

Severity-Related Changes of Bronchial Microbiome in Chronic Obstructive Pulmonary Disease

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Bronchial colonization by potentially pathogenic microorganisms (PPMs) is often demonstrated in chronic obstructive pulmonary disease (COPD), but culture-based techniques identify only a portion of the bacteria in mucosal surfaces. The aim of the study was to determine changes in the bronchial microbiome of COPD associated with the severity of the disease. The bronchial microbiome of COPD patients was analyzed by 16S rRNA gene amplification and pyrosequencing in sputum samples obtained during stable disease. Seventeen COPD patients were studied (forced expiratory volume in the first second expressed as a percentage of the forced vital capacity [FEV₁%] median, 35.0%; interquartile range [IQR], 31.5 to 52.0), providing a mean of 4,493 (standard deviation [SD], 2,598) sequences corresponding to 47 operational taxonomic units (OTUs) (SD, 17) at a 97% identity level. Patients were dichotomized according to their lung function as moderate to severe when their FEV₁% values were over the median and as advanced when FEV₁% values were lower. The most prevalent phyla in sputum were *Proteobacteria* (44%) and *Firmicutes* (16%), followed by *Actinobacteria* (13%). A greater microbial diversity was found in patients with moderate-to-severe disease, and alpha diversity showed a statistically significant decrease in patients with advanced disease when assessed by Shannon ($\rho = 0.528$; $P = 0.029$, Spearman correlation coefficient) and Chao1 ($\rho = 0.53$; $P = 0.028$, Spearman correlation coefficient) alpha-diversity indexes. The higher severity that characterizes advanced COPD is paralleled by a decrease in the diversity of the bronchial microbiome, with a loss of part of the resident flora that is replaced by a more restricted microbiota that includes PPMs.

Bronchial colonization by potentially pathogenic microorganisms (PPMs) is a frequent finding in stable chronic obstructive pulmonary disease (COPD) (1, 2). Positive sputum cultures are found in a significant proportion of patients, *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis* being the most frequently recovered bacteria (3, 4). Currently available culture-based techniques are, however, unsuitable for the identification of a significant portion of the bacteria inhabiting human mucosal surfaces, which do not grow in the most commonly selective cultures used and may be masked by fast-growing bacteria (5–7). Culture-independent techniques such as PCR amplification and sequencing of the 16S rRNA gene determine the composition of the bronchial microbiome (1) and may be useful for defining the relationships between colonizing bacteria and COPD. The use of these techniques on samples of bronchial secretions from COPD patients has demonstrated a rich microbiome in addition to previously recognized PPMs, with *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* being the main recovered phyla (8–10).

In this study, we aimed to identify severity-related modifications of the bronchial microbiome in COPD. Bronchial secretions from stable COPD patients were examined by amplification and pyrosequencing of the 16S rRNA gene, relating microbiome patterns to lung function.

MATERIALS AND METHODS

Design and population. A cross-sectional study focusing on the bronchial microbiome of stable COPD was performed in outpatients diagnosed

according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria (11). Participants were examined during stable conditions for >4 weeks, and their sociodemographic and clinical data were recorded (smoking habits, respiratory symptoms, exacerbation frequency, lung function, and treatments). Exclusion criteria were age <40 years, diagnosis of asthma, bronchiectasis, cystic fibrosis, neoplasia, chronic treatment with oral corticosteroids or immunosuppressive drugs, and admission to a hospital for respiratory symptoms in the previous 6 months.

Ethics statement. Written informed consent was obtained from each of the participants in this study. The present study was reviewed and approved by the Corporació Sanitària Parc Taulí research ethics committee in Catalonia, Spain.

Functional variables. Functional characteristics were assessed at enrollment by forced spirometry and reversibility testing (12), the 6-min walking test (13), body mass index, and the multidimensional BODE index (body mass index, airflow obstruction, dyspnea, and exercise capacity, as assessed by the 6-min walking test) (14). Forced vital capacity

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TABLE 1 Chronic obstructive pulmonary disease patient characteristics

Characteristic ^a	All patients	Patients with moderate-to-severe disease	Patients with advanced disease	P value ^b
<i>n</i>	17	8	9	
Age (median [IQR]) (yr)	69 (66–75)	68 (62–69)	74 (68–77)	0.059
Male (<i>n</i> [%])	16 (94)	7 (87)	9 (100)	0.279
Smoking (median [IQR]) (pack-yr)	67.5 (40–105)	75 (52–110)	55 (35–117)	0.504
FEV _{1 postbd} (median [IQR]) (%)	35.0 (31.5–52.0)	52.0 (41.5–69.0)	32.0 (29.5–35.0)	0.001
BODE index (median [IQR])	5 (2–7)	2 (0.25–4)	7 (5.5–8)	0.001
Exacerbations ^c (median [IQR]) (<i>n</i> /yr)	1.8 (1.3–2.5)	1.7 (1.3–2.5)	1.9 (1.2–2.5)	0.841
Inhaled corticosteroid use (<i>n</i> [%])	14 (82)	5 (62.5)	9 (100)	0.082
Antibiotic courses ^{c,d} (median [IQR]) (<i>n</i> /yr)	3.5 (1.9–6.0)	3.6 (2.5–5.0)	3.3 (1.6–6.0)	0.797
Positive PPM sputum culture ^e (<i>n</i> [%])	12 (62.5)	5 (62.5)	7 (77.8)	0.625
<i>Haemophilus influenzae</i>	4 (23.5)	1 (12.5)	3 (33.3)	
<i>Pseudomonas aeruginosa</i>	5 (29.4)	3 (37.5)	2 (22.2)	
<i>Streptococcus pneumoniae</i>	2 (11.8)	1 (12.5)	1 (11.1)	
<i>Moraxella catarrhalis</i>	2 (11.8)	2 (25.0)	0	
Other	2 (11.8)	0	2 (22.2)	

^a FEV_{1 postbd} forced expiratory volume in the first second, postbronchodilator; IQR, interquartile range; BODE, multidimensional scale that includes the items body-mass index, degree of airflow obstruction, dyspnea, and exercise capacity (14); PPM, potentially pathogenic microorganism.

^b Fisher's exact test, Student *t* test, or Mann-Whitney U test used as required.

^c Average of previous 2 years.

^d Antibiotic prescriptions related to any cause (respiratory and nonrespiratory).

^e Polymicrobial colonization in three patients.

(FVC) and forced expiratory volume in the first second (FEV₁) were measured with the same dry rolling seal spirometer (Sibelmed, Sibelgroup, Barcelona, Spain) and expressed as absolute values (ml) and percentages of the reference values obtained from age- and height-adjusted selected volunteers from the Barcelona province (15).

Sputum collection. Sputum was collected for microbiological tests from patients in a clinically stable condition. Murray-Washington criteria were used to identify and select samples representative of bronchial secretions (16, 17). Sputum samples were cultured within 60 min of collection, and the remaining sample was processed for culture-independent microbiological techniques (18). Quantitative cultures for PPMs were performed through serial dilutions in selective media according to standard methods (19) and considered positive when PPMs were recovered at a concentration of ≥ 100 CFU/ml. Gram-negative and Gram-positive bacteria recognized as agents causing respiratory infections, such as *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterobacteriaceae*, were considered PPMs.

DNA extraction. Sputum samples were treated for 15 min with an equal volume of Sputasol (Oxoid, Hampshire, United Kingdom) followed by centrifugation for 15 min at $13,000 \times g$, and genomic DNA extraction from sputum was performed using a Qiagen DNA blood kit (Qiagen, Crawley, United Kingdom). Briefly, samples were treated with an in-house lysis solution containing 47,700 U/ml of lysozyme (Sigma, Poole, United Kingdom), 100 U/ml of mutanolysin (Sigma, Poole, United Kingdom), and 2 U/ml of lysostaphin (Sigma, Poole, United Kingdom) as previously described (8). DNA was quantified in the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) and stored at -80°C for further determinations.

PCR amplification of the V1/V2 region of the 16S rRNA gene. The hypervariable regions V1/V2 of the 16S rRNA gene were amplified with universal bacterial primers and with different bar codes specific for each sample, following the procedure of McKenna et al. (20). After amplification, the products were visualized in 2% agarose gel, purified, and quantified with the QuantiT PicoGreen double-stranded DNA (dsDNA) assay kit (Invitrogen, Life Technologies, Carlsbad, CA, USA). Samples with different bar code sequences were pooled in equimolar amounts, and pyro-

sequencing was carried out using the Roche 454 GS-FLX system Titanium chemistry.

Sequences library analysis. As a first quality step, 16S rRNA raw sequences were analyzed with the mothur software package 1.27 (21) to remove sequences shorter than 200 bp and chimeras. The quantitative insights into

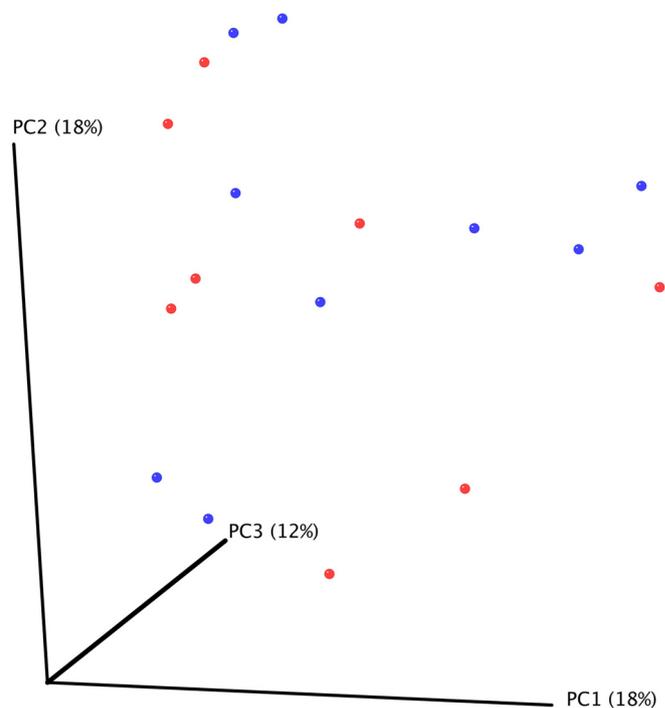


FIG 1 Principal coordinate (PC) analysis with Bray-Curtis dissimilarity index. Red and blue dots represent moderate-to-severe and advanced COPD patients, respectively (Adonis test, $P = 0.221$).

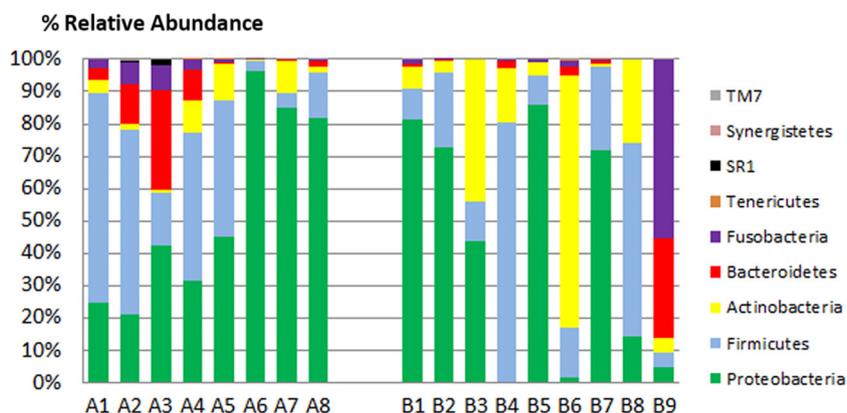


FIG 2 Taxonomic identification at phylum level in each sample from moderate-to-severe (labeled with A) and advanced (labeled with B) chronic obstructive pulmonary disease (COPD) patients.

microbial ecology (QIIME) pipeline (22) was used for de-bar-coding and quality filtering based on the characteristics of each sequence as a second step, removing any low-quality or ambiguous reads with default parameters in order to obtain taxonomic information. Operational taxonomic units (OTUs) present in the samples at 97% identity were determined and classified according to the taxonomy of the Greengenes database (23).

Statistical analysis. Results for categorical variables were expressed as absolute and relative frequencies, and continuous variables were expressed as means and standard deviations (SD) in normal distributions or as medians and interquartile ranges (IQR) in abnormal distributions.

Statistical analyses were performed using the SPSS statistical software package, version 18 (SPSS, Inc., Chicago, IL, USA), the linear discriminative analysis effect size (LEfSe) (24), and R (<http://www.r-project.org>), using the Vegan package to cluster and construct abundance heat maps. The beta-diversity Bray-Curtis dissimilarity index was used for principal coordinate analyses and analysis of similarity (Adonis) in order to identify and compare statistically the bacterial communities visualized in both groups. Alpha bacterial biodiversity was assessed on the basis of the number of OTUs observed, using the Chao1 (25) and Shannon (26) indexes, which estimate the richness and homogeneity, respectively, of the microbiome. Alpha- and beta-diversity metrics were calculated after rarefaction in QIIME to avoid sequencing effort bias (431 sequences).

Patients were categorized into two groups according to median FEV₁% (35%), considering patients with a higher value as having moderate-to-severe disease and the reference and patients with an equal or lower FEV₁% as suffering from advanced disease. First, results of sputum cultures in the two subgroups were compared (chi-square test). Second, after manual removal of the OTUs present in only one sample from the OTU table, the LEfSe method was employed to compare relative abundances (RAs) of bacterial OTUs between the groups for all taxonomic levels. Finally, correlations among relative abundances, bacterial diversity, and lung function were assessed (Spearman correlation test). Statistical tests used in the study were two sided, and a *P* value of ≤ 0.05 was reported as statistically significant. The predetermined threshold on the logarithmic linear discriminant analysis (LDA) score for discriminative features was 2.0.

Nucleotide sequence accession numbers. Bacterial 16S rRNA data sets from this study are accessible in the European Nucleotide Archive under the study accession number PRJEB4144 (accession numbers ERS255709, ERS255711, ERS255712, ERS255714, ERS255716, ERS255720, ERS255723, ERS255724, ERS255726, ERS255731, ERS255733, ERS255735, ERS255736, ERS255738; see <http://www.ebi.ac.uk/ena/data/view/PRJEB4144>) and in the MG-RAST server (see <http://metagenomics.anl.gov/>) under accession numbers 4481647.3, 4481649.3, 4481655.3, 4481657.3.

RESULTS

Patient characteristics. Seventeen COPD patients, former smokers with an average age of 69 years (IQR, 66 to 65 years) were

included in the study (Table 1). Sputum cultures were positive for PPMs in more than half of the patients, with a similar prevalence between moderate-to-severe and advanced disease patients (5/8 [62.5%] versus 7/9 [77.82%]; *P* = 0.062, Fisher's exact test).

Bronchial microbiome. A mean of 4,493 (SD, 2,598) sequences were obtained in the analyzed sputum samples, which corresponded to 125 OTUs (mean of 47 bacterial OTUs [SD 17] per sample), at a 97% identity level. Principal coordinate analysis (PCoA) with the Bray-Curtis beta-diversity metric was performed to assess differences between patients with moderate-to-severe and advanced disease and did not show clustering differences between the microbial communities of both groups (Adonis statistics, *P* = 0.221) (Fig. 1).

The most prevalent phyla in the sputum samples were *Proteobacteria* (44%) and *Firmicutes* (16%) followed by *Actinobacteria* (13%), with *Bacteroidetes*, *Fusobacteria*, *Tenericutes*, SR1 incertae, TM7, and *Synergistetes* identified in lower proportions (<3%) (Fig. 2). Relative abundances of >50% were found only for *Firmicutes* in 4 patients (23%), and *Proteobacteria*, which include the majority of pathogens related to COPD, were found in 7 patients (41%), with an inverse correlation between both phyla (ρ = -0.629; *P* = 0.007, Spearman correlation coefficient) (Fig. 3).

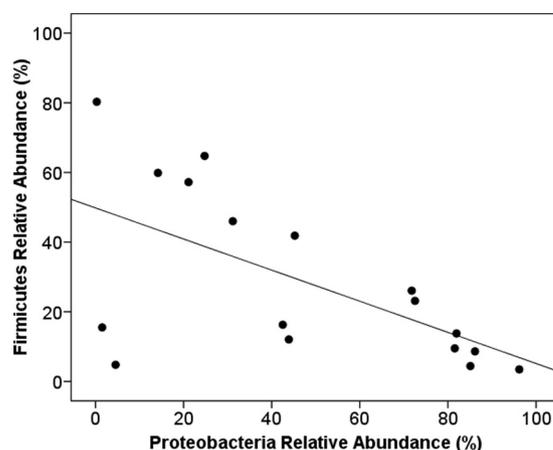


FIG 3 Scatterplot of *Proteobacteria* relative abundances and *Firmicutes* relative abundances (Spearman correlation coefficient, ρ = -0.629 and *P* = 0.007) in chronic obstructive pulmonary disease (COPD) patients.

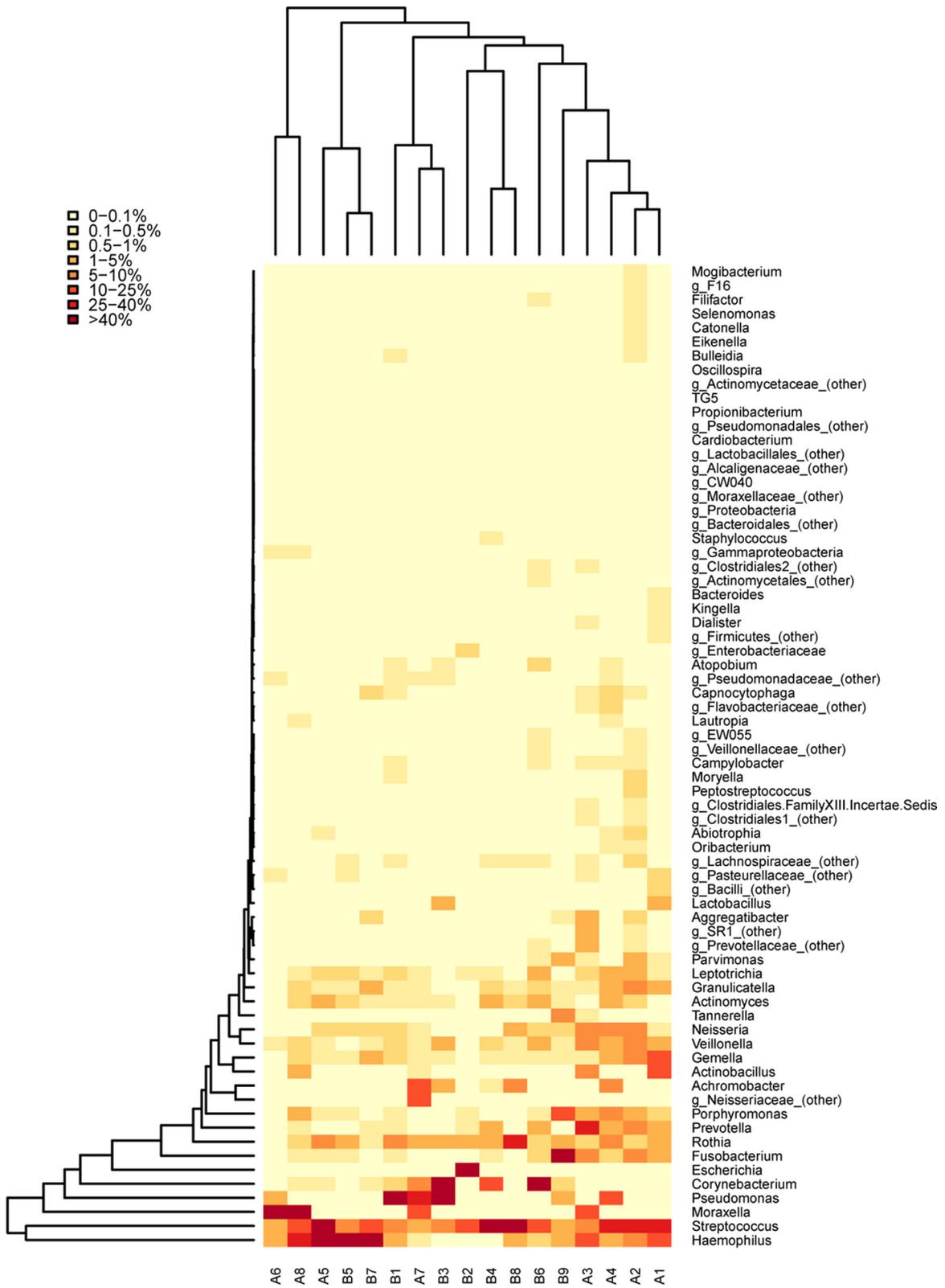


FIG 4 Heat map showing clustering of genera communities and the most predominant genera in chronic obstructive pulmonary disease (COPD) patients with moderate-to-severe (A) and advanced (B) disease. Sample numbers are listed horizontally along the bottom. The genera are listed vertically on the right. The relative abundance of each genus is represented by the color key.

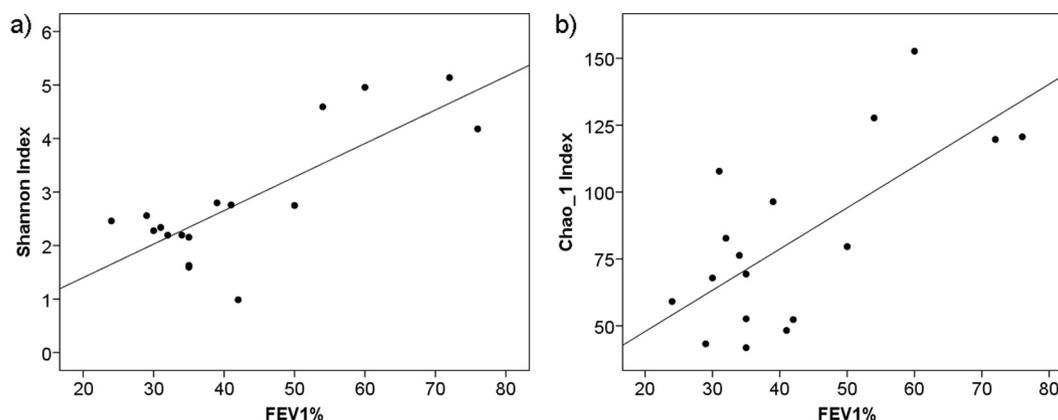


FIG 5 Microbiome diversity in chronic obstructive pulmonary disease (COPD) patients according to lung function by the Shannon index (a) and Chao1 index (b) ($P = 0.029$, $\rho = 0.53$; $P = 0.028$, Spearman correlation test).

Patients with moderate-to-severe disease showed greater microbial diversity than patients with advanced disease (Fig. 4), as shown by the statistically significant correlation between FEV₁% and the bacterial diversity in the sputum, expressed as either Shannon ($\rho = 0.528$; $P = 0.029$, Spearman correlation coefficient) or Chao1 ($\rho = 0.53$; $P = 0.028$, Spearman correlation coefficient) alpha-diversity indexes (Fig. 5). The higher diversity in moderate-to-severe COPD patients was paralleled by a statistically significant lower relative abundance of the predominant bacterial OTUs in the sample (median, 36.7, IQR, 34.4 to 44.0%), compared with patients with advanced disease (median, 78.6; IQR, 56.8 to 71.9%; $P = 0.016$, Mann-Whitney test). The comparative analysis of bacterial relative abundance (RA) identified 10 OTUs that showed a significantly higher relative abundance in moderate-to-severe COPD patients ($P < 0.05$) (Table 2; Fig. 6). These OTUs belonged

to 5 genera overrepresented in moderate-to-severe COPD, which reached a median relative abundance of 7.56% (IQR, 0.01 to 43%), much higher than the 0% (IQR, 0 to 0.1%) found in advanced COPD for these genera.

A total of 3.2% (4/125) of the bacterial OTUs identified in COPD patients were shared by all participants, independently of their impairment level. These bacterial OTUs were part of the genera *Rothia*, *Streptococcus*, and *Veillonella* and represented 12.1% of the observed relative abundance as a whole.

DISCUSSION

This study related bronchial microbiome and the severity of disease in COPD patients through 16S rRNA gene pyrosequencing and demonstrated an important decline in bacterial diversity in patients with lung function impairments that reach the level of

TABLE 2 Discriminative features at different taxonomic levels between moderate-severe and advanced chronic obstructive pulmonary disease (COPD) patients

Taxonomic level ^a	Relative abundance (median [IQR]) (n) in:		P
	Patients with moderate-to-severe disease	Patients with advanced disease	
Phylum/class/order/family			
<i>Proteobacteria/Gammaproteobacteria/Pseudomonadales/Moraxellaceae</i>	7.36 (0.004–37.11)	0 (0)	0.010
Phylum/class/order/family/genus			
<i>Firmicutes/Clostridia/Lachnospiraceae/Oribacterium</i>	0.04 (0–0.2)	0 (0–0.009)	0.022
<i>Proteobacteria/Betaproteobacteria/Neisseriales/Neisseriaceae/Eikenella</i>	0.02 (0–0.07)	0 (0)	0.020
<i>Proteobacteria/Gammaproteobacteria/Pseudomonadales/Moraxellaceae/Moraxella</i>	7.36 (0.004–37.11)	0 (0)	0.01
<i>Proteobacteria/Gammaproteobacteria/Pasteurellaceae/Actinobacillus</i>	0.14 (0.005–5.61)	0 (0)	0.002
Phylum/class/order/family/genus/bacterial OTU ^b			
<i>Firmicutes/Clostridia/Lachnospiraceae/Oribacterium</i> unidentified	0.04 (0.004–0.18)	0 (0–0.06)	0.01
<i>Proteobacteria/Betaproteobacteria/Neisseriales/Neisseriaceae/Eikenella/E. corrodens</i>	0.02 (0–0.07)	0 (0)	0.02
<i>Proteobacteria/Gammaproteobacteria/Pseudomonadales/Moraxellaceae/Moraxella/Moraxella</i> unidentified	7.36 (0.004–37.11)	0 (0)	0.01
<i>Proteobacteria/Gammaproteobacteria/Pasteurellaceae/Actinobacillus/Actinobacillus</i> unidentified	0.13 (0.005–5.44)	0 (0)	0.002
<i>Proteobacteria/Gammaproteobacteria/Pasteurellaceae/Actinobacillus/Haemophilus paraaemolyticus</i>	0.01 (0–0.24)	0 (0)	0.02

^a Only results with a statistically significant difference between moderate-to-severe and advanced COPD groups (Mann-Whitney test, $P < 0.05$).

^b Bacterial operational taxonomic unit (OTU) identification according to Greengenes database.

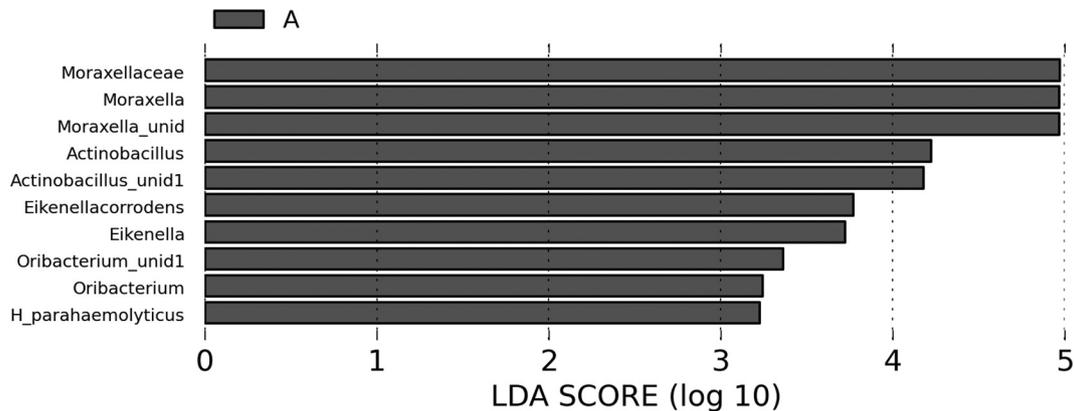


FIG 6 LEfSe (24) rank plot of differentially abundant taxa (bacterial OTUs) at different taxonomic levels in moderate-to-severe versus advanced COPD patients. Statistical analysis found 10 bacterial OTUs overrepresented in moderate-to-severe COPD patients (A) compared with patients with advanced disease. unid, unidentified.

advanced disease. The bronchial microbiome in these patients changed to a restricted flora that included an overrepresentation of the bacterial OTU portion of the *Proteobacteria* phylum, which included most of the bacteria considered PPMs, and paralleled a decline in the relative abundance of the bacterial OTU portion of the *Firmicutes* phylum.

Data from our patients confirm the complex bacterial community harbored in bronchial secretions from stable COPD patients, with *Proteobacteria*, *Firmicutes*, and *Actinobacteria* as the main colonizing phyla. This characteristic microbial pattern disrupts the continuity of the microbiome pattern observed from the oropharynx to the bronchial tree in normal subjects (27), with the appearance of PPM usually not found in healthy people (10). The magnitude of this change parallels the severity of the disease, as suggested by the inverse correlation between the relative abundances of *Firmicutes* and *Proteobacteria*, which include the main PPMs for the respiratory system, with an overrepresentation of this pathogenic flora in patients with advanced disease.

Bacterial diversity was clearly higher in the moderate-to-severe COPD patients, who showed a statistically significant overrepresentation of the genera part of the most prevalent phyla. These genera represented 12% of the relative abundance in this subgroup and <1% in patients with advanced COPD. Our results do not correlate with previous data from Pragman and colleagues (28), who did not find severity-related differences in the microbial diversity of the bronchial tree in COPD, a discrepancy probably attributable to patient selection and sampling. Our study included patients who showed a wider range of lung function impairment, increasing the power for identification of differences between COPD subgroups. Additionally, Pragman et al. analyzed bronchoalveolar lavage samples, which have shown similarity to bronchial biopsy specimens but clear-cut differences in their microbiome composition compared with upper bronchial samples such as sputum and bronchial aspirates (8). Sputum, a sample with easier recovery and a standardized processing procedure (16, 17), was selected for our study because of the greater representation of the whole bronchial tree, considering studies that have found significant differences in the microbiome pattern in the bronchial surface of different bronchi from the same patient (9).

We have shown that the loss of bacterial diversity in advanced COPD was paralleled by a substitution of the original flora by colonizing bacteria only marginally present in the bronchial mi-

crobiome of COPD patients with less severe lung function impairment, a change shown by the decrease in alpha diversity found in patients with advanced disease. This observation supports a severity-related change in the bronchial microbiome in COPD, from a more diverse flora to a restricted microbiome, with an overrepresentation of genera which included PPMs, paralleling the lung function decline that characterizes advanced COPD. This pattern of decreased diversity has also been reported in cystic fibrosis (29–31), suggesting a change to a bacterial flora with a predominance of some bacterial species that dominate the community (31).

In our study, we have identified a group of bacterial OTUs present in all patients suffering from this disease, restricted to 3% of the detected bacterial OTUs, which included bacterial OTUs in microorganisms from the genera *Rothia*, *Streptococcus*, and *Veillonella* and in the main components of the bronchial microbiome in healthy subjects (27). Microbial components shared by patients with moderate-to-severe and advanced disease may be considered part of the residential flora of their upper bronchial tree or oropharyngeal contamination of the sputum sample, a differentiation that may be only of marginal importance, considering that a continuity pattern in the bronchial flora from oropharynx to trachea has been clearly described in a healthy population (27).

Potentially significant limitations of the study are the limited number of samples and the differences in historical antibiotic use in COPD patients according to their severity, which may influence the observed microbiome. To minimize this effect, we have included in the study only patients who had not been treated with antibiotics in the previous month, an approach supported by the demonstrated recovery of bronchial colonization a few weeks after antibiotic treatment for an exacerbation (20). We cannot exclude effects of the technical difficulties inherent in the detection of certain specific bacteria due to the DNA extraction protocols and/or amplification primers on the observed results. Accordingly, our results must be considered an initial insight into the microbiome patterns in COPD, which would need to be confirmed in larger cohorts.

In conclusion, we confirm the presence of a rich bronchial microbiome in COPD in the absence of symptoms of bronchial infection, with a high prevalence of microorganisms belonging to the *Proteobacteria*, *Firmicutes*, and *Actinobacteria* phyla. Moreover, our data support the hypothesis that the bronchial microbiome in patients suffering from advanced COPD shows a decline

in bacterial diversity, with the substitution of the wide range of bacteria harbored in patients with less severe impairment by a more restricted microbiome that includes well-known PPMs that have only a marginal presence in moderate-to-severe COPD patients. The substitution of the originally resident microbiome observed in advanced COPD may be partly due to the structural changes of the bronchial tree that characterize the disease at this severity stage and may be influenced by the recurrent need of antibiotic courses for exacerbation treatment. The greater abundance of genera that include PPMs in patients with advanced COPD may contribute to the maintenance of chronic inflammatory response in the bronchial mucosa, which would further impair the changes in bronchial mucosa of advanced disease. This vicious circle, accordingly, may be important for the natural history of the disease.

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