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Gut microbiota in chronic obstructive pulmonary disease varies by CT-verified emphysema status

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ABSTRACT

Background and aim: The association of the gut microbiota to chronic obstructive pulmonary disease (COPD) phenotypes is underexplored. We aimed to compare stool samples from patients with COPD and subjects without COPD and relate findings to emphysema status, exacerbation rate, blood eosinophil levels, symptom score, and lung function.

Methods: We report findings from a single-centre case–control study with 62 current and former smoking patients with COPD and 49 subjects without COPD. DNA was extracted from stool samples, and the V3V4-region of the bacterial 16S-rRNA gene was sequenced. Emphysema was defined based on thoracic computed tomography (CT thorax) low attenuating areas ≥/<10% at threshold -950 and -910 hounsfield units, respectively. Differential abundance of taxa was evaluated using Analysis of Composition of Microbes with Bias Correction (ANCOM-BC). Beta diversity was compared using a distance-based permanova-test.

Results: The genus *Veillonella* was decreased and a genus belonging to class *Clostridia* was increased in COPD compared with controls without COPD. The composition of microbes (beta diversity) differed in emphysema compared to controls, and 27 genera were differentially abundant in emphysema vs. controls. Nine of these genera belonged to the family *Lachnospiraceae*. Lung function, blood counts and COPD assessment test score correlated with several genera's relative abundance. Of the genera showing significant correlation to lung function, nine belonged to the family *Lachnospiraceae*.

Conclusion: The gut microbiota in COPD differs from that in healthy individuals, even more so in emphysema. In particular, future studies should look into the mechanisms and therapeutic potential of dysbiosis affecting the family *Lachnospiraceae*.

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Microbiota; microbiome; COPD; emphysema; *Lachnospiraceae*

Introduction

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death worldwide [1]. COPD is characterized by persistent airflow limitation and is a heterogeneous condition with multiple phenotypes [2]. The pathogenesis is complex with several mechanisms interacting. Identifying biomarkers associated with specific endotypes would aid in developing new preventive or therapeutic strategies [3].

One potential biomarker is the gut microbiota, which can be defined as the collective of microbes residing in the gut. Microbiota studies have been made possible by the emergence of high-throughput sequencing methods. The gut microbiota has been correlated to many disorders, ranging from inflammatory bowel disease to cancer and major depressive disorders [4]. To a degree, there is evidence that both lung and gut microbiota impact asthma development, phenotype, and severity [5]. For instance, in a seminal study, Arrieta and co-workers showed how the decreased abundance of certain gut microbes in infants 3 months of age was associated with atopy and wheezing illness, highly predictive of asthma [6]. They further substantiated their findings by demonstrating a preventive effect of these microbes in a murine model. However, less is known about the role of the gut microbiome in COPD.

To our knowledge, only a few studies have investigated the gut microbiota of patients with COPD. Bowerman et al. found 146 species to be differentially

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abundant in 28 COPD patients compared with 29 healthy controls [7], while Lai et al. found 15 differentially abundant genera in 37 COPD patients compared to 35 healthy controls [8]. Li et al. underscored the relevance of the distinct gut microbiome of 99 COPD patients in their cohort by demonstrating its effect on inflammation and COPD development by faecal microbiota transplantation to murine models [9]. Illustrating the lack of established gold standard methodology in microbiome studies, the bioinformatic and statistical methods applied in these studies, which all included a control group, varied substantially. Adding to the exploratory nature of these studies, this underscores the need for these associative findings to be replicated in different cohorts, preferably consistently across different methodological methods, to validate the findings. In a study without control subjects, Chiu et al. demonstrated a correlation between the abundance of a *Bacteroides* operational taxonomic unit (OTU) and low blood eosinophils in 60 COPD patients [10], as well as some community shifts in the gut microbiota associated with lung function decline in patients with COPD [11]. Wei et al. used summary data from two large international studies, applied Mendelian randomisation techniques and identified nine gut microbiota taxons with a nominal causal relationship to COPD [12]. In spite of their large numbers, this latter study lacks demographic and clinical data, underlining the need for a study with comprehensive clinical data.

These clinical data are relevant to the renewed interest in COPD as a disease with distinct phenotypes, connected to what some authors refer to as treatable traits (e.g. emphysema, frequent exacerbators, eosinophilic COPD). In our review of the literature, we have not found any well-powered studies with clinical data on the major phenotypes of COPD as well as a control group.

The MicroCOPD study collected gut microbiota samples from a Norwegian cohort with both patients with COPD and controls without COPD [13,14]. Clinical data enabled us to differentiate COPD-related phenotypes. With this material at hand, we aimed to investigate how the gut microbiota was related to COPD and COPD phenotypes such as emphysema, frequent exacerbators, eosinophilic COPD and COPD patients with high symptom burden.

Methods

Study population

The MicroCOPD study is a single-centre observational study with data collection performed at the Department of Thoracic Medicine, Haukeland University Hospital, Bergen, in 2013–2015. Subjects were recruited mainly from two previous study cohorts, the Bergen COPD Cohort Study [15] and the GeneCOPD study [16]. All participants in the MicroCOPD study were 40 years or older and included never, former, and current smokers. Participation was postponed if subjects had received antibiotics or oral corticosteroids in the 2 weeks preceding participation, or if the patients had symptoms indicating an ongoing COPD exacerbation. While the primary aim of the MicroCOPD study was to investigate the lower airway microbiota with bronchoscopy, stool samples were also collected to explore the gut microbiota. The detailed protocol for the MicroCOPD study has previously been published [13].

For inclusion in the current study, only MicroCOPD participants who had delivered a fresh stool sample the day after the clinical visit were included.

Data

At inclusion, blood sampling and structured interviews, including smoking history and symptom burden according to the COPD assessment test (CAT score), were performed. Blood eosinophils were measured by routine laboratory methods. Spirometry was performed with a Viasys Vmax ENCORE 30 minutes after bronchodilation with 400 mcg salbutamol administered through a large-volume expander. Fresh stool samples were delivered the day after inclusion. It was frozen less than 24 hours after defecation and stored at −80 degrees Celsius.

Both the COPD diagnosis and status as a control subject without COPD ('controls') were confirmed by experienced pulmonologists based on medical history and post-bronchodilator spirometry. Subjects with increased bleeding risk, cardiac valve prosthesis, known acute coronary syndrome in the preceding 6 weeks, known cancer in the last five years, or on strong immunosuppression were ineligible for inclusion in the MicroCOPD study. All patients with COPD had forced expiratory volume in 1 second (FEV_1)/forced vital capacity (FVC) ratio <0.7 and FEV_1 <80% of predicted [17]. Controls had no prior history of lung or airway disease.

Thoracic computed tomography (CT thorax) was taken using a Siemens Somatom Definition Flash. For the classification of emphysema, 3D Slicer software was used for density mask analysis [18]. Irrespective of COPD status, emphysema was defined as >10% of the area below the density threshold −950 hounsfield units (Hu), the so-called low attenuating area (LAA) [19,20]. We chose a stricter <10% of the area below the density

threshold −910 hu when we identified subjects without emphysema [21].

Only former or current smokers were included in the final analyses of this study. Participants diagnosed with asthma were excluded (Figure 1). The asthma diagnosis was confirmed by three experienced pulmonologists based on lung function, clinical history and CT thorax to ensure the diagnosis was correct before the participants were removed from the analysis.

DNA extraction and 16S ribosomal RNA gene sequencing

The detailed laboratory protocol is published [22]. Briefly, bacterial DNA was extracted by both enzymatic and mechanical lysis methods. PCR amplification of the V3V4 region of the 16S rRNA gene was followed by index PCR allowing 96 samples in each run. Samples were DNA quantified and normalized before sequencing according to the protocol for 16S Metagenomic Sequencing Library Preparation for the Illumina MiSeq System (Part # 15044223 Rev. B). Sterile water, used as diluting fluid in PCR and sequencing, was used as negative controls to account for the risk of bacterial DNA contamination in the samples.

Bioinformatic processing

FASTQ files were imported into Quantitative Insights Into Microbial Ecology version 2 (QIIME 2) for upstream analysis [23]. Denoising, dereplication and chimera-removal were performed using the Divisive Amplicon Denoising Algorithm version 2 (DADA2) [24]. A median quality score >30 resulted in forward- and reverse-read lengths of 281 and 235bp for all runs. Additional chimaeras were identified using VSEARCH with de novo method [25] and removed. Taxonomy, which is classifying the data with names in a hierarchical structure (ranging from domain to kingdom, phylum, class, order, family down to genus for our data), was assigned with the q2-feature-classifier [26] using a self-trained naïve-Bayes classifier based on the Silva database version 138.1 [27,28]. Amplicon sequence variants (ASVs) representing archaea or unclassified beyond kingdoms were discarded. We also discarded ASVs that were present in less than two samples and/or represented less than 0.005% of all reads. Further contaminants were identified using the Decontam package [29] in R [30] and removed together with negative control samples. At this stage, we removed samples from 12 asthma patients, whose samples had been sequenced along with the current study material. A phylogenetic tree was generated using MAFFT-aligned FastTree.

Diversity

Description of microbiota data usually contains a description of both diversity and taxonomy. Diversity measures are ways to describe the complexity of the composition of ecological data. Alpha diversity describes the complexity within a sample, while beta diversity compares the complexity between samples. For alpha diversity, we calculated Shannon index, which attempts to reflect both the number of microbes (richness) and their abundance and Faith´s Phylogenetic diversity, which combines the richness with phylogenetic distances as a way to measure the complexity of the sample. For beta diversity, we calculated Bray–Curtis distance, which reflects both the presence and abundance of species in each sample and weighted UniFrac distance, which also accounts for phylogenetic distances in addition to presence and abundance. Diversity measures were calculated after rarefaction at 46,709 sequences per sample in QIIME 2. This level was set to retain as many features as possible without losing many samples and retained 41.7% of features in 98.5% of samples. An alpha rarefaction plot confirmed that the rarefaction level was acceptable.

Statistical analyses

Statistical testing was done in R. Differences in alpha diversity between groups were tested with the Wilcoxon rank sum test, while beta diversity in groups was compared by distance-based permanova-test [31]. We compared diversity between COPD and controls, and between participants with and without emphysema. Additionally, we did COPD subgroup analysis according to the level of eosinophils in peripheral blood ($\geq$ vs $< 0.3 \times 10^9$/L), exacerbation rate ($\geq$2 vs none exacerbations previous year) and symptom score (CAT score $\geq$ vs $<$ 10). The analysis of the exacerbation rate was also stratified by the level of blood eosinophils ($\geq/< 0.3 \times 10^9$/L).

While diversity measures reduce the complexity to a collective number for each sample or each pair of samples, it does not consider the differences in abundance of each microbe. Differential abundance compares the abundances of each microbe or taxon in the samples of different groups. Because of the inherited structure of compositional microbiome abundance data, which are overdispersed, non-normally distributed, and high in zeroes, differential abundance is often analysed with specialized statistical tests, and we

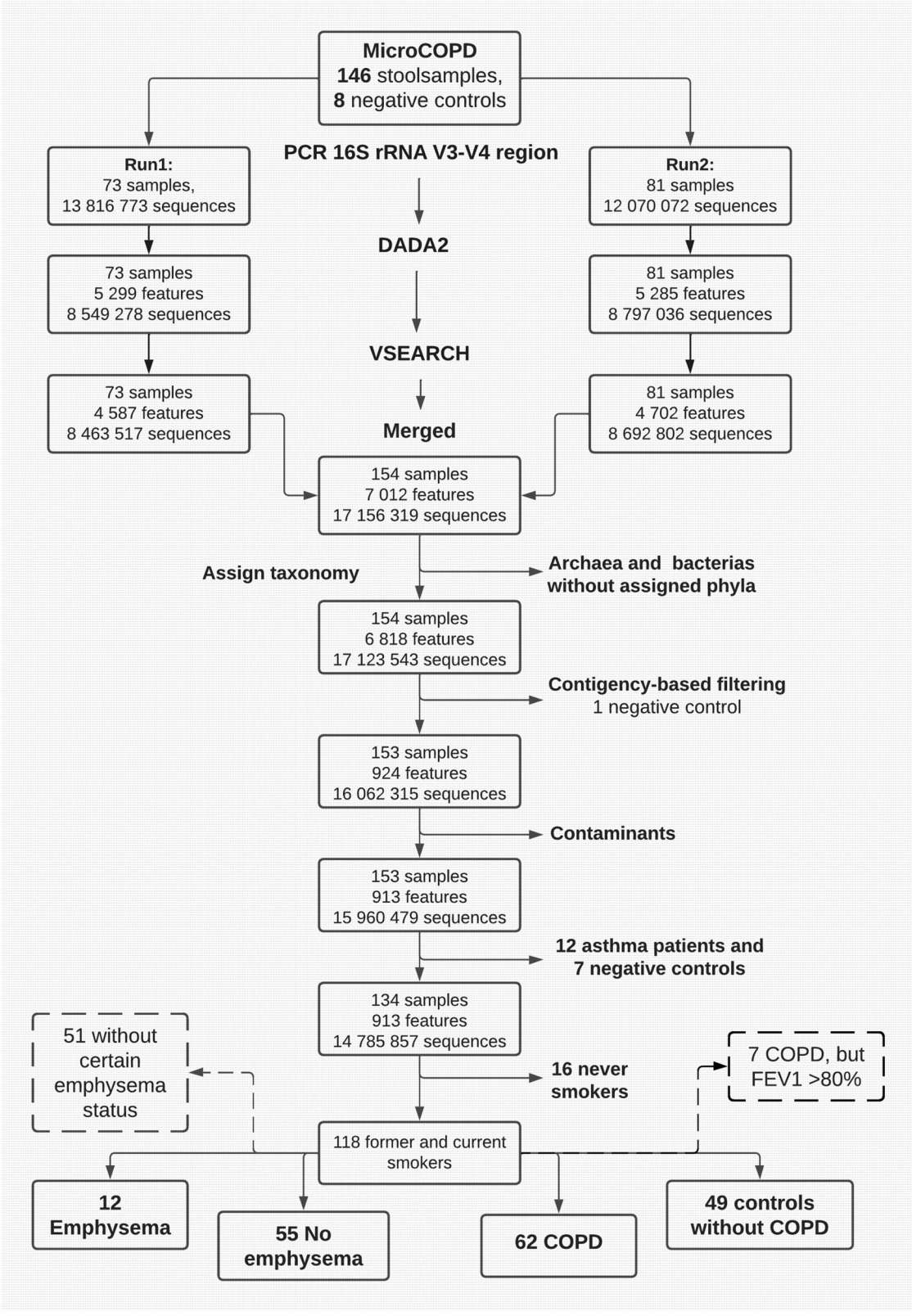

Figure 1. Flowchart showing the workflow from sequencing, bioinformatic processing, and filtering as well as grouping in to features (i.e. amplicon sequence variants (ASVs), here thought of as a group of similar sequences representing the same microbial organism). DADA2, divisive amplicon denoising algorithm version 2. VSEARCH, vectorized search.

evaluated differential abundance using Analysis of Composition of Microbiomes with Bias Correction (ANCOM-BC) [32]. The test compensates for the bias introduced by the unknown sampling fractions and provides a statistical test for compositional microbiome data while still acknowledging that sometimes the absence of a microbe has biological meaning. Structural zeroes (when differences are the result of absence in one group) were classified by ANCOM-BC using each group's asymptotic lower bound at the phylum and genus level [33]. P-values were corrected using the Holm – Bonferroni method to counter for multiple testing [34]. Multiple group testing was done with the addition of age and body mass index (BMI) as continuous variables in ANCOM-BC.

Correlation between centre log ratio (CLR) transformed abundance of genera and lung function (as % predicted by Global Lung Initiative (GLI)), blood counts and symptom burden (CAT score) was calculated using Spearman's correlation test.

Ethics

The study was conducted in accordance with the declaration of Helsinki and guidelines for good clinical practice. The study was approved by the regional committee of Medical Ethics Norway's north division (REK Nord, application number 2011/1307). All participants provided informed oral and written consent.

Results

Stool samples were collected from 118 former and current smokers, of which 62 were classified as having COPD and 49 were controls without COPD (Table 1, Figure 1). The participant groups matched well in age, sex, BMI, and smoking status (Table 1). Among our 118 participants, 12 participants had emphysema, 55 participants had no emphysema, and 51 participants did neither satisfy the criterion for emphysema (>10% LAA as defined by −950 hu) nor the criterion for no emphysema (<10% LAA as defined by −910 hU). All participants with emphysema were in the COPD group, as were 19 participants with no emphysema. Table 2 shows the characteristics of subjects with/without emphysema.

Diversity

We found no significant difference in alpha (within-sample) diversity between COPD patients and controls and between participants with emphysema and without emphysema. Patients with frequent exacerbations had lower Shannon index (p-value = 0.02), while there was no difference in Faith's PD, which incorporates phylogenetic information. When we stratified exacerbation frequency by eosinophils level, the difference in Shannon index remained significant for subjects with low eosinophils (p-value = 0.015), while it was no longer significant in subjects with high eosinophils (p-value = 0.92). Analysis of Faith PD also showed a statistically significant difference according to exacerbation frequency in those with *low* serum eosinophil levels (p-value = 0.035).

While there were no differences in beta (between sample) diversity between COPD patients and controls, we found significant differences in both Bray-Curtis and weighted UniFrac distance between those with emphysema and those without (p-values 0.02 and 0.038). However, the R^2 (a measure of explained variation) was low (0.02 and 0.03, respectively), and principal coordination plots of both Bray-Curtis and weighted UniFrac distance showed no certain separation of the groups (Figure 2). Eosinophilic COPD patients (B-eosinophils ≥0.3 × 10^9/L), had a significant difference in beta diversity weighted UniFrac distance (R^2 0.03, p-value = 0.03), but not Bray-Curtis distance, compared with non-eosinophilic COPD patients. We found no significant differences in

Table 1. The demographic and clinical characteristics of the study participants with COPD and control subjects without COPD.

	Control, $n = 49$	COPD, $n = 62$
Age (year)	67.5 (±7.3)	66.3 (±6.7)
Sex (male)	31 (63%)	36 (58%)
BMI (kg/m^2)	27.3 (±4.2)	26.5 (±4.9)
Smoking status		
Current	10 (20%)	14 (23%)
Former	39 (80%)	48 (77%)
Smoking, pack years	22.6 (±15)	34.3 (±21)
FEV_1 % of predicted	103.8 (±10.9)	52.5 (±16.3)
FVC % of predicted	112.8 (±12.6)	92.0 (±16.6)
FEV_1/FVC-ratio	0.73 (±0.05)	0.45 (±0.11)
Blood eosinophils		
≥0.3 × 10^9 /L	8 (16%)	24 (39%)
<0.3 × 10^9 /L	41 (84%)	38 (61%)
Exacerbations previous year		
≥2		11 (18%)
1		12 (19%)
0		39 (63%)
CAT score	6.6 (±5.1)	18.5 (±13.0)
CAT score		
≥10		51 (82%)
<10		11 (18%)
Inhaled corticosteroid use	0 (0%)	38 (61%)
Medication for GERD	3 (6%)	10 (16%)
GERD	5 (10%)	10 (16%)

Data presented as n (%) or mean ± SD. COPD, chronic obstructive pulmonary disease. BMI, body mass index. Pack years were defined as smoking one package of prefabricated cigarettes a day for one year. FEV1, forced expiratory volume in 1 second. FVC, forced vital capacity. CAT, COPD assessment test. GERD, gastroesophageal reflux disease. Medication for GERD included proton pump inhibitors and H2 blockers.

Table 2. The demographic and clinical characteristics of the study participants with and without emphysema.

	No emphysema, *n* = 55	Emphysema, *n* = 12
Age (year)	65.6 (±7.0)	70.6 (±5.0)
Sex (male)	29 (53%)	8 (67%)
BMI (kg/m^2)	27.5 (±4.7)	24.8 (±3.0)
Smoking status		
Current	14 (25%)	1 (8%)
Former	41 (75%)	11 (92%)
Smoking, pack years	22.8 (±13.9)	45.9 (±25.4)
FEV_1 % of predicted	90.3 (±22.9)	40.2 (±16.3)
FVC % of predicted	105.4 (±15.8)	89.9 (±17.5)
FEV_1/FVC-ratio	0.67 (±0.12)	0.34 (±0.08)
Blood eosinophils		
$\geq 0.3 \times 10^9$	13 (24%)	7 (58%)
$<0.3 \times 10^9$	42 (76%)	5 (42%)
Exacerbations previous year		
≥2	2 (4%)	3 (25%)
1	3 (5%)	4 (33%)
0	50 (91%)	5 (41%)
CAT score	8.9 (±6.5)	20.9 (±8.3)
CAT score		
≥10	24 (44%)	12 (100%)
<10	31 (56%)	0 (0%)
Inhaled corticosteroid use	10 (18%)	9 (75%)
Medication for GERD	4 (7%)	3 (25%)
GERD	5 (9%)	1 (8%)

Data presented as n (%) or mean ± SD. COPD, chronic obstructive pulmonary disease. BMI, body mass index. Pack years defined as smoking one package of prefabricated cigarettes a day for one year. FEV1, forced expiratory volume in 1 second. FVC, forced vital capacity. CAT, COPD assessment test. GERD, gastroesophageal reflux disease. Medication for GERD included proton pump inhibitors and H2 blockers.

beta diversity in COPD patients with frequent exacerbations (≥2 during the previous year) or with a large symptom burden (CAT score ≥ 10).

Differential abundance

We observed the genus *Veillonella* to be less abundant, and a genus from the class *Clostridia* to be more abundant, in COPD compared with controls. These taxa were among the least abundant taxa, being the 148th and the 137th most prevalent genera of the 159 in our material, both with a mean relative abundance less than 10^{-5}, and both present in only 13 samples. Both taxa were classified as structural zeroes by ANCOM-BC, meaning their absence in one of the groups was likely driving the result. This result remained significant when correcting for age and BMI. We found no differences at the phylum level or among the most prevalent genera (Figure 3) between COPD patients and controls.

We found 27 differentially abundant genera comparing those with and without emphysema. All but two of these were differentially abundant based on structural zeroes. The genera *Lachnospiraceae ND3007 group* and *Eubacterium hallii group* were both enriched in subjects without emphysema compared with patients with emphysema. *Eubacterium hallii group* was the most prevalent of the differentially abundant genera and the 29th most prevalent genus in our material with a mean relative abundance of 0.008. *Lachnospiraceae ND3007 group* was the 64th most prevalent genus with a mean relative abundance of 0.001. Both these genera belong to the family *Lachnospiraceae*, as did seven others of the differentially abundant genera. The result remained significant when correcting for age and BMI. The relative and bias-adjusted abundances of these two genera are shown in Figure 4, and the log-fold change of all differentially abundant genera in Figure 5. A negative log-fold difference means that the genera are less prevalent in participants with emphysema. None of the 20 most prevalent genera were differentially abundant in participants with emphysema (Figure 6). At the phylum level, we found two differentially abundant phyla between emphysema and no emphysema; *Synergistota* and *Cyanobacteria*. Both of these phyla were only represented by one genus each, *Cloacibacillus* and *Gastranaerophilales* respectively. Also, they were both low prevalent with a mean relative abundance below 10^{-4}, and were deemed differentially abundant based on structural zeroes.

Correlation with lung function, blood counts and symptom score

Altogether, 49 genera were significantly (p-value <0.05) correlated with lung function (as expressed in percentage of expected), blood counts, and/or symptoms according to CAT score (Figure 7). Nine of the significantly correlated genera to both lung function and/or CAT score belonged to the family *Lachnospiraceae*.

Discussion

We found associations between the gut microbiota and COPD, CT-verified emphysema, symptom score and airflow limitation. There were modest differences in the abundance of some microorganisms between patients with COPD and controls. More pronounced differences in the gut microbiota were observed when groups were divided according to emphysema status. Additionally, lung function, CAT score, and blood counts were significantly correlated with the gut microbiota. In several of our analyses, the significant findings were related to the *Lachnospiraceae* family, highlighting its potential role in COPD pathogenesis and progression.

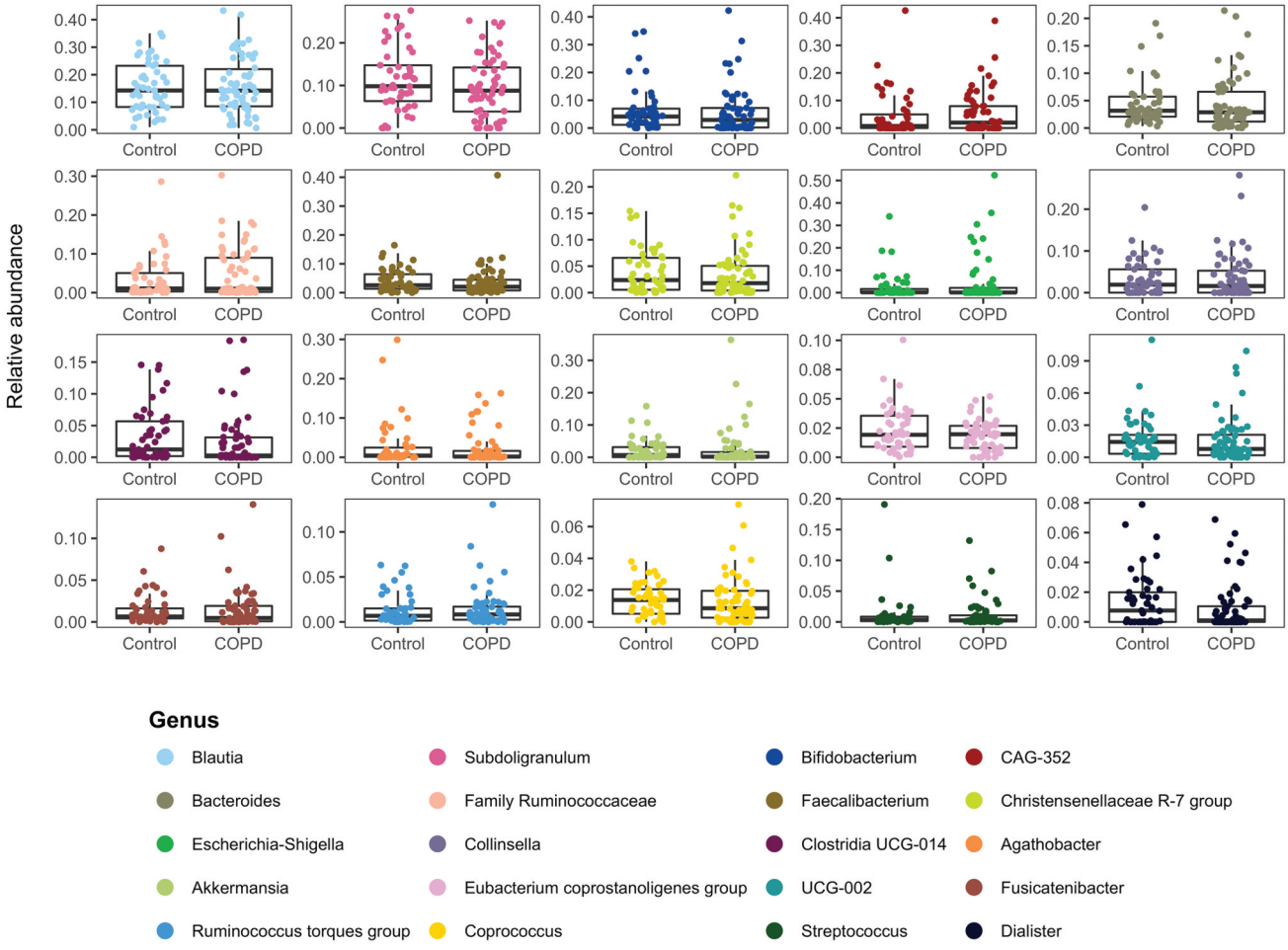

Figure 2. Principal coordinate analysis (PCoA) of beta diversity measures Bray-Curtis (BC) and weighted UniFrac distance (WUniF), coloured by emphysema status. The circles mark 95% confidence intervals if assuming t-distribution. Both measures differed statistically (Bray-Curtis R^2 0.02, p-value 0.02 and weighted UniFrac R^2 0.03 and p-value 0.034). The low R^2 (indicating that a low fraction of variation is explained) aligns with no apparent visual separation of the groups in this PCoA-plot.

Gut microbiota in COPD

We found no significant difference in alpha diversity between COPD and controls. Nor could we, nor Lai [8], replicate the significant difference in beta diversity measured by the Bray-Curtis distance reported by Bowerman et al. [7] and Li et al. [9]. Nevertheless, alpha and beta diversity are overall measures of diversity and although different diversity measures will complement each other, they are not sensitive to changes in abundance of specific microbes. *Veillonella*, which we found decreased in COPD patients, is an obligate anaerobe and a producer of short-chained fatty acids (SCFAs) [35], which can interact with the immune system. The other differentially abundant genus (in the class *Clostridia*) which we found increased in COPD, was unclassified beyond the class level, which makes it hard to speculate on further interpretation. The number of differentially abundant genera in the gut microbiota of patients with COPD compared with controls in our study is considerably lower than the 107 genera Bowerman et al. identified as either significantly enriched or depleted between patients with COPD and controls. These results are not directly comparable since Bowerman et al. have analysed data from shotgun metagenomic sequencing and used the DESeq2 method for testing differential abundance, as opposed to our 16S rRNA gene sequencing data being tested with ANCOM-BC. DESeq2 does not take compositionality into full account and has been shown to lead to high false discovery rates [36]. Li et al. found differences in relative abundances at the phylum and family levels, while Lai et al. found differences at the genus level. However, differences in bioinformatic processing and statistical methodologies make the differences in abundance between these studies difficult to evaluate.

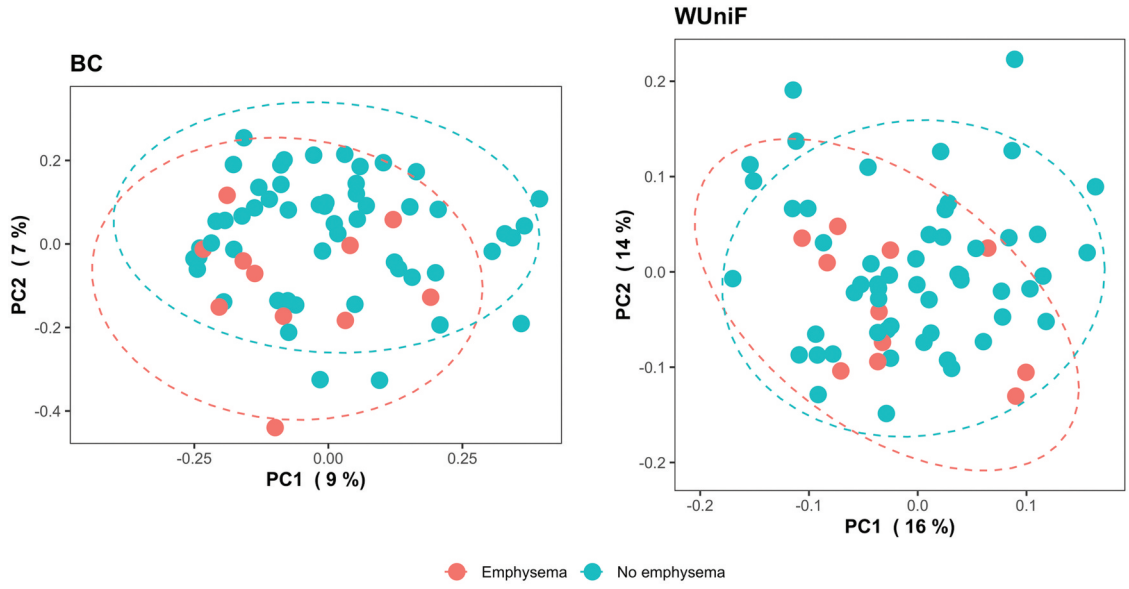

Figure 3. Taxonomic box plot by COPD status. The relative abundances of the 20 most prevalent genera in samples, divided by COPD status and sorted by relative abundance. None of these high-abundant genera were differentially abundant in COPD patients compared with controls (ANCOM-BC).

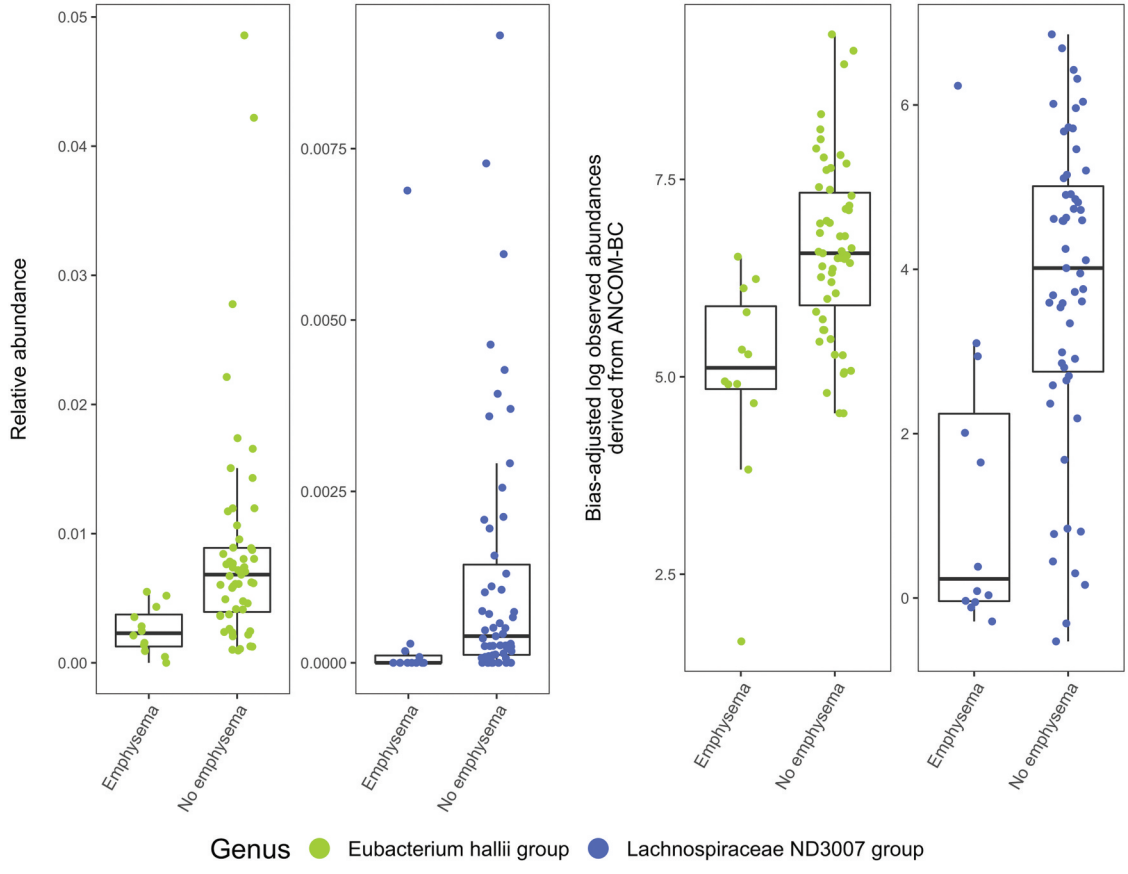

Figure 4. The relative and bias-adjusted abundance of the *Lachnospiraceae ND3007 group* and the *Eubacterium hallii group* were differentially abundant in participants with emphysema (as compared with participants without emphysema), but not structural zeroes. Bias-adjusted abundance was derived from the analysis of composition of microbiomes with bias correction (ANCOM-BC).

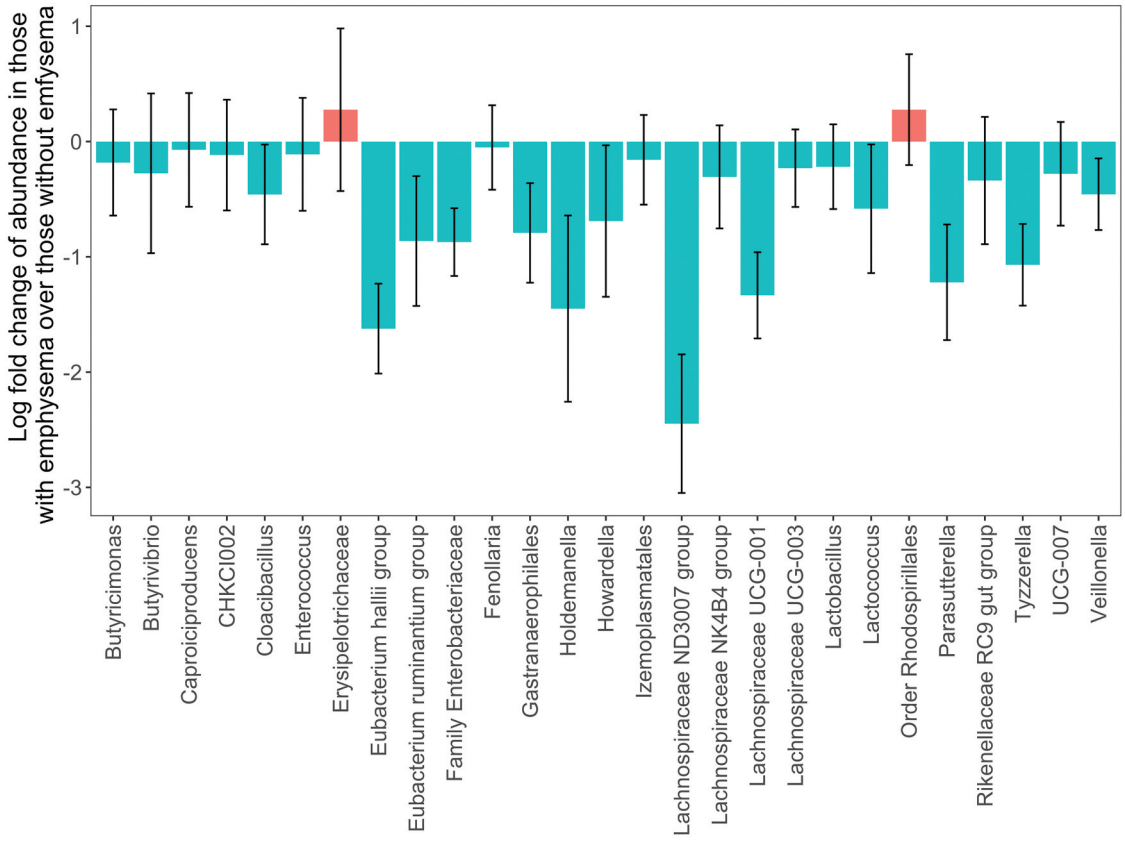

Figure 5. Waterfall plot displaying log fold difference and standard errors of all differential abundant genera when comparing samples from participants with emphysema and without emphysema. A negative log-fold difference means that the genera are less prevalent in participants with emphysema.

Emphysema and gut microbiota

This study is, to our knowledge, the first study to investigate the gut microbiota in patients with CT-verified emphysema. *Lachnospiraceae ND3007 group* and *Eubacterium hallii group*, both significantly reduced in patients with emphysema, are known producers of SCFAs, and have been found reduced in other disease context. SCFAs, and particularly butyrate, are potent regulators of immune function. *Lachnospiraceae ND3007 group* was reduced in Parkinson patients [37], and oral treatment with *Eubacterium hallii group* improves insulin sensitivity in mice [38]. Also, both were reduced in a cohort of patients with major depressive disorder and schizophrenia, compared with healthy controls [39].

Furthermore, the association of the gut microbiota with emphysema is also supported by studies in mouse models. Lai et al. showed that an anti-inflammatory lipopolysaccharide (LPS) purified from a commensal bacteria could ameliorate COPD and emphysema in a mouse cigarette-smoking model [40]. Li et al. [9] have found that faecal microbiota transplantation (FMT) from patients with COPD to COPD mice increased lung inflammation compared to FMT from healthy controls. After concurrent FMT and biomass fuel smoke exposure, mice with FMT from COPD showed an accelerated decline in lung function, severe emphysematous changes, airway remodelling and mucus hypersecretion. In another study, Jang et al. [41] found that high-fibre diets, modulating the gut microbiota, attenuated emphysema development and inflammatory response in a cigarette smoke-exposed emphysema mouse model. Gut-derived microbial metabolites and products, are present in human lungs and can influence the local immune tone and cell metabolism in mice [42]. Altogether, this indicates a potential mechanism for emphysema development associated with the gut microbiota, and the current study has shown that the gut microbiota might be changed in human subjects with emphysema.

Lachnospiraceae

Both in our study and in Bowerman's COPD cohort [7], several members of the family *Lachnospiraceae*

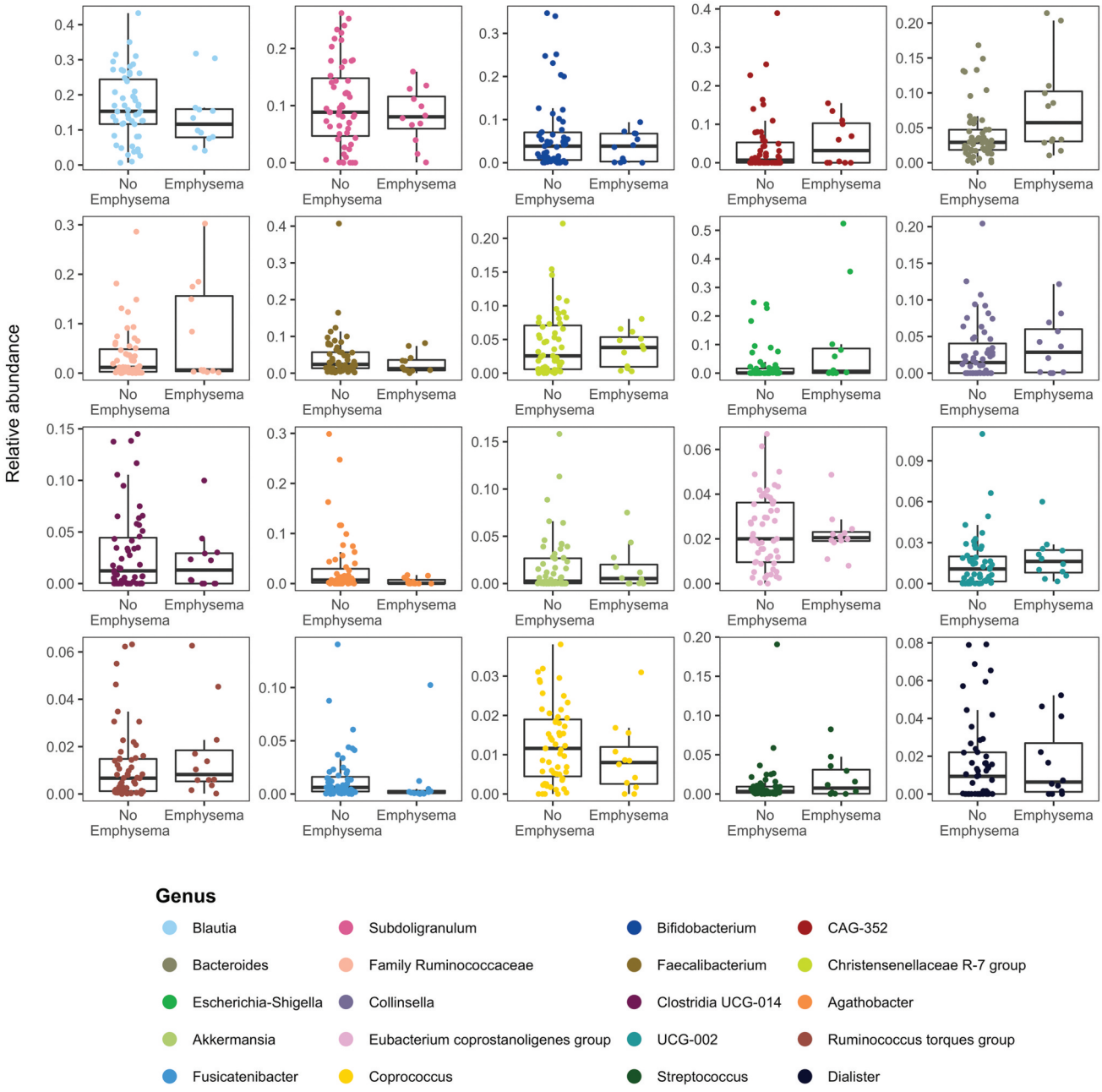

Figure 6. Taxonomic box plot by emphysema status. The relative abundances of the 20 most prevalent genera in samples, divided by emphysema and sorted by relative abundance. None of these high-abundant genera were differentially abundant in participants with emphysema compared with those without.

were significantly reduced. *Lachnospiraceae* is a family of obligate anaerobic microbes, with members among the main producers of short-chain fatty acids [43]. Allais et al. showed an increase in *Lachnospiraceae* in mice after a 24-week smoking exposure [44]. Their impact on the host physiology is inconsistent across different studies, but *Lachnospiraceae* seems to be increased in metabolic syndrome, obesity and diabetes [43]. The inconsistencies between studies might also be explained by a substantial degree of inter- and intra-species genomic and functional heterogeneity within the *Lachnospiraceae* family [45].

We observed a lower prevalence of some *Lachnospiraceae* in patients with emphysema. However, the emphysema patients had smoked more than the non-emphysema patients and included a high percentage of ex-smokers. Whether the association between *Lachnospiraceae* and emphysema is dependent on smoking, is not possible to derive from our data. Nevertheless, the associations between *Lachnospiraceae*

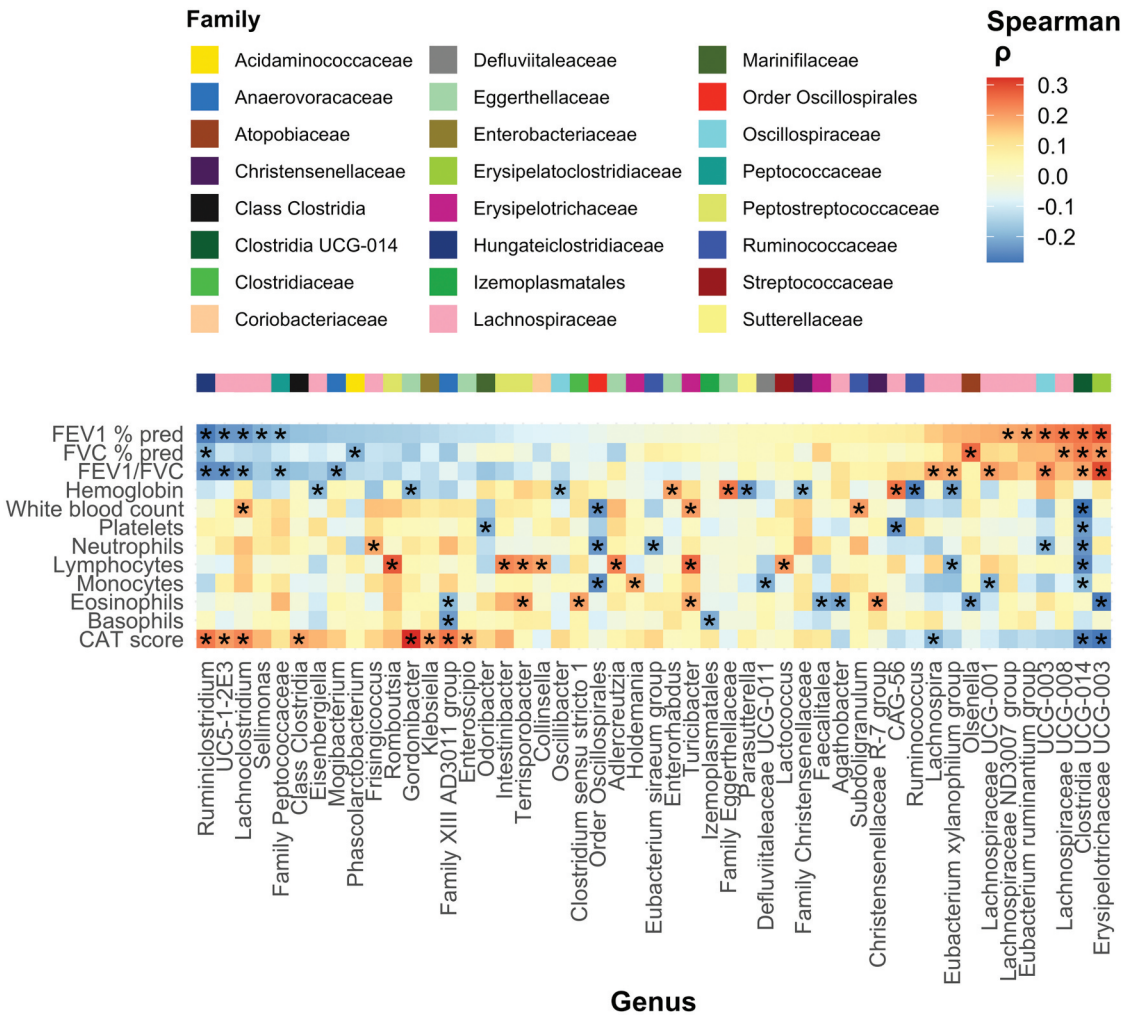

Figure 7. Heatmap of correlations (Spearman´s ρ) between centre log-ratio transformed abundance of genera, and lung function (as % of predicted by GLI), blood counts and COPD assessment test (CAT) score. Only genera that were significantly correlated (p-value < 0.05) to either lung function or blood counts are shown, and each significant correlation (p-value <0.05) is marked with a * . Family is displayed above.

and emphysema in our opinion deserve further exploration.

Exacerbation frequency, eosinophils and gut microbiota

Our study also revealed an interesting association between blood eosinophil levels and the gut microbiota, particularly concerning exacerbation frequency. We found that the exacerbation rate was associated with alpha diversity as measured by the Shannon index, but not if including phylogenetic information with Faith's PD. Thus, the difference is related to evenness or richness assessed with the Shannon index. The difference was most likely driven by those with low blood eosinophils, as the difference remained significant in those with blood eosinophils $< 0.3 \times 10^9$/L. It is also known that exacerbation frequency varies by level of eosinophilic granulocytes [46]. Measurement of blood eosinophils has since 2019 been recommended in the follow-up of COPD and guides the prescription of inhaled glucocorticoids [47]. Blood eosinophils have also been found to be positively associated with the risk of developing obstructive lung disease, but the mechanism remains unclear [48]. Chui et al. [10] found a significant correlation between blood eosinophils and the abundance of some genera in the gut, while Bowerman et al. [7] did not. In our study, we found an association between eosinophils and phylogenetic, quantitative beta diversity. Further studies are needed to confirm and elaborate on the gut microbiome's role in eosinophilic COPD and the potential role of microbiome-targeted treatment of frequent COPD exacerbators.

Lung function, symptom score and gut microbiota

We found correlations between several genera with both post-bronchodilator lung function and respiratory symptoms (Figure 7). An association of the gut microbiota in COPD and lung function has previously been indicated by two studies [7,10], but we do not know of previously published studies on the gut microbiota's correlation to respiratory symptoms in COPD. Some of the genera that showed the strongest correlations to lung function and CAT score belonged to the *Lachnospiraceae family*.

Gut-lung axis and therapeutic implications

The relationship between gut microbiota and lung health has gained considerable attention in recent years, driven by evidence supporting the gut-lung axis, which suggests that gut-derived metabolites and immune signals can influence lung function and inflammation. Our study provides further evidence for the potential role of the gut microbiota in COPD and emphysema pathogenesis. The observation that genera such as *Lachnospiraceae* are significantly reduced in patients with emphysema suggests that gut microbiota modulation could be an attractive therapeutic avenue for COPD management.

Our study supports the hypothesis that microbiota-based interventions, such as FMT, prebiotics, or antimicrobial therapies, could potentially alter the progression of COPD or improve symptom control. Future randomized controlled trials could investigate whether these interventions can influence disease progression, reduce exacerbation rates, or improve lung function in COPD patients.

Study strengths and limitations

This study benefits from a robust dataset, including a cohort of patients with clinically relevant disease and carefully controlled confounding factors. Our use of 16S rRNA gene sequencing and strict bioinformatic processing enhances the reliability of our findings. However, several limitations should be acknowledged. First, we lack data on participants' diet, a key factor influencing gut microbiota composition. Future studies could incorporate dietary questionnaires or biomarkers to account for this potential confounder. Second, we focused on a cohort of ever-smokers with moderate-to-severe COPD, which limits the generalizability of our findings to other COPD subgroups, such as never-smokers or those with milder disease. Third, while we demonstrate associations between the gut microbiota and disease phenotypes, the cross-sectional nature of this study limits our ability to establish causality. Longitudinal studies will be necessary to determine whether gut dysbiosis precedes or results from COPD progression. Fourth, there are other phenotypes of COPD that we have not investigated, such as small airway disease and chronic bronchitis. Fifth, a 14-day preceding period in which participants could not receive any antibiotics might be too short, as the effect of antibiotics on gut microbiome could extend far beyond this period. However, this was a practical decision to not exclude many potential participants and to reflect the real-life microbiome of actual COPD patients. Time since last antibiotics were recorded and 101 of 118 included in the statistical analysis had not used any antibiotics the previous 12 weeks before inclusion. Sixth, we choose strict predefined criteria both for defining patients with and without emphysema. While this allowed us to evaluate two biologically different populations, it may also have caused a loss of power and therefore influenced our results. Seventh, we used sequencing of 16S rRNA gene sequencing, limiting us to go beyond the resolution of the genus level. Finally, we lack quantitative PCR measurements, and can only compare relative abundances in the gut microbiota.

Conclusion

We have shown that specific members of the gut microbiota were differentially abundant in COPD compared with subjects without COPD and that these differences are more pronounced when you give attention to phenotypes such as emphysema, lung function, elevated eosinophils and symptom burden. This supports that the postulated lung-gut-axis is also relevant in COPD. There is a need for large-scale studies on the associations between COPD, COPD phenotypes and the microbiota.

Ideally, the next step should be a longitudinal, high-powered study on the association between COPD, COPD phenotypes and the microbiota. This study should employ shotgun sequencing to conduct functional metagenomics, as well as classify taxa at the species and strain level. In addition, animal models and experiments should be utilized to investigate the role of specific members of the *Lachnospiraceae* family. Ultimately, understanding the intricate relationship between the microbiota and COPD could pave the way for novel therapeutic strategies, offering hope for improved disease management and better patient outcomes.

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Authors' contributions

AØR – Formal analysis, Writing.
TME – Concept and design. Data collection. Supervision. Writing – review and editing
ST – Supervision. Writing – review and editing
GRH – Data collection. Writing – review and editing
KO – Data curation. Writing – review and editing
RN – Concept and design. Supervision. Data collection. Writing – review and editing

Abbreviations

ANCOM-BC	analysis of composition of microbiomes with bias correction
ASVs	amplicon sequence variants
BMI	body mass index
CAT	COPD assessment test
CLR	centre log ratio
COPD	chronic obstructive pulmonary disease
CT thorax	Thoracic computed tomography ()
DADA2	Divisive Amplicon Denoising Algorithm version 2
FEV_1	forced expiratory volume in 1 second
FMT	faecal microbiota transplantation
FVC	forced vital capacity
GERD	gastroesophageal reflux disease
GLI	Global lung initiative
Hu	Hounsfield units
LAA	low attenuating areas
LPS	lipopolysaccharide
MicroCOPD	The Bergen COPD Microbiome Study
OTU	operational taxonomic unit
PCoA	principal coordinate analysis
PD	phylogenetic diversity
SCFA	short-chained fatty acids
VSEARCH	vectorized search

Disclosure statement

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Data availability statement

The data that support the findings of this study are openly available in the Dryad repository at https://doi.org/10.5061/dryad.m37pvmd6c.

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