Lung microbiome in chronic obstructive pulmonary disease

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Lung microbiome in chronic obstructive pulmonary disease

Directed by Professor Ji Ye Jung

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Doctor of Philosophy in Medical Science

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This certifies that the Doctoral Dissertation of
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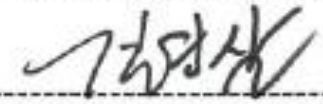

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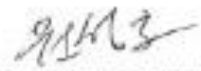

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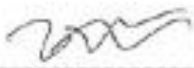

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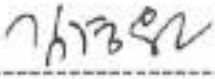

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ABSTRACT

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Introduction: The profile of the lung microbiome when sampled longitudinally over extended time frames remain poorly understood. In this study, we aimed to compare the lung microbiome of chronic obstructive pulmonary disease (COPD) to that of healthy subjects and how it differs according to various clinical characteristics by serial data collection from the same participants.

Methods: Ten healthy males and 43 males with COPD were recruited between February 2017 and August 2021 at Severance Hospital. We collected 129 sputum samples annually for 2 years from the participants with COPD. Sputum was analyzed using 16S ribosomal ribonucleic acid gene sequencing. We investigated the association of recent exacerbation (acute exacerbation within 3 months), inhaled corticosteroid (ICS) use, smoking status (current or ex-smoker), and lung function with the respective genera of the lung microbiome by fitting multiple negative binomial mixed models (NBMM).

Results: There were no significant differences between the microbial diversity of patients with COPD and that of healthy controls, or according to clinical characteristics. However, *Parvimonas*, *Selenomonas*, *Peptostreptococcus*, *Bulleidia*, and *PAC000661_g* were significantly more frequently identified in patients with COPD. In the NBMM adjusted for age, post-bronchodilator ratio of forced expiratory volume in 1 s, (FEV_1) % predicted, and smoking status, abundances of the genera *PAC001141_g*, *PAC001354_g*, and *Slackia* were significantly lower in the patients who suffered from recent exacerbations. In the model adjusted for age, FEV_1 % predicted, and smoking status, the abundances of the genera *PAC001141_g*, *AB494828_g*, and *Veillonella* were significantly lower in ICS users, while

the abundances of *PAC000661_g*, *Capnocytophaga*, *Phocaeicola*, and *Paludibacter* were significantly higher in ICS users. In the model adjusted for age and FEV_1 % predicted, the abundances of the genera *Actinomyces*, *Atopobium*, *Eubacterium_g11*, *Neisseriaceae_G*, *Bulleidia*, *Fretibacterium*, *Slackia*, *Dialister*, and *PAC001354_g* were significantly higher in current smokers than in ex-smokers. In the model adjusted for age and smoking status, significantly lower abundances of the genera *Bacteroides*, *Pasteurellaceae_G*, and *Aggregatibacter*, and significantly higher abundances of the genera *Prevotella*, *Leptotrichia*, *Megasphaera*, *AB494828_g*, and *Butyrivibrio* were observed according to increasing FEV_1 % predicted.

Conclusion: Using serial data collected over years, we demonstrated that the lung microbiome in the patients with COPD differs significantly according to recent exacerbations, ICS use, smoking status, and lung function. These findings expand the current understanding of the microbiome in patients with COPD.

Key words: chronic obstructive pulmonary disease, lung microbiome, smoking, lung function, exacerbation, inhaled corticosteroid

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I. INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disorder resulting in an irreversible decline in lung function due to inhalation of tobacco smoke or other irritants.[1] Worldwide, COPD affects 300 million people and is the third leading cause of death.[2] COPD is characterized by largely irreversible airflow limitation, mucus hypersecretion, small airway fibrosis, and destruction of the alveolar space.[1,3] Over the past decade, there has been a tremendous surge in interest in discovering novel biomarkers for COPD; however, in most cases, the value of novel biomarkers in guiding COPD phenotyping, prognosis, and management has been limited.[4]

From the initial description of COPD, there has been considerable controversy about the role of microbiome in its pathogenesis.[5] The human microbiome compromises an estimated 100 trillion microbes and our understanding of it has increased dramatically in recent years. [6] The healthy human lung contains a variety of commensal microbiota throughout the respiratory tract, which shows substantial heterogeneity between, and over time, within individuals and across regions within the lung.[7,8]

The lung microbiome represents an emerging opportunity for understanding the heterogeneity and exacerbation of COPD. Alterations in the taxonomic composition of the lung microbiome, known as dysbiosis, have been associated with multiple lung diseases, and in particular may play a functional role in disease severity and exacerbation.[9] There is emerging evidence showing that the lung microbiome is associated with clinical outcome and mortality of patients with COPD.[10]

Most studies on the lung microbiome in COPD were cross-sectional[11] or short-term studies.[9,12] COPD is a persistent, often progressive disease,[3] and investigating the microbiome over the long-term is crucial for the clinical utility of the microbiome as a diagnostic or predictive tool. However, the profile of the lung microbiome sampled over longer time frames remains poorly understood.[13] By observing the lung microbiome in COPD over years, researchers could gain a better understanding of how the microbial composition may be linked to various clinical characteristics of COPD, including disease severity, exacerbation frequency, response to treatment, and overall prognosis.

In this prospective study, sputum samples, which are reported to be consistent with the respiratory microbiome detected in bronchoalveolar lavage and bronchial samples[14,15], were collected annually for two years from patients with COPD. Through serial sampling of the lung microbiome using a sensitive molecular diagnostic technique, we aimed to compare the lung microbiome of COPD to that of healthy subjects and identify the differences according to various clinical characteristics.

II. MATERIALS AND METHODS

1. Study population

At baseline, 10 healthy male participants who smoked cigarettes and 54 (53 male and 1 female) patients with COPD were recruited between February 2017 and July 2018 at Severance Hospital.

The inclusion criteria for patients with COPD were as follows: post-bronchodilator ratio of forced expiratory volume in 1 s (FEV_1) % predicted to forced vital capacity (FVC) < 0.7, and absence of respiratory diseases other than COPD (e.g., previous pulmonary resection, tuberculosis-affected lung, and bronchiectasis).

We excluded the one recruited female patient from the analysis because we did not have enough female participants to determine the influence of sex on the lung microbiome. Additionally, 10 male patients with COPD were excluded from the

study due to loss to follow-up (n = 9) or incomplete clinical information (n = 1). Finally, 10 healthy male subjects without COPD and 43 patients with COPD were included in this study.

After an initial enrollment visit, the patients were followed-up every year for two years, and demographic data, exacerbations, pulmonary function tests, COPD assessment test (CAT),[16] modified medical research council (mMRC) dyspnea scale, [17] St George's Respiratory Questionnaire (SGRQ) scores, sputum samples, and laboratory tests were collected or performed (Figure 1). Recent exacerbation was defined as the aggravation of one of three symptoms (dyspnea, cough, or sputum) for two or more days requiring an unscheduled hospital visit for additional treatment with systemic steroids or antibiotics, emergency room visits, or hospitalization within the past 3 months.

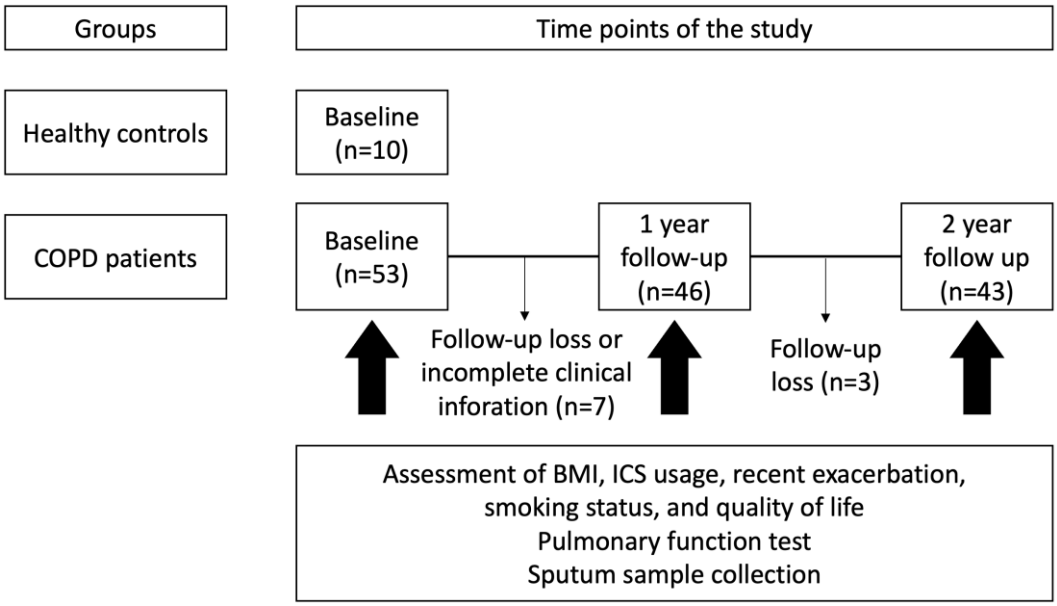


Figure 1. Data collection timeline. COPD, chronic obstructive pulmonary disease; BMI, body mass index; ICS, inhaled corticosteroids.

All procedures were conducted in accordance with the principles of the Declaration of Helsinki. The study protocol was approved by the Institutional Review Board of the Severance Hospital (Institutional Review Board approval number: 2016-2133-001). Written informed consent was obtained from all patients.

2. Sputum sample acquisition

Sputum was induced using 3% saline solution and samples were collected in a deoxyribonucleic acid (DNA)-free container. After collection, fresh sputum samples were transferred to sterile containers and stored at -80 °C until processing.

3. Bacteriome (16S rRNA gene amplicon) sequencing and analysis

To decrease viscosity, 20 μL of β-mercaptoethanol (Sigma Aldrich, USA) was added to 1 mL of induced sputum, and the sample was shaken at 200 rpm for 0.5 to 1 h at 37 °C. The pretreated samples were subjected to DNA extraction using a FastDNA SPIN Kit for soil DNA extraction (MP Biomedicals, USA), according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification of the V3-V4 region of the 16S rRNA gene was performed using 2× KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Italy) with primers (318F: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′ and 806R: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′). The PCR cycling conditions were as follows: 3 min at 95 °C; 25 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C; 5 min at 72 °C; and holding at 4 °C. PCR products were purified using AMPure XP beads (Beckman Coulter, High Wycombe, UK). The amplicon sequencing library was constructed according to the 16S metagenomic sequencing library preparation method (Illumina, USA), and 300 bp paired-end sequencing (2 × 300 bp) was conducted using an Illumina MiSeq Reagent Kit v3

(Illumina, USA).

Raw sequencing data files were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) 2 pipeline (https://qiime2.org; version 2021.4). DADA2 in the QIIME 2 package was used to denoise the raw sequence data. Quality filtering of the paired-end FASTQ files was performed based on Phred quality scores. A feature table consisting of amplicon sequence variants was constructed after merging paired-end sequences and removing chimeras. Taxonomic analysis was carried out using q2featureclassifier in QIIME[18] with the EzBioCloud 16S database.[19] A pre-specified exclusion criteria was samples with zero counts confirmed in specimens of more than 50% of the total for the negative binomial mixed model (NBMM; described in Statistical analysis section).

4. Statistical analysis

Statistical analysis was performed using R version 4.1.3, SPSS version 21 (IBM Inc., Armonk, NY, USA), and OriginPro (Version 2023b. OriginLab Co. USA). Descriptive data were presented as means with standard deviations or relative abundances (%). Alpha diversity indices [observed operational taxonomic units (OTUs)[20], abundance-based coverage estimators (ACE)[21], Chao1[22], Shannon[23], and Simpson[24]] were computed for each rarefied table, and the Kruskal–Wallis test was implemented in R to detect significant differences. Principal coordinates analysis was based on Bray-Curtis dissimilarity. Differences in taxa abundance were calculated using Wilcoxon tests. The Spearman's rank correlation coefficient was used to analyze the correlation between the microbiota and clinical characteristics, and visualization was performed in the form of a heat map. We then investigated effects of recent exacerbation, inhaled corticosteroid (ICS) usage, smoking status, and FEV_1 % predicted, on the respective genus by fitting multiple NBMM[25] (R package 'NBZIMM') with age, ICS usage, smoking status, FEV_1 % predicted, and time (year), respectively. $P < 0.05$ was considered

statistically significant.

III. RESULTS

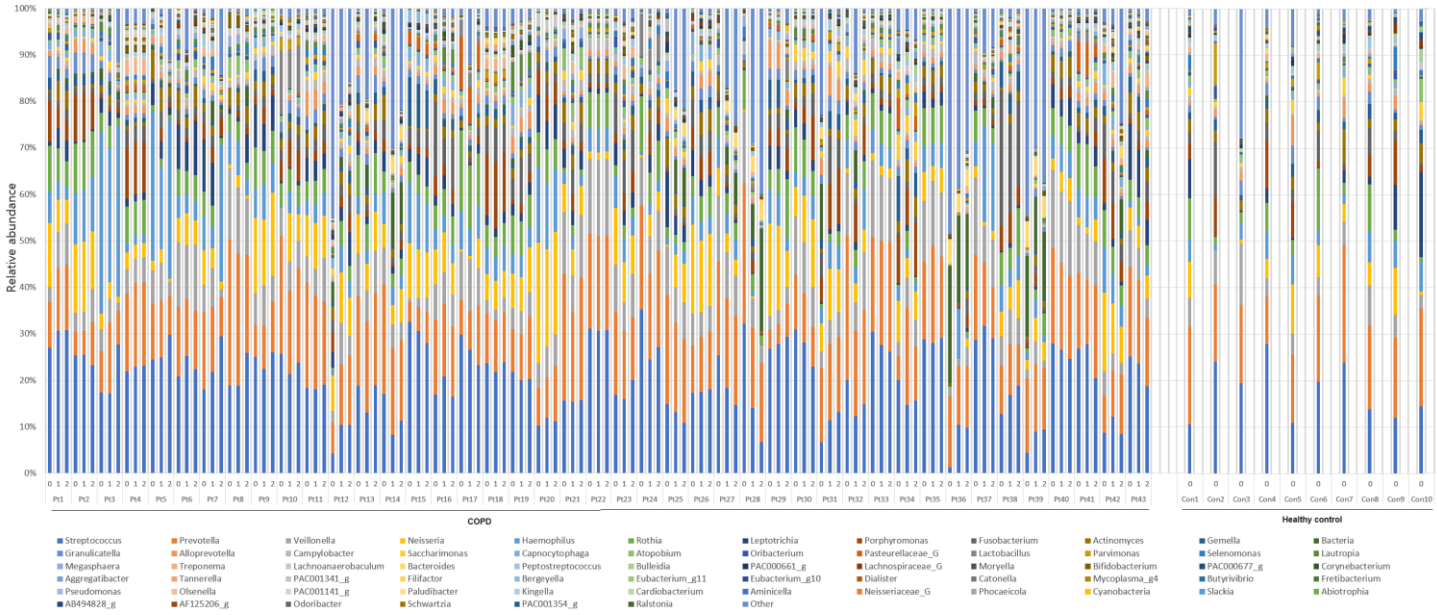
1. Baseline characteristics

One hundred and twenty-nine sputum samples from patients with COPD and ten sputum samples from healthy control subjects were included in the study. The clinical characteristics of the cohort are shown in Table 1. Compared to the healthy controls at baseline, the patients with COPD were older (62.6 years vs 72.0 years, P =0.001), had lower FEV_1 % predicted (102.6 % vs 63.7 %, P <0.001), and lower FEV_1/FVC (77.8 % vs 44.2 %, P <0.001). Among the patients with COPD, 23.3% were current smokers, 69.8% were categorized into global initiative for COPD group A, 25.6% were using ICS, and 39.5% and 48.8% of the subjects reported that they had CAT and SGRQ scores higher than 10 and 65, respectively. The taxonomic classification of the bacterial communities present in sputum is presented in Figure 2. Across the sample population, the most abundant genera were *Streptococcus* (28.9%), *Prevotella* (21.8%), and *Veillonella* (10.9%).

Table 1. Summary characteristics of participants included in the study

Baseline characteristics		Control (n=10)	COPD (n=43)		
		Baseline	Baseline	1-year follow up	2-year follow up
Age, years		62.6 ± 10.3	72.0 ± 6.6	73.0 ± 6.6	74.0 ± 6.6
BMI, kg/m^2		22.8 ± 3.3	23.8 ± 3.5	24.0 ± 3.5	24.1 ± 3.3
Smoking					
	Ex-smoker	8 (80%)	33 (76.7%)	35 (81.4%)	35 (81.4%)
	Current smoker	2 (20%)	10 (23.3%)	8 (18.6%)	8 (18.6%)
Pulmonary function tests					
	FVC, L	3.97 ± 0.39	3.37 ± 0.65	3.40 ± 0.71	3.29 ± 0.67
	FVC, % predicted	97.8 ± 12.1	94.2 ± 15.0	93.7 ± 16.0	91.5 ± 16.3
	FEV_1, L	3.02 ± 0.55	1.56 ± 0.41	1.52 ± 0.43	1.49 ± 0.45
	FEV_1 % predicted	102.6 ± 10.6	63.7 ± 16.0	64.1 ± 19.7	61.9 ± 19.1
	FEV_1/FVC, %	77.8 ± 2.8	44.2 ± 10.0	42.6 ± 11.0	42.3 ± 11.0
	FEV_1 % predicted <50%	0 (0%)	7 (16.3%)	8 (18.6%)	8 (18.6%)
GOLD group	A	n/a	30 (69.8%)	24 (55.8%)	28 (65.1%)
	B,E	n/a	13 (30.2%)	19 (44.2%)	15 (34.9%)
Average CAT score		n/a	13.3 ± 7.8	13.1 ± 8.1	13.7 ± 9.0
CAT score	< 10	n/a	26 (60.5%)	24 (55.8%)	25 (58.1%)
	≥ 10	n/a	17 (39.5%)	19 (44.2%)	18 (41.9%)
Acute exacerbation within 3 months		n/a	5 (11.6%)	5 (11.6%)	4 (9.3%)
ICS use, yes		n/a	11 (25.6%)	13 (30.2%)	14 (32.6%)

COPD, chronic obstructive pulmonary disease; BMI, body mass index; FVC, functional vital capacity; FEV_1, forced expiratory volume in 1 s; CAT, COPD assessment test; SGRQ, St George's Respiratory Questionnaire; GOLD, Global Initiative for Chronic Obstructive Lung Disease; ICS, Inhaled corticosteroid



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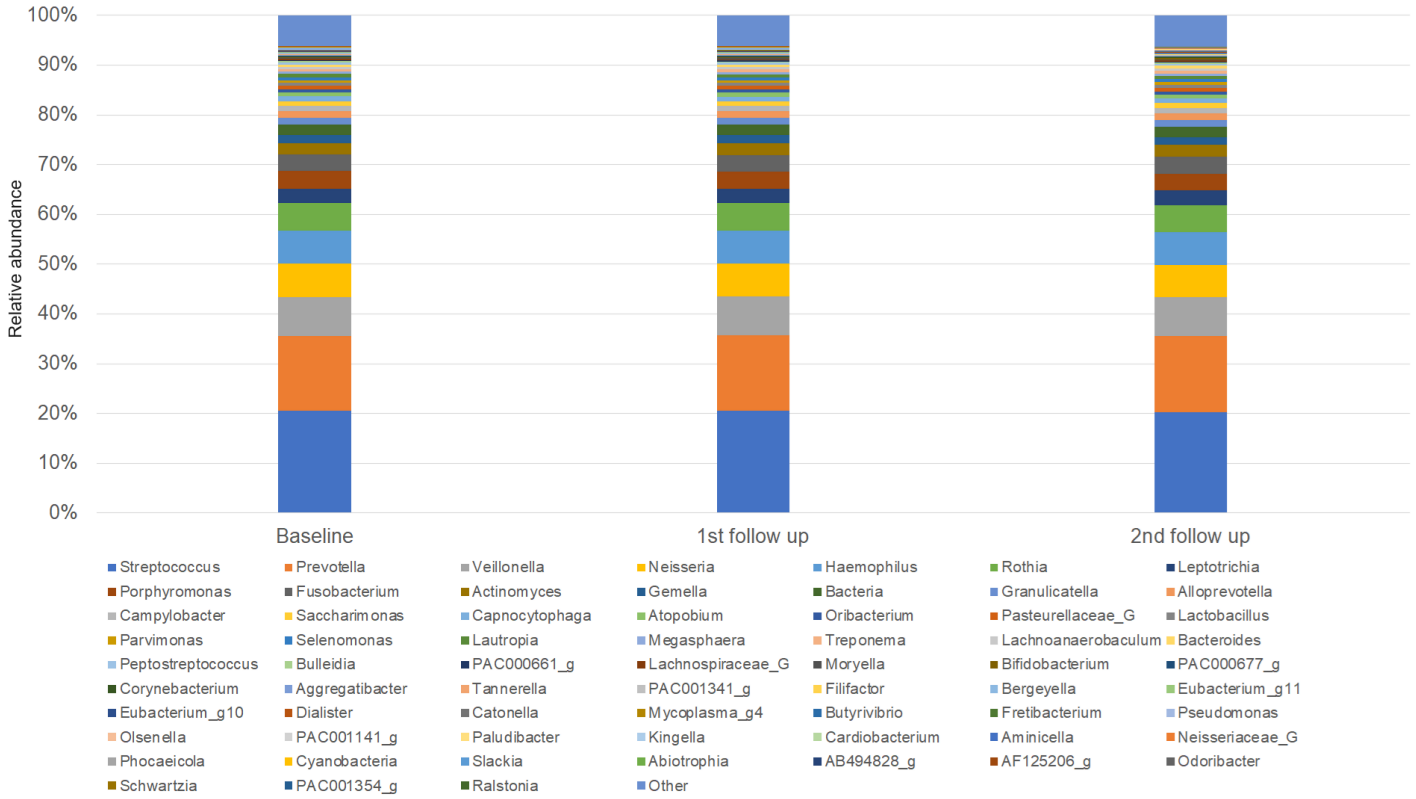
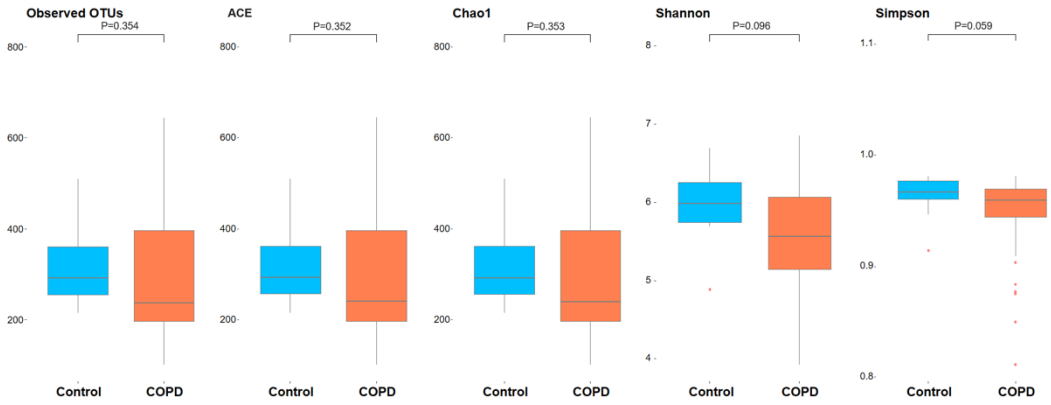


(b)

Figure. 2 Bacterial composition of sputum samples at the genera level; (a) in all samples and (b) by annual average.

2. Lung microbiome in patients with COPD and healthy controls

No significant differences in microbial diversity were observed between the patients with COPD and healthy control lungs (Figure 3) using each of the alpha diversity metrics (observed OTUs, ACE, Chao1, Shannon, and Simpson; all $P > 0.05$) or Bray-Curtis analysis ($P = 0.127$). However, at the genus level, *Parvimonas*, *Selenomonas*, *Peptostreptococcus*, *Bulleidia*, and *PAC000661_g* were significantly more abundant in the lung microbiome of patients with COPD than in that of healthy controls (Figure 4).



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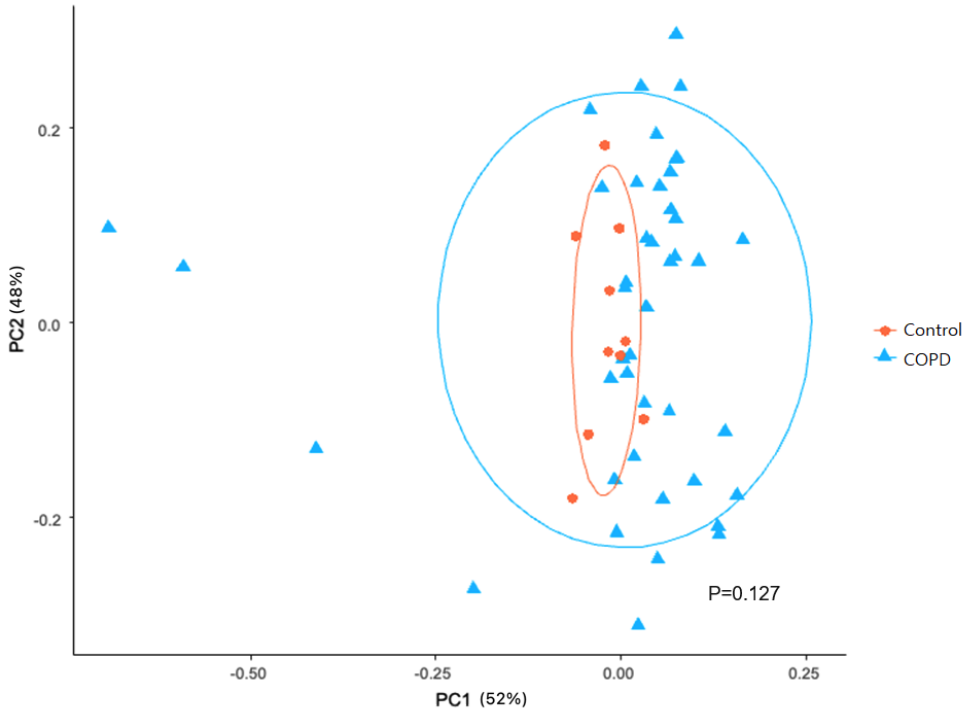

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Figure 3. Comparison of the lung microbiome between patients with chronic obstructive pulmonary disease and healthy controls; (a) alpha diversity indices, and (b) principal coordinates analysis plot with Bray-Curtis dissimilarity.

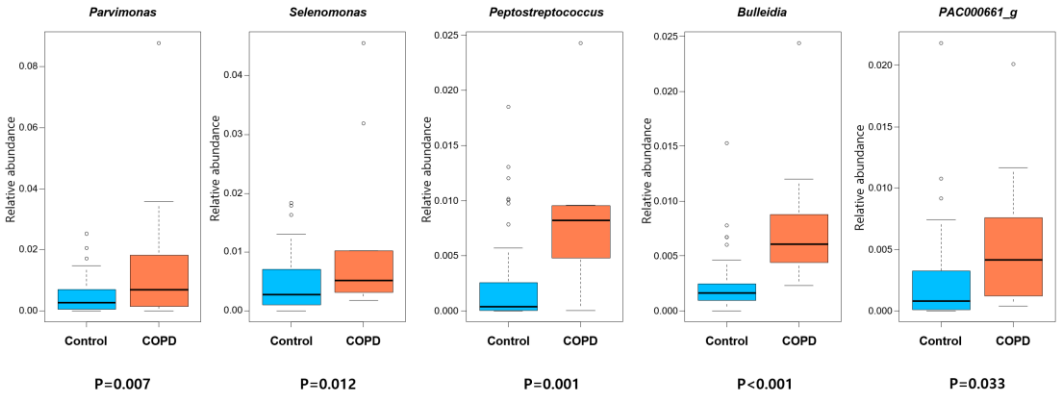


Figure 4. Differences in the lung microbiome between patients with chronic obstructive pulmonary disease and healthy controls at the genera level.

3. Lung microbiome in COPD according to clinical characteristics at baseline

Figure 5 shows the results of the Spearman correlation analyses between the variables and lung microbiome in COPD. *Prevotella, Rothia, Actinomyces, Atopobium, Lactobacillus, Megasphaera, Moryella,* and *Kingella* were positively, *Gemella, Lachnospiraceae_g,* and *Abiotrophia* were negatively correlated with age. *Porphyromonas, Lachnoanaerobaculum, Bergeyella, PAC001141_g,* and *Schwartzia* were positively, *Lachnospiraceae_g and PAC001341_g* were negatively correlated with FEV_1 % predicted. *Lactobacillus, Selenomonas, Treponema, Bifidobacterium, and Phocaeicola* were positively, *Haemophilus and Pseudomonas* were negatively correlated with quality of life (CAT).

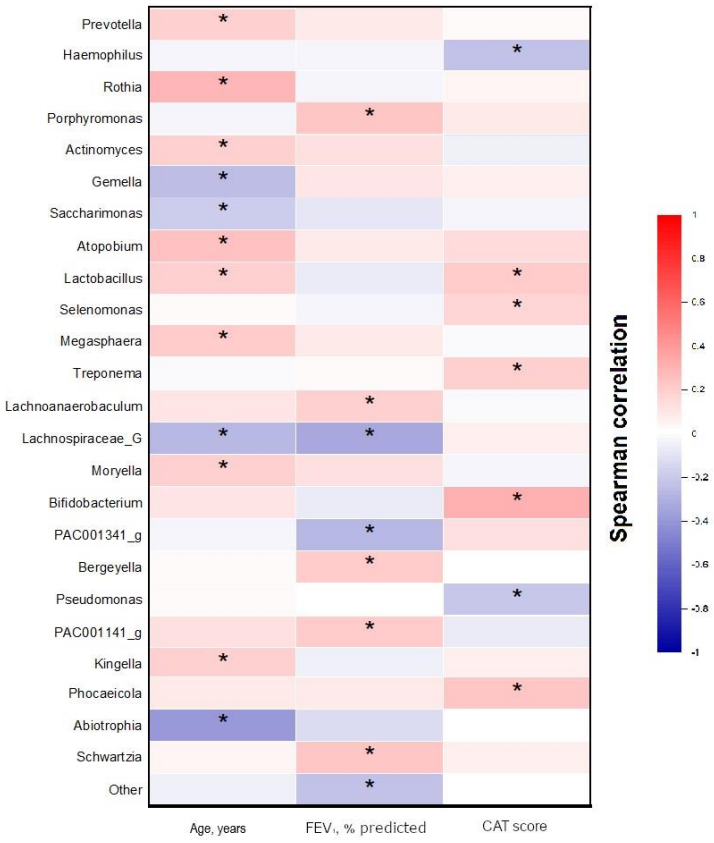
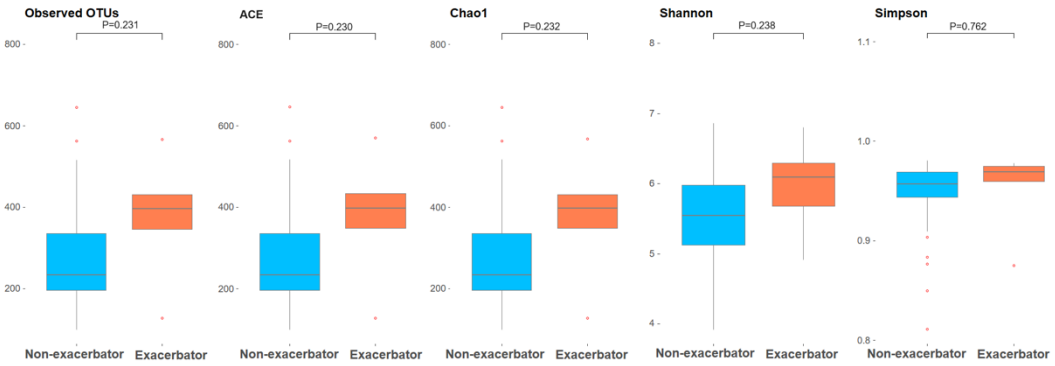


Figure 5. Spearman correlation heatmap based on genera and clinical variables. Only significant genera are shown and P <0.05 is represented as *

FEV_1, forced expiratory volume in 1 s; CAT, chronic obstructive pulmonary disease assessment test.

The comparison of the lung microbiome among patients with COPD were not significantly different using the alpha diversity metrics (observed OTUs, ACE, Chao1, Shannon, and Simpson) or Bray-Curtis analysis according to recent exacerbation (Figure 6), current smoking (Figure 7), ICS use (Figure 8), and FEV_1 % predicted value of 50% (Figure 9). At the genus level, *Capnocytophaga* and *Lautropia* were more abundant in those who suffered from recent

exacerbations. *Capnocytophaga* was more abundant in ICS users. *Actinomyces*, *Saccharimonas*, and *Pasteurellaceae* were more abundant in current smokers, and *Rothia* and Pasteurellaceae_G were more abundant in the patients with a FEV_1 % predicted lower than 50% (Figure 10).



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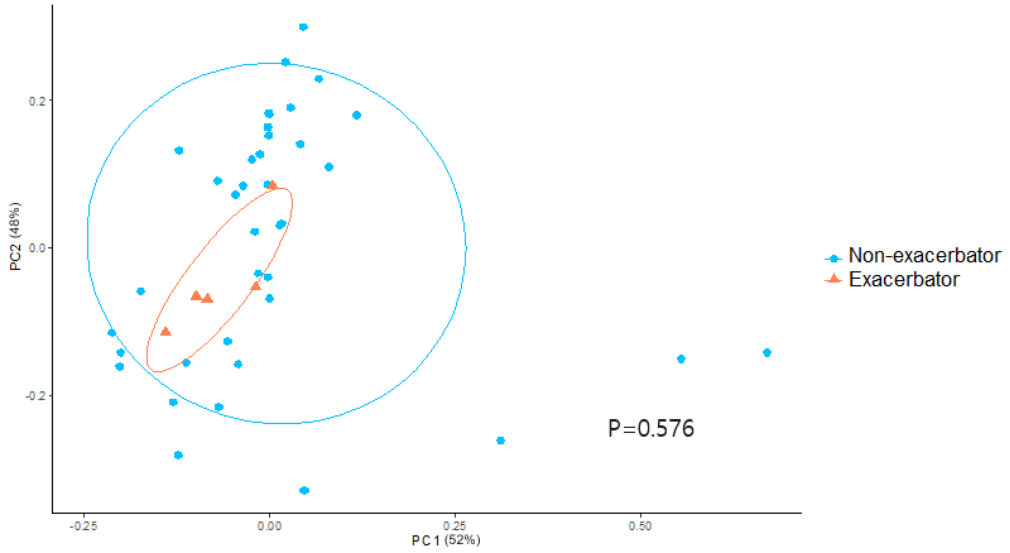

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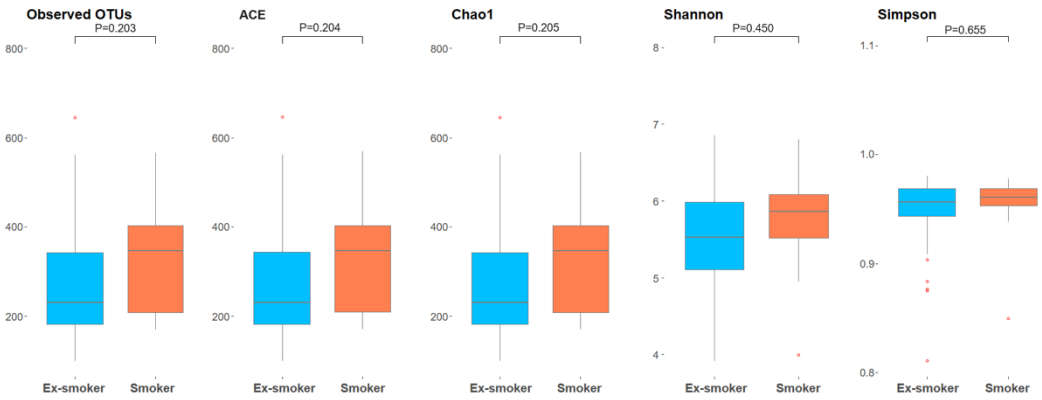
Figure 6. Microbiome profile according to recent exacerbation within 3 months at baseline; (a) alpha diversity indices, and (b) principal coordinates analysis plot with Bray-Curtis dissimilarity.



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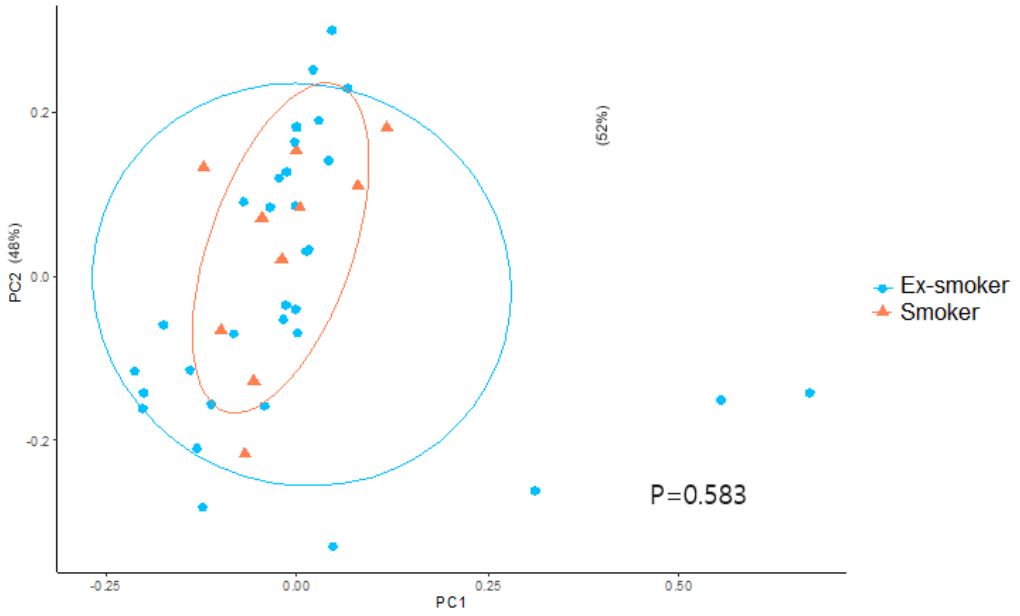

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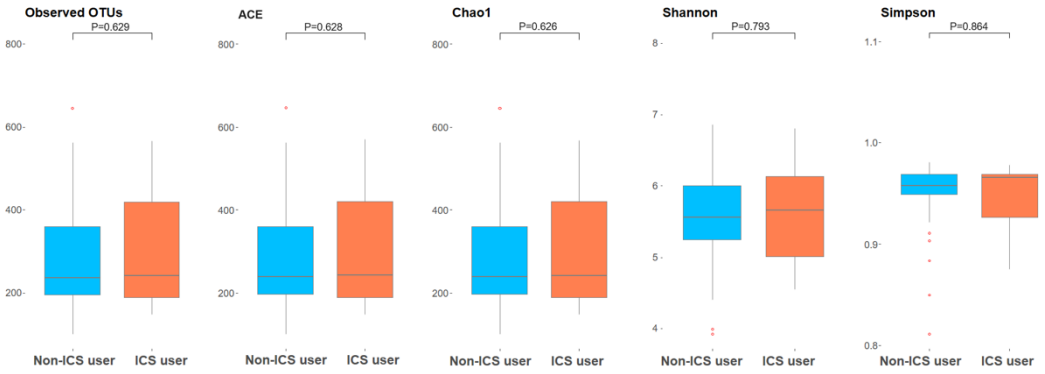
Figure 7. Microbiome profile according to smoking status at baseline; (a) alpha diversity indices and (b) principal coordinates analysis plot with Bray-Curtis dissimilarity.



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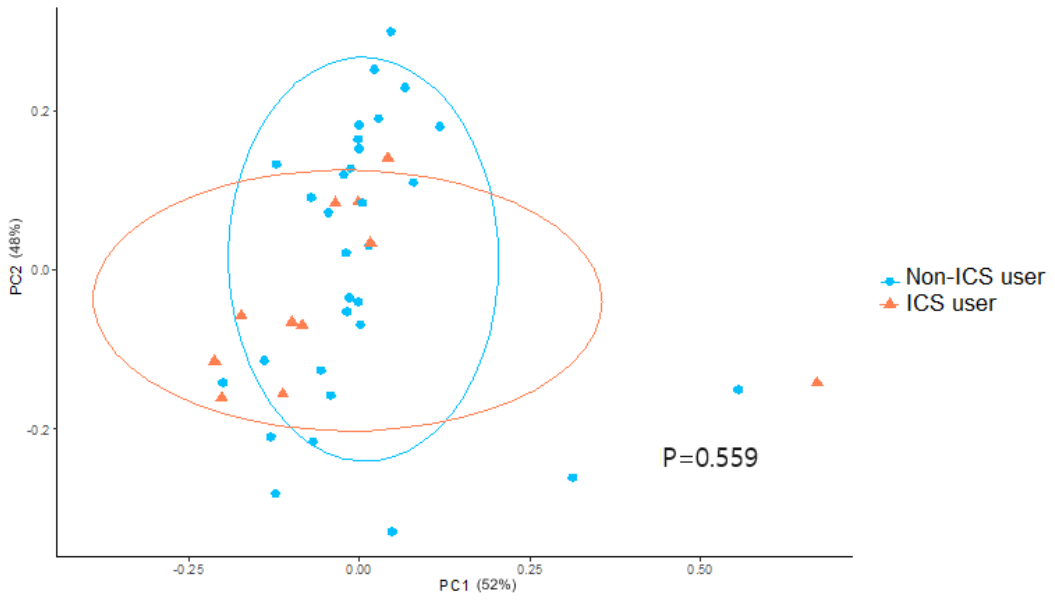

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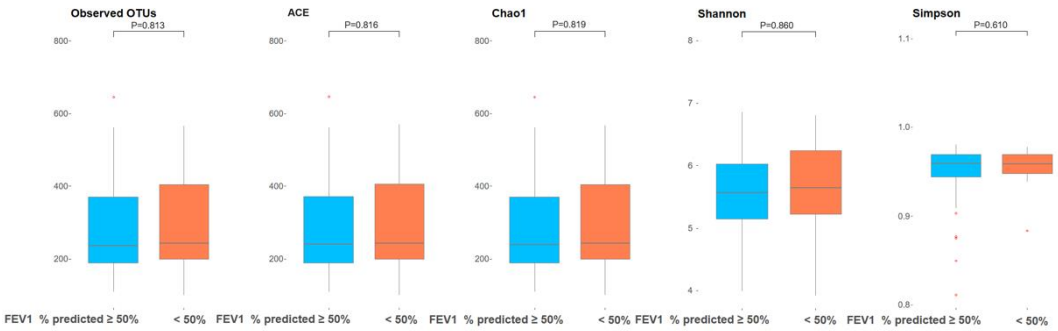
Figure 8. Microbiome profile according to inhaled corticosteroid use at baseline; (a) alpha diversity indices and (b) principal coordinates analysis plot with Bray-Curtis dissimilarity.



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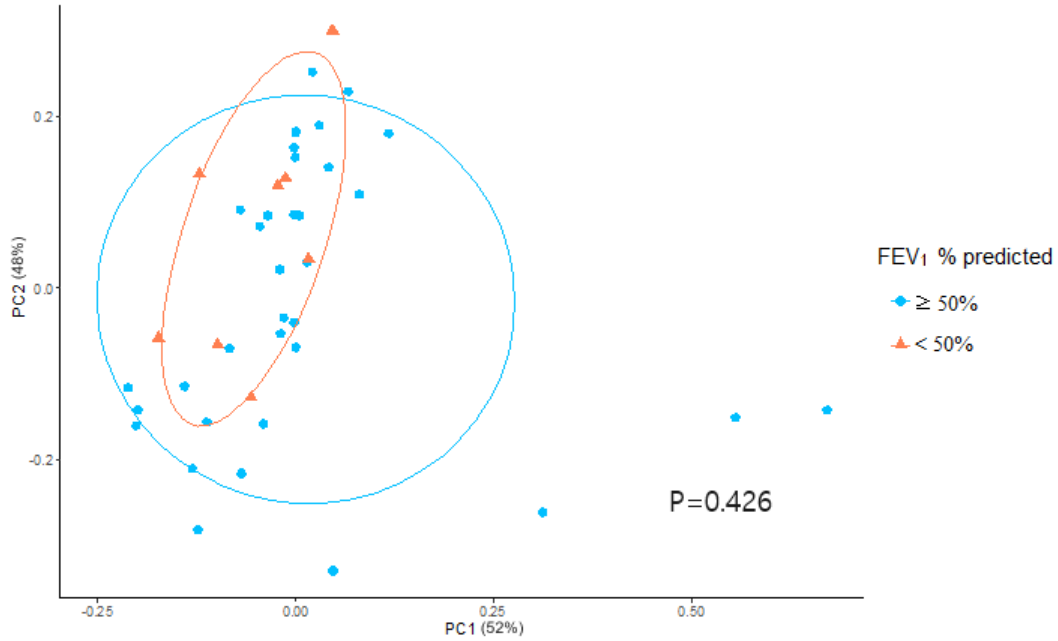

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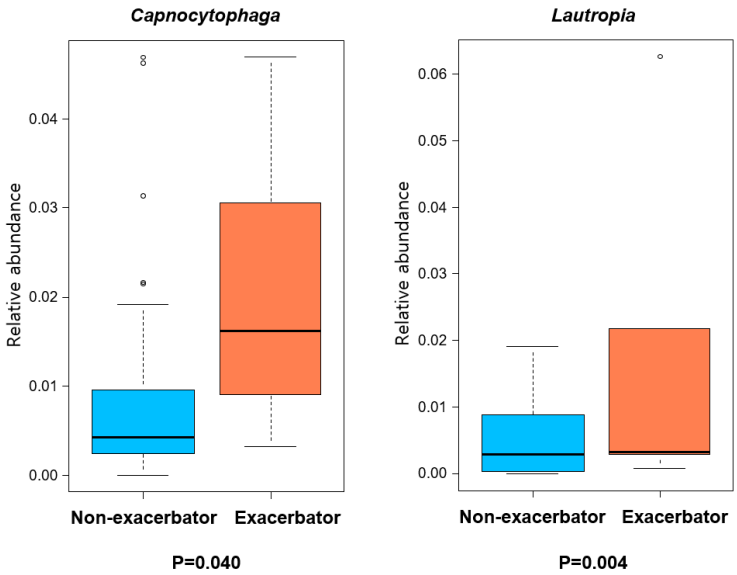
Figure 9. Microbiome profile according to forced expiratory volume in 1 s % predicted value of 50% at baseline; (a) alpha diversity indices and (b) principal coordinates analysis plot with Bray-Curtis dissimilarity.



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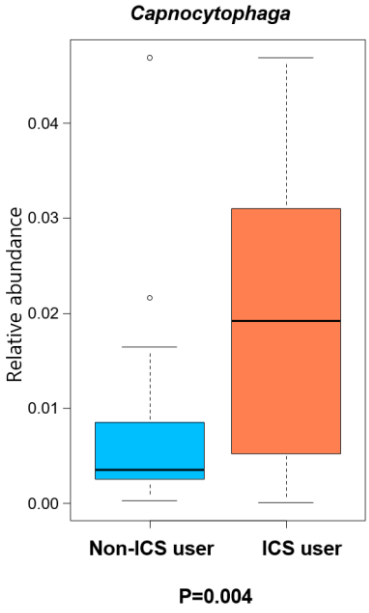

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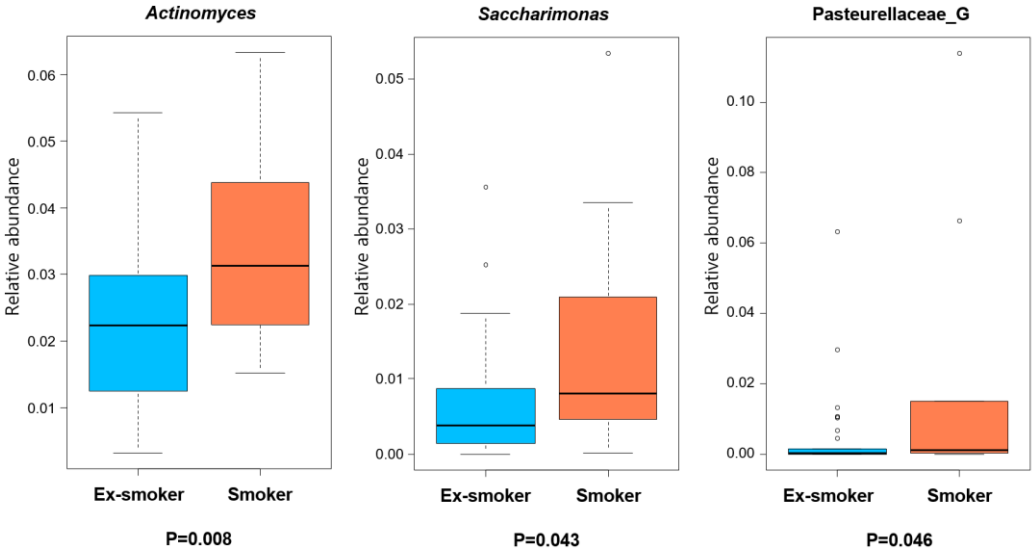

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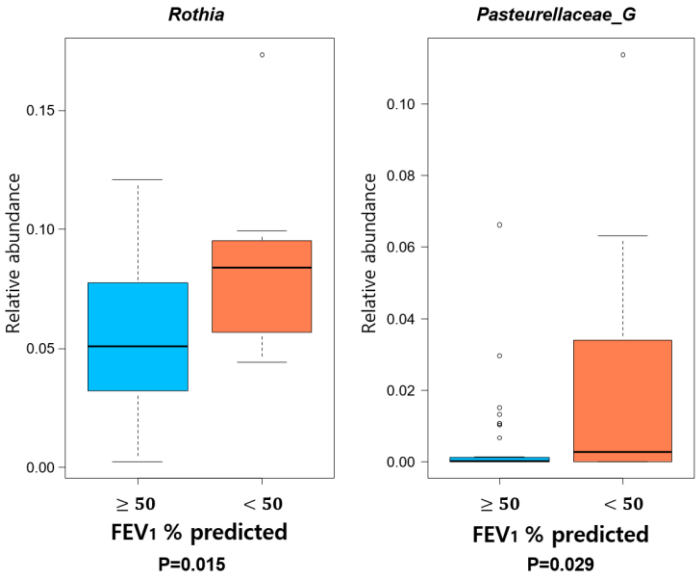

(d)

Figure 10. Significant differences in the microbiome of patients with chronic obstructive pulmonary disease at the genera level according to (a) recent exacerbation, (b) inhaled corticosteroid use, (c) smoking status, and (d) forced expiratory volume in 1 s (FEV_1) % predicted value of 50%.

4. Association between repeatedly collected lung microbiome and various clinical characteristics in COPD

The genera that exhibited significant differences in their respective NBMM are shown in Table 2. In the model adjusted for age, FEV_1 % predicted, and smoking status, abundances of the genera *PAC001141_g*, *PAC001354_g*, and *Slackia* were significantly lower in the patients who suffered from recent exacerbations (model 1). In the model adjusted for age, FEV_1 % predicted, and smoking status, the abundances of the genera *PAC001141_g*, *AB494828_g*, and *Veillonella* were significantly lower in ICS users, while the abundances of *PAC000661_g*, *Capnocytophaga*, *Phocaeicola*, and *Paludibacter* were significantly higher in ICS users (model 2). In the model adjusted for age and FEV_1 % predicted, the abundances of the genera *Actinomyces*, *Atopobium*, *Eubacterium_g11*, *Neisseriaceae_G*, *Bulleidia*, *Fretibacterium*, *Slackia*, *Dialister*, and *PAC001354_g* were significantly higher in current smokers (model 3). In the model adjusted for age and smoking status, significantly lower abundances of the genera *Bacteroides*, *Pasteurellaceae_G*, and *Aggregatibacter*, and significantly higher abundances of the genera *Prevotella*, *Leptotrichia*, *Megasphaera*, *AB494828_g*, and *Butyrivibrio* were observed according to increasing FEV_1 % predicted (model 4).

Table 2. Negative binomial mixed models for genera with significantly different abundances according to various clinical characteristics

	Estimate	S.E.	t-value	P-value
Model 1 - AE within 3 months[1]				
PAC001141_g	-1.211	0.602	-2.013	0.047
PAC001354_g	-0.848	0.389	-2.178	0.032
Slackia	-0.632	0.300	-2.105	0.038
Model 2 - ICS use[2]				
PAC001141_g	-1.134	0.432	-2.624	0.010
AB494828_g	-0.843	0.396	-2.130	0.036
Veillonella	-0.278	0.113	-2.451	0.016
PAC000661_g	0.522	0.219	2.376	0.020
Capnocytophaga	0.612	0.187	3.279	0.002
Phocaeicola	0.784	0.333	2.353	0.021
Paludibacter	0.933	0.284	3.291	0.001
Model 3 – Current smoking[3]				
Actinomyces	0.402	0.173	2.320	0.023
Atopobium	0.520	0.231	2.247	0.027
Eubacterium_g11	0.592	0.257	2.307	0.024
Neisseriaceae_G	0.638	0.274	2.325	0.022
Bulleidia	0.664	0.254	2.613	0.011
Fretibacterium	0.673	0.325	2.072	0.041
Slackia	0.715	0.358	1.994	0.049
Dialister	0.800	0.368	2.175	0.032
PAC001354_g	0.863	0.375	2.300	0.024
Model 4 - FEV_1 % predicted[4]				
Bacteroides	-0.048	0.014	-3.303	0.001
Pasteurellaceae_G	-0.023	0.006	-3.711	0.000
Aggregatibacter	-0.020	0.005	-4.142	0.000
Prevotella	0.005	0.002	2.085	0.040

Leptotrichia	0.013	0.006	2.203	0.030
Megasphaera	0.020	0.007	2.894	0.005
AB494828_g	0.020	0.007	2.796	0.006
Butyrivibrio	0.025	0.009	2.832	0.006

[1]Model 1 - Adjusted for age, FEV_1 % predicted, smoking status

[2]Model 2 - Adjusted for age, FEV_1 % predicted, smoking status

[3]Model 3 - Adjusted for age, FEV_1 % predicted

[4]Model 4 - Adjusted for age, smoking status

AE, recent acute exacerbation; ICS, inhaled corticosteroid; FEV_1, forced expiratory volume in 1 s; NBMM, negative binomial mixed model; S.E., standard errors

We investigated the differences in the genera that were significant in the NBMM analysis according to recent exacerbation (Figure 11), ICS use (Figure 12), smoking status (Figure 13), and FEV_1 % predicted (Figure 14). The abundance of *PAC00141_g* differed significantly among patients with no recent exacerbation during the follow-up period and did not differ among those with recent exacerbation. The abundance of *AB494828_g* differed significantly during follow-up only in those who did not use ICS and the abundance of *PAC001141_g* differed significantly during follow-up only in those who used ICS. The abundances of *Neisseriaceae_G*, *Fretibacterium*, *Slackia*, and *Dialister* differed significantly during follow-up only in ex-smokers. No significant differences in genera were observed during follow-up according to FEV_1 % predicted value of 50%.

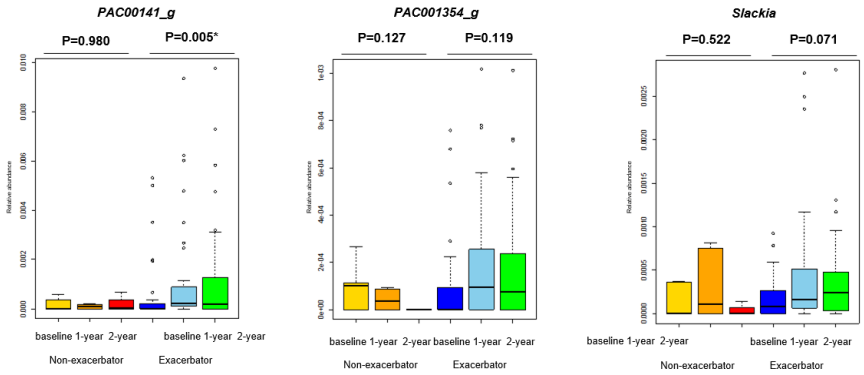

Figure 11. Difference in abundance during the follow up according to recent exacerbation.

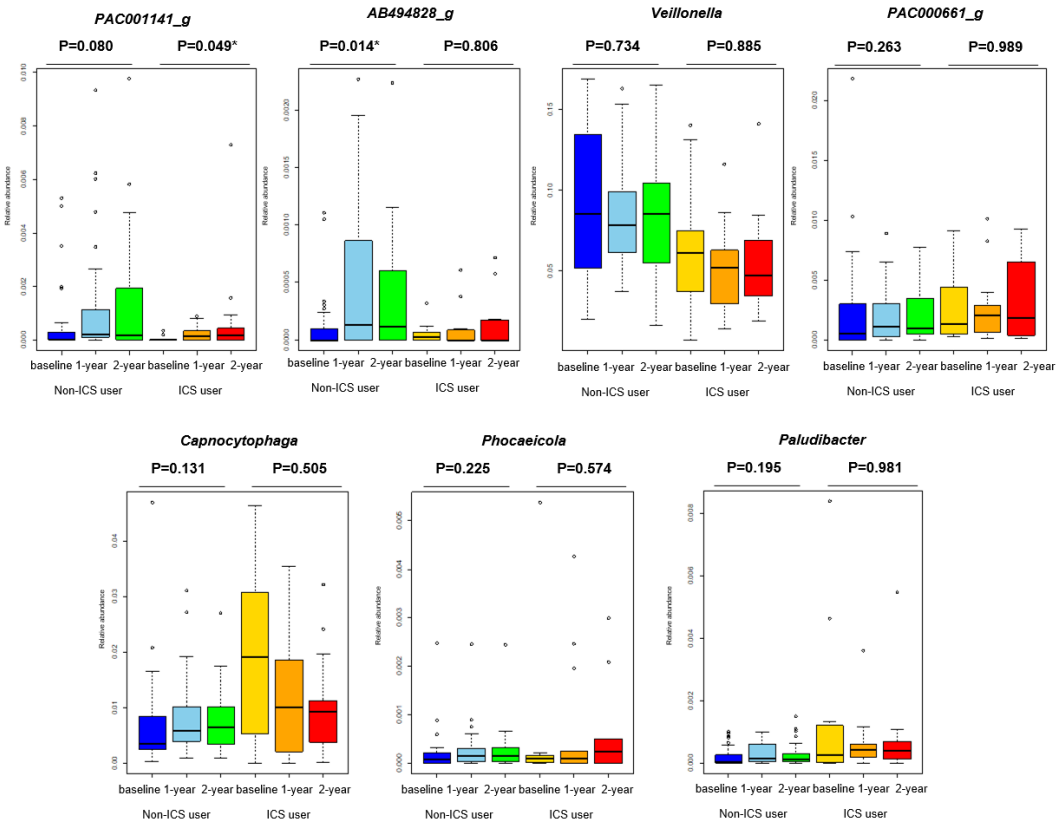

Figure 12. Difference in abundance during the follow up according to inhaled corticosteroid usage.

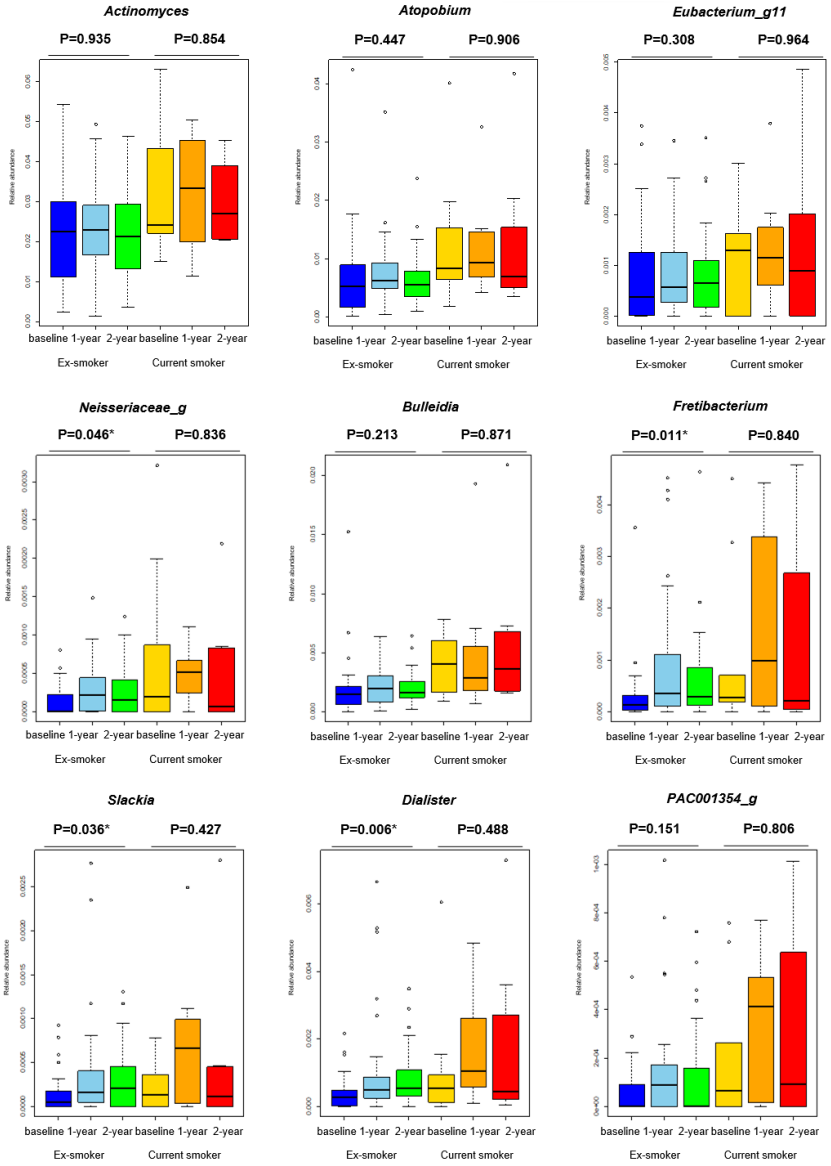

Figure 13. Difference in abundance during the follow up according to smoking status.

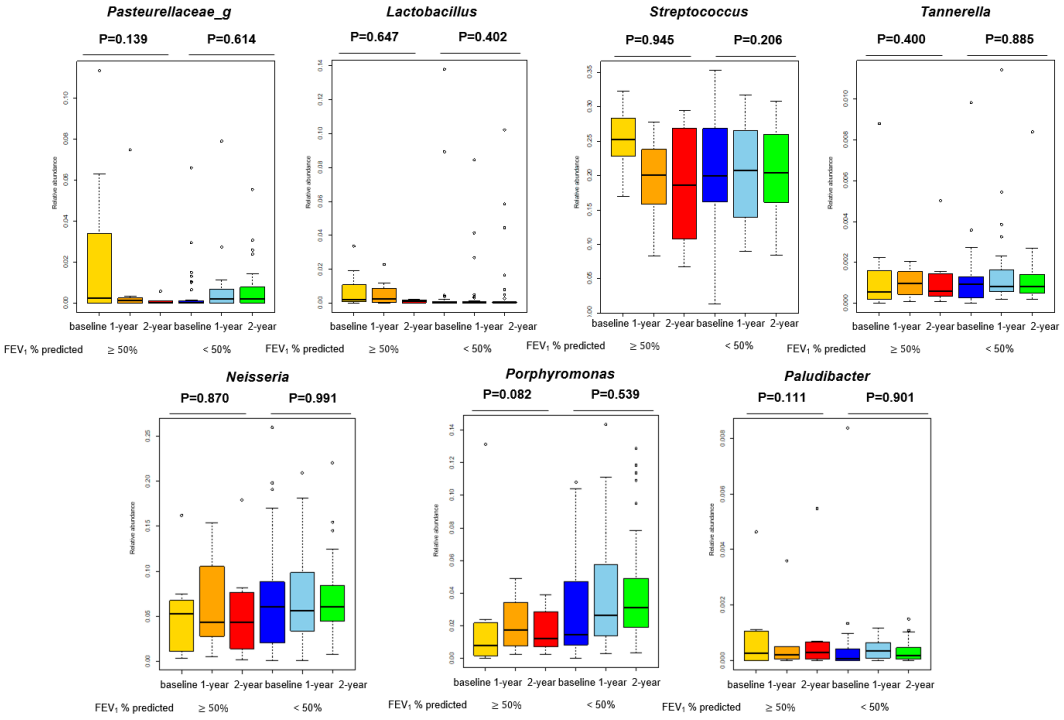

Figure 14. Difference in abundance during the follow-up according to FEV_1 % predicted value of 50%.

IV. DISCUSSION

The clinical significance of the microbiome lies in its potential to serve as a biomarker for disease progression and therapeutic outcomes[26,27] In our study, the lung microbiome was associated with factors such as recent exacerbation of COPD, ICS use, smoking status, and lung function.

The lung microbiome in patients with COPD is known to be significantly different from that of healthy lungs.[15,28] In our study, the genera *Parvimonas*, *Selenomonas*, *Peptostreptococcus*, *Bulleidia*, and *PAC000661_g* were more abundant in patients with COPD than in healthy controls. These results are consistent with those of previous studies. *Selenomonas* is known to be related to smoking[29] and acute exacerbation[30] in

COPD; *Peptostreptococcus* is known to be related to the progression of COPD[31], and an increased prevalence of *Bulleidia* in COPD has been reported.[32]

In the results of lung microbiome at baseline according to clinical characteristics, many of the results were in line with previous studies; more abundant *Capnocytophaga* in steroid usage[33] and exacerbation[34], more abundant *Lautropia* in exacerbation[35], more abundant *Actinomyces*[36], *Saccharimonas* and *Pasteurellaceae_G*[37,38] in smokers. Decreased lung function was associated with more abundant *Rothia* and *Pasteurellaceae_G* in this study. This result is inconsistent with that of a previous study in which *Rothia* was shown to have an inhibitory effect on pathogen-induced inflammatory responses.[39] Further studies are needed on this matter.

Our results from the NBMM are also in line with previous studies; more abundant *Capnocytophaga* with steroid usage[32], more abundant *Actinomyces*, *Dialister*, and *Atopobium*[39] with smoking, and less abundant *Bacteroides* with increased lung function.[40] In contrast, the predominance of *Neisseriaceae* in smokers is inconsistent with that of previous study for *Neisseriaceae* that reported a reduced relative abundance in the upper gastrointestinal tract of smokers.[41] The smoking may result in different response to the relative abundance between gastrointestinal tract and respiratory tract. Less abundance of *Neisseriaceae* in gastrointestinal tract in smokers is explained by alterations in duodenal bicarbonate secretion and lower pH[42] in smokers as *Neisseriaceae* is a capnophile with sensitivity to acidic conditions.[43]

The association between *Veillonella*, *Phocaeicola*, and *Paludibacter* and ICS usage and *Leptotrichia* and *Megasphaera* and lung function has not been well studied, indicating that this is a relatively novel finding in this study. Interestingly, these genera are members of the oral commensal/pathogenic bacteria and among the genera that exhibited significant differences in the NBMM, *Slackia, Veillonella, Capnocytophaga, Actinomyces, Atopobium, Bulleidia, Fretibacterium, Bacteroides, Pasteurellaceae, Prevotella,* and *Leptotrichia* are members of the oral flora, and *Slackia, Eubacterium, Capnocytophaga, Aggregatibacter, Phocaeicola,* and *Megasphaera* are oral pathogens

associated with periodontitis.[44-46] We identified *Slackia* as being significantly less abundant in patients with COPD that experienced recent exacerbation and more abundant in patients who are currently smoking. *Leptotrichia* and *Megasphaera* were more abundant and *Aggregatibacter* was less abundant as FEV_1 % predicted increased. Periodontal disease has been reported to be a significant and independent risk factor for COPD[47] and our study provides evidence of associations between COPD and oral commensal/pathogenic bacteria.

There were no significant differences in any diversity measurements (Observed OTUs, ACE, Chao1, Shannon, Simpson, and Bray-Curtis) between patients with COPD and healthy controls or according to clinical characteristics. This observation is in agreement with previous COPD studies[18,48,49], and the associations between lung microbiome diversity and chronic lung disease remain a matter of debate. [50] A meta-analysis reported that the measurement of alpha-diversity does not suffice to fully understand the link between microbiota and health in patients with COPD. [50] It is also important to note that the healthy controls were all smokers. As there are differences in the microbiomes of smokers and non-smokers,[51] cautious interpretation is warranted. This result may also be explained by the distinct sampling methods used or different geographic regions compared to other studies.[52]

In this study, we chose NBMM to analyze our longitudinal repetitively collected data. The Poisson mixed-effects models can also be used for longitudinal repetitively collected data[53]; however, this model was not realistic in this study because of the restriction that the mean and variance are equal.[53,54] In practice, repetitively collected data are often over-dispersed, that is, the variance is greater than the average.[54] NBMM can effectively adjust this overdispersion[53,55] and was more appropriate for this study.

This study has some limitations. First, it was conducted at a single Korean center, and the results may not be generalizable to other samples or populations. Second, it should be noted that sputum, although widely used in the studies of the lung microbiome, has limitations in that it is often an intermediate between bronchoalveolar lavage and upper

airway swabs, and therefore contains populations from both the upper and lower airways. However, it is noteworthy that the predominant bacterial constituents of the sputum microbiome were consistent with the lung microbiome detected in bronchoalveolar lavage and bronchial samples reported in previous studies.[26,56] This suggests that our observations are representative of the bacterial composition of the lung microbiome. Third, we did not perform longitudinal repetitive sampling in healthy participants to demonstrate the reproducibility of the sputum microbiome over time in the healthy lung. Therefore, it is unclear whether the results from the annual follow-ups are specific to patients with COPD or whether they differ from those in healthy subjects. Fourth, only the compositional relative abundances of the taxa were used in this study. Changes in the absolute abundance of a single taxon can alter the relative abundances of all taxa, and testing hypotheses regarding mean relative abundance may not be equivalent to testing hypotheses regarding mean absolute abundance.[57] Finally, we did not characterize the viral and fungal communities in this study, and they may have an important role in COPD.

V. CONCLUSION

Our longitudinal study that collected repetitive data from the same patients annually over 2 years provides unique insights into the long-term adaptations of the lung microbiome in COPD. We demonstrated that the lung microbiome in COPD differed significantly according to clinical characteristics of recent exacerbation, smoking status, ICS use, and lung function. These findings will expand the current understanding of the microbiome in patients with COPD and may facilitate its use as a biomarker in the future.

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ABSTRACT (IN KOREAN)

만성폐쇄성폐질환에서 폐 미생물 군집

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문 성 우

서론: 폐 미생물 군집의 프로파일은 장기적인 데이터에서는 여전히 잘 연구되지 않았다. 본 연구에서는 반복적으로 폐 미생물 군집을 확인함으로써, COPD 폐 미생물 군집이 임상적 특성에 따라 어떤 차이가 있는지 확인하고자 한다.

방법: 2017년 2월부터 2021년 8월까지 세브란스 병원에서 10명의 건강한 남성과 43명의 만성폐쇄성폐질환 환자를 모집하였다. 43명의 만성폐쇄성폐질환 환자로부터는 2년 동안 매년 총 129개의 객담 샘플을 수집하여 16S rRNA 유전자 시퀀싱을 진행하였다. 최근 3개월내 급성 악화력, 흡입용 스테로이드 사용, 흡연 상태 및 폐기능 (FEV_1 % predicted)이 각각의 균주에 미치는 영향을 조사하기 위해서 negative binomial mixed model (NBMM)을 이용하여 분석하였다.

결과: 건강한 대조군과 만성폐쇄성폐질환 환자의 폐 미생물 군집의 비교 및 만성폐쇄성폐질환 환자의 임상적 특성에 따른 폐 미생물 군집의 비교에서 모두 알파-다양성과 베타-다양성의 차이가 없었다. 건강한 대조군과 만성폐쇄성폐질환 환자의 폐 미생물 군집 비교한 결과, *Parvimonas, Selenomonas, Peptostreptococcus, Bulleidia, PAC000661_g* 의 균주들이 건강한 대조군에서 유의하게 더 많이 관찰되었다. 반복적으로 수집한 임상적 특성에 따른 폐 균주의 차이를 확인하는 NBMM 분석에서는, 나이, FEV_1 % predicted, 그리고 흡연 여부를 보정한 모델에서 *PAC001141_g, PAC001354_g, Slackia* 균주들의 빈도가 3개월 내 급성 악화력이 있는 경우 낮았다. 나이, FEV_1 % predicted, 그리고 흡연 여부를 보정한 모델에서 PAC001141_g, AB494828_g, 그리고 *Veillonella* 균주들의 빈도는 흡입용 스테로이드 사용하는 경우 유의하게

낮았고, *PAC000661_g, Capnocytophaga, Phocaeicola, Paludibacter* 균주들의 빈도는 유의하게 높았다. 나이와 FEV_1 % predicted 을 보정한 모델에서 *Actinomyces, Atopobium, Eubacterium_g11, Neisseriaceae_G, Bulleidia, Fretibacterium, Slackia, Dialister, PAC001354_g* 균주들의 빈도는 현재의 흡연자에서 유의하게 높았다. 나이와 흡연 여부를 보정한 모델에서 FEV_1 % predicted 의 증가에 따라 *Bacteroides, Pasteurellaceae_G, Aggregatibacter* 균주들의 빈도는 유의하게 낮았고, *Prevotella, Leptotrichia, Megasphaera, AB494828_g, Butyrivibrio* 균주들의 빈도는 유의하게 높았다.

결론: 본 연구는 반복적으로 수집한 데이터를 기반으로 만성폐쇄성폐질환 환자에서 임상적 특성에 따른 폐 미생물 군집의 차이를 보임을 확인하였다. 이러한 결과는 만성폐쇄성폐질환 환자의 미생물 군집에 대한 이해를 확장시킬 것이다.

핵심되는 말 : 만성폐쇄성폐질환, 폐 미생물 군집, 악화, 흡입용 스테로이드, 흡연, 폐기능,

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