing the taxa in all BAL from patients with COPD against all BAL from controls, *Firmicutes* and nine genera were significantly differentially abundant (table 2 and figure 3A). The three genera enriched in patients with COPD all belonged to the *Firmicutes* phylum. *Solobacterium*, *Prevotella*, *Prevotella_7* and *Alloprevotella* representing the *Bacteroidota* phylum were enriched in controls. The largest log-fold differences were found for *Leptotrichia* belonging to the *Fusobacteriota* phylum, and *TM7x* belonging to the *Patescibacteria* phylum. Figure 3B shows the mean relative abundances of the nine genera in controls, patients with mild/moderate COPD (COPD I/II) and patients with severe/very severe COPD (COPD III/IV). *Granulicatella* and *Gemella* both had their highest mean relative abundances in BAL from patients with mild/moderate COPD.

For the characteristics sex, age and pack years, we found no differentially abundant taxa when including all 194 participants (table 2). While *Alloscardovia* was absent in female patients with COPD, it was found in female controls. The *Bacteroidota* phylum decreased with age in the control group only, but no genera belonging to the *Bacteroidota* phylum were found to be differentially abundant with age. Three low-abundant genera were more abundant in smokers than in non-smokers. Of these, *Filifactor* was the most frequently seen with the presence in only 34 of 194 samples. *Oribacterium* was absent in smoking patients with COPD, whereas no significant difference was linked to smoking in the control group. Pack years were associated with differentially abundant genera only in controls. With increasing smoking history in the control group, *Lachnoanaerobaculum* and *Haemophilus* both diminished. *Lautropia* was never seen in BAL from smoking controls, but *Lautropia* was also present in

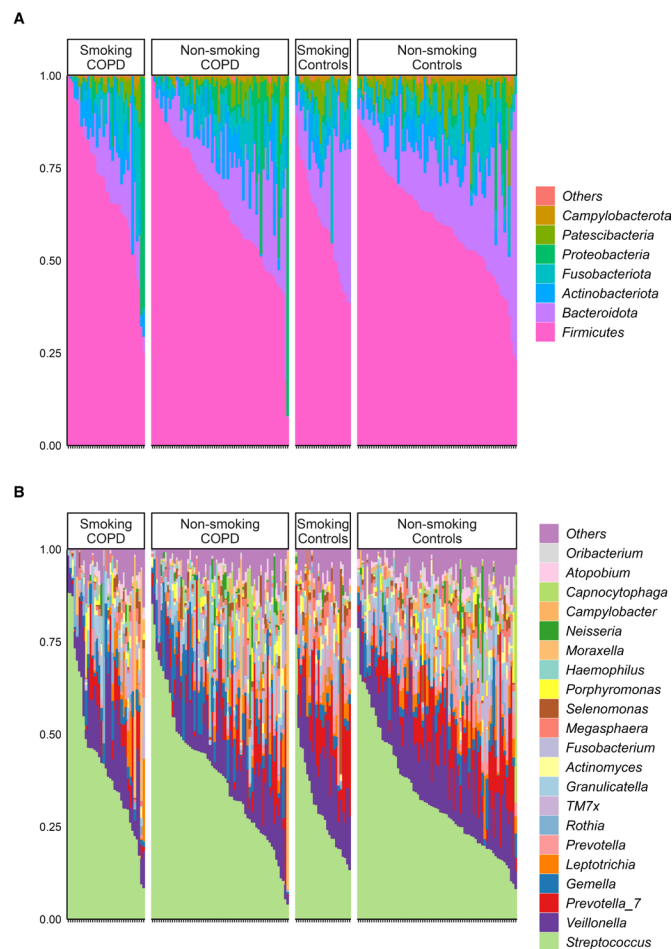


Figure 2 Relative abundances of (A) phyla and (B) genera in bronchoalveolar lavage from participants with and without chronic obstructive pulmonary disease (COPD) stratified by smoking. Non-smoking was defined by smoking cessation >1 year prior to inclusion. Others: taxa with mean relative abundance <1% of the total sequencing data.

only 22 of 194 samples. In the COPD cohort, disease severity and ICS were not associated with the differential abundance of any taxa. In those with frequent exacerbation, both *Catonella* and *Actinobacillus* were absent. These genera were observed in 65 and 22 samples, respectively. *Leptotrichia* had lower abundances in those with frequent exacerbation (table 2).

DISCUSSION

This is to our knowledge the largest bronchoscopy study to date comparing the lower airway microbiota in BAL between patients with COPD and controls, and the first using protected BAL with a sterile inner catheter inserted in the working channel of the bronchoscope. We confirmed a loss in alpha-diversity in COPD compared with controls and found it linked to lesser evenness rather than a loss of richness. We identified nine genera that differed between patients with COPD and controls, of which *Streptococcus* is an important potential pathogenic taxon. Smoking quantified by pack years was associated with a significant reduction in *Haemophilus* and *Lachnoanaerobaculum* in healthy controls. Such associations within the COPD cohort were not found. Neither diversity nor taxonomic abundances differed in BAL fluid with the use of ICS or with increased COPD severity.

Table 2 Differentially abundant* phyla and genera in bronchoalveolar lavage samples from patients with COPD and controls

Comparison†	Taxa‡	LFD	SE	q	Zero§
All participants n=194					
COPD (n=97) vs control (n=97)	Firmicutes	0.76	0.23	0.008	—
	<i>Alloprevotella</i>	−1.02	0.28	0.01	—
	<i>Prevotella</i>	−0.97	0.29	0.02	—
	<i>Prevotella_7</i>	−1.02	0.28	0.009	—
	<i>Solobacterium</i>	−0.81	0.22	0.006	—
	<i>Granulicatella</i>	0.90	0.23	0.004	—
	<i>Streptococcus</i>	0.46	0.14	0.03	—
	<i>Gemella</i>	0.84	0.23	0.01	—
	<i>Leptotrichia</i>	−1.13	0.29	0.004	—
	<i>TM7x</i>	−1.35	0.33	0.002	—
Sex	—	—	—	—	—
Age	—	—	—	—	—
Smoking	—	—	—	—	—
Pack years	—	—	—	—	—
Smoking vs non-smoking	<i>Alloscardovia</i>	0.82	0.25	0.04	—
	<i>Peptoniphilus</i>	0.81	0.23	0.01	—
	<i>Filifactor</i>	0.99	0.24	0.001	—
COPD	—	—	—	—	—
Sex	<i>Alloscardovia</i>	—	—	—	Males only
Age	—	—	—	—	—
Smoking	—	—	—	—	—
Pack years	—	—	—	—	—
Smoking vs non-smoking	<i>Oribacterium</i>	−1.46	0.43	0.03	—
	—	—	—	—	—
	—	—	—	—	—
Frequent exacerbation	—	—	—	—	—
Yes vs no	<i>Catonella</i>	—	—	—	No only
	<i>Leptotrichia</i>	−2.16	0.61	0.02	—
	<i>Actinobacillus</i>	—	—	—	No only
Control	—	—	—	—	—
Sex	—	—	—	—	—
Age	Bacteroidota	−0.05	0.02	0.03	—
Smoking	—	—	—	—	—
Pack years	<i>Lachnoanaerobaculum</i>	−0.04	0.01	0.03	—
	<i>Haemophilus</i>	−0.05	0.02	0.048	—
Smoking vs non-smoking	<i>Lautropia</i>	—	—	—	Non-smoking only

*Analysis of Compositions of Microbiomes with Bias Correction: a differential abundance analyses where the unknown sampling fractions are estimated, and the bias induced by their differences corrected. Significance level $q < 0.05$.

†Positive LFD means higher abundance in the first listed of compared groups.

‡Phyla in bold, genera in non-bold.

§Zero: taxa identified as structural zeros, meaning they are present in the group mentioned and absent in the group not mentioned.

COPD, chronic obstructive pulmonary disease; LFD, log-fold difference; q, Holm-adjusted p values.

Alpha-diversity has previously been reported to be decreased in airway samples from patients with COPD compared with controls, and to be further lowered with disease progression^{13 14 20} and during COPD exacerbation.²³ In our study, we demonstrate that while evenness was reduced, both richness and PD were sustained in the lower airways of patients with COPD compared with healthy controls. The lower airway microbiota is proposed

to be constantly modulated by the addition of bacteria through inhalation and micro-aspiration, local replication and survival, and removal through mucociliary clearance, cough, and immune responses.^{5 34} It is conceivable that the decrease in evenness but maintenance of richness and PD can be attributed to a continuous influx of upper airway microbiota, with certain taxa thriving more in the inflamed airways, thus diminishing evenness.

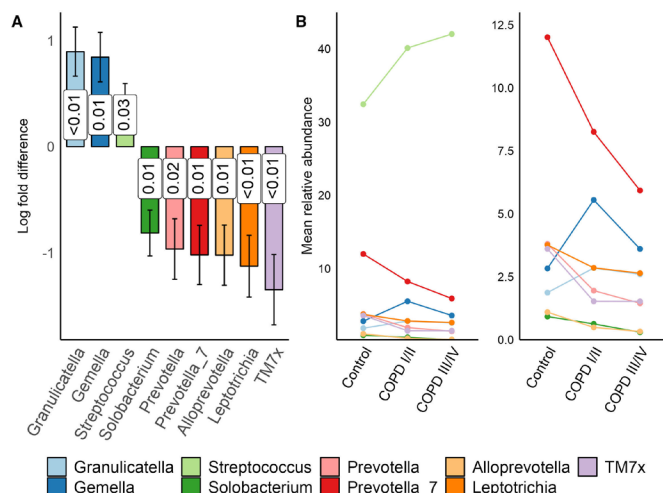


Figure 3 Comparing abundances of genera across COPD and controls. (A) Analysis of Compositions of Microbiomes with Bias Correction found nine genera significantly differentially abundant comparing BAL from participants with and without COPD (positive log-fold difference=more abundant in COPD, q values for each taxon in plot: significance level $q < 0.05$ after Holm correction). (B) Mean relative frequencies of the significantly differentially abundant genera in BAL from controls and patients with COPD with mild/moderate (GOLD stage I/II) and severe/very severe disease (GOLD stage III/IV). The right panel with cropped y-axis is not displaying *Streptococcus* to emphasise the remaining eight. BAL, bronchoalveolar lavage; COPD, chronic obstructive pulmonary disease; GOLD, Global Initiative for Chronic Obstructive Lung Disease.

The lack of difference in beta-diversity between BAL from patients with COPD and controls agrees with previous studies.^{13 35} COPD is a progressive and heterogeneous disease, with a gliding transition from a healthy state. Differences can be diluted due to this characteristic, and further due to an insufficient number of participants. The beta-diversity analyses comparing smoking and non-smoking patients with COPD showed that differences in abundance and relatedness of ASVs were not independently sufficient to separate between smokers and non-smokers. Only weighted UniFrac taking both aspects into account identified a significant difference. Meanwhile, smoking could only explain 4% of the separation of the samples, indicating a relatively small effect of smoking on beta-diversity.

As expected from the alpha-diversity results, the current study identified several differentially abundant taxa comparing patients with COPD and controls. We observed an increase in *Granulicatella*, *Gemella* and *Streptococcus* in COPD, and a successive increase in *Streptococcus* with increased disease severity. This confirms the results from Opron *et al* and Wang *et al* in BAL and sputum, respectively.^{13 14} *Gemella*, *Streptococcus*, *Prevotella* and *Leptotrichia* were among several genera found to be increased in COPD compared with controls in a study by Ramsheh *et al* using bronchial brush samples.³⁵ Whether the lower airway in COPD has an impaired inflammatory profile in which *Firmicutes* are allowed to multiply at the cost of other taxa should be investigated further.

The remodelling in COPD airways includes different degrees of emphysema, an increased bronchial mucus production, impairment of the mechanisms necessary to remove mucus, remodelling of the airway wall and altered inflammatory activity.^{36 37} This can create an environment in which certain bacteria thrive. One can also imagine that overgrowth of pathogenic bacteria could contribute to the remodelling seen in COPD

airways. Thus, one can imagine a scenario where smokers with an adverse composition of the lower airway microbiota would be more likely to develop disease than other smokers. Madapoosi *et al* examined matched BAL microbiome and metabolomic data in smokers with no known COPD, and with mild and moderate COPD, and suggested that elements from both the lung microbiome and metabolome may jointly influence the pathophysiological mechanisms already in milder stage COPD.³⁸

Sex and age were not strongly associated with the lower airway microbiota in our study. For smoking, consistent changes in the microbiota across the COPD cohort and controls were not found, in line with findings from Opron *et al*.¹³ In a study by Pfeiffer *et al* specifically investigating the effect of smoking on the airway microbiota in 33 healthy smokers and 13 healthy never-smokers, a non-significant negative correlation between pack years and relative abundances of *Haemophilus* in BAL samples was found. The only significant differences in mean relative abundances of genera were found for *Campylobacter* and *Neisseria*.³ Although COPD is a multifactorial disease, the main risk factor for disease development is undoubtedly inhalation of harmful substances, of which tobacco smoking is the most significant. The effect of smoking on the lower airway microbiota in healthy individuals is thus an urgent research question but remains understudied.

In the GOLD strategy documents, ICS is recommended for a carefully selected subpopulation of patients with COPD. The reason is the known increase in the risk of pneumonia and infectious exacerbation.⁹ Keir *et al* conclude their review of ICS and the lung microbiota by stressing that ICS use should be founded on leucocyte endotypes to avoid prescription to patients at a higher risk of *Streptococcus* and *Haemophilus* overgrowth.¹¹ During COPD exacerbation, two longitudinal sputum studies have shown that alpha-diversity decreased when patients were treated with oral corticosteroids.^{39 40} Whether ICS use impacts the lower airway microbiota in COPD was specifically addressed in a randomised controlled trial (the DISARM Study) from Vancouver, Canada.^{12 41} In that study, 56 patients with COPD were sampled with unprotected cytology brushes before and 12 weeks after use or non-use of ICS.¹² Lower richness and Shannon entropy were observed in the ICS-treated. However, the intraindividual differences in alpha-diversity between sampling time points resemble what we observed in a study on the microbiota in induced sputum samples collected across stable state and exacerbation in a COPD cohort.⁴² Whether these intraindividual differences are linked to ICS, or within what to be expected in repeated samples is arguably yet unknown. Leitao Filho *et al* found no links between ICS and *Streptococcus*, but *Haemophilus* was reduced after 12 weeks of treatment with fluticasone.¹² It is notable that the host transcriptome was found to be affected by fluticasone, but not in the non-ICS study arm.⁴¹

In our study, 60 of 97 patients with COPD used ICS. In stable COPD, we did not find evidence of a significant influence of ICS on the lower airway microbiota, including the abundances of *Streptococcus* and *Haemophilus*. These genera were also not linked to ICS in the two bronchoscopy studies by Opron *et al* and Ramsheh *et al*.^{13 35}

Combined, there is yet little current evidence of a link between ICS treatment and unfavourable changes in the lower airway microbiota in patients with COPD.

Frequent exacerbation in COPD was linked to reduced abundance of *Leptotrichia*, a genus belonging to the *Fusobacteriota* phylum. A publication focusing on its pathogenic potential concludes that it rarely causes severe infections, and mainly in immunosuppressed patients. The *Fusobacteriota* phylum is commonly found as a commensal bacterium

in the oral cavity,⁴³ and its role in those with frequent exacerbation is yet elusive.

Despite being one of the most extensive bronchoscopy studies on the lower airway microbiota in COPD, our study has several limitations. First, the cross-sectional design does not permit causal inferences. Future longitudinal and mechanistic studies are needed to determine whether the differences in microbiota between patients with COPD and controls are a cause or a result of disease development and progression. Additionally, the sequencing of target genes like the 16S rRNA only provides information at the genus level, limiting species-level evaluation.⁴⁴ Second, our study relied on self-reported smoking habits, which could have been improved by using biomarkers such as cotinine. Third, we sampled BAL from the right middle lobe in all participants ensuring standardisation. However, this precludes analysing geographical variation within the airway microbiota, which is likely to exist.^{15 20} Ventilation-to-perfusion ratio differs from the upper to lower parts of the lungs, and some airways may receive more micro-aspiration than others. Whether this potential variation is differentially impacted by ICS use is unknown. Fourth, a 2-week wash-out of antibiotics before inclusion may have been too narrow. All medication use was recorded, and out of the 194 study participants, 89 patients with COPD and 90 healthy controls had not used any antibiotics the previous 12 weeks to inclusion. Finally, the control group was not age or smoking matched to the patients with COPD.

CONCLUSION

Differences in the lower airway microbiota related to COPD are evident. The difference lies in an altered microbial balance in which bacteria from the *Firmicutes* phyla appear favoured. This could not be linked to smoking or to the use of ICS in patients with COPD. Whether differences in the lower airway microbiota observed in patients with COPD compared with healthy controls are pathogenic in COPD or are the results of COPD must be further investigated in longitudinal studies.

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Data availability statement Data are available in a public, open access repository. Data are available from the time of publication and without end date at DRYAD depository: <https://doi.org/10.5061/dryad.rfj6q57f>. Submitted data include de-identified participant data (metadata) in .xlsx and .txt format, ASV and representative sequences tables, phylogenetic tree (rooted), and taxonomy in .qza format from QIIME 2, QIIME 2 code and R code. The data have been generated by ST and TME. The study protocol is published and linked to in the manuscript.

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