의학박사 학위논문

소변 내 세균 유래 소포체로 분석한 정상인과 만성폐쇄성폐질환의 체내 미생물체의 차이

The difference of bodily microbiota between COPD and healthy population using bacteria-derived extracellular vesicles in urine

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이 논문을 의학박사 학위 논문으로 제출함

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요약

배경: 미생물체(microbiome)는 면역체계의 발달과 염증 반응 등을 조절하며, 만성 염증성 질환의 발생에 기여하는 것으로 알려져 있다. 선행 연구들에서 특정한 세균의 소포체를 주입했을 때 염증반응의 증가 및 폐기종의 발생이 관찰되었고, 만성 폐쇄성 폐질환이 있는 환자에서 정상인에 비해 폐의 미생물체 구성이 다르다는 점이 알려져 있다. 그러나, 만성 폐쇄성 폐질환(COPD) 환자에서 체내 미생물체의 구성에 대한 정보는 잘 알려져 있지 않다.

연구방법: 본 연구는 후향적 코호트 연구로, 한국인 유전체역학조사 사업(Korean Genome Epidemiology Study, KoGES)의 일환으로 구축된 지역사회 기반 코호트 자료를 이용하였다. 소변 내에 분비되는 소포체(extracellular vesicles)의 메타유전체분석을 통해 만성 폐쇄성 폐질환이 있는 환자에서 정상인에 비해 더 많이 나타나는 균이 있는지를 비교해보았다.

결과: 2001 년부터 모집된 환자 중 14 년간 추적한 3484 명을 분석하였고, COPD 의 유병률은 3.0%로 관찰되었다. 14 년의 추적 기간 동안 COPD 는 144 명(5.0%)에서 발생하였다. COPD 군은 정상군에 비해 나이가 많고, 남성이 많았으며, 흡연자 및 과거 흡연자의 비율도 높았다. COPD 군에서 미생물의 다양성이 정상군에 비해 감소되었고, COPD 군의 미생물체 조성은 정상군과 달랐다. 성향점수 매칭된 동수의 정상군과 비교했을 때에도 COPD 군에서 39 개의 균(taxon)이 정상군보다 자주 관찰되었다. Actinobacteria 문(phylum)이 가장 빈번하게 관찰되었으며, 그 다음으로 Firmicutes, Bacteroidetes 순이었다. 23 개의 종이 더 자주 발견되었고, Cellulomnas, Rothia, Lactobacillus 종과 family Enterobactericeae 가 포함되었다. 상대존재비 분석에서 COPD 군에서 정상군에 비해 Firmicutes 문이 더 많았고, Firmicutes/Bacteroidetes 비가 낮았다.

결론: 정상인의 미생물체 구성은 COPD 가 있는 사람과 다르고, 특정문의 균들이 더 자주 관찰되었다. 신체의 미생물체 구성의 차이가 만성폐쇄성폐질환의 이환에 영향을 줄 가능성이 있다.

차 례

도표목차

Abstract

BACKGROUND: The microbiome has an important role in maintaining health and regulating the various inflammatory responses. Bacteria excrete extracellular membrane vesicles (EVs) which can promote airway inflammation and induce emphysema. Lung microbiota of healthy subjects is different from that of chronic obstructive lung disease (COPD). However, is not well understood whether the composition of bodily microbiota in COPD has distinct characteristics compared to a healthy population.

OBJECTIVE: To evaluate the difference in microbiome composition between the patients with COPD and healthy population.

DESIGN: A retrospective cohort study.

RESULTS: We included 3,484 patients with a 14-year follow up period since 2001 from pre-existing population-based cohort built in Ansan. COPD prevalence was 3.0%. During follow up, COPD occurred in 5.0% of the participants. COPD patients were older and had lower BMI. They had a higher proportion of males and smokers and worse pulmonary function compared to the normal group. In COPD patients, the diversity of the microbial community was decreased. The microbial composition of COPD patients was different from that of the healthy population. Compared with propensity score-matched cohort, 39 taxa were more frequently detected in COPD. Actinobacteria was the most abundant phylum, followed by Firmicutes and Bacteroidetes. We observed an increased portion of the Bacteroidetes phylum among identified genera. Twenty-three genera were more frequently

found in COPD including genus Cellulomonas, Rothia, Lactobacillus, and family Enterobactericeae. The relative abundance of phylum Firmicutes was increased and Firmicutes/Bacteroidetes ratio was decreased in COPD.

CONCLUSIONS: The microbial composition of COPD was different from those of healthy population. The difference in bodily microbiota composition may affect morbidity of COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death among non-communicable diseases (1). COPD is characterized by an airflow limitation that is not fully reversible and caused by an abnormal inflammatory response of the airways to noxious inhaled gases and particles. The best example of this noxious insult is smoking, however, a only fraction (9~31%) of smokers develop COPD (2). Moreover, airway inflammatory response is not fully recovered in COPD patients who no longer smoke (3). These findings suggest that there is more to it than noxious stimuli such as smoking to cause COPD. Recent evidence indicate that disrupted innate and adaptive immune response contributes to the pathogenesis of COPD, but the causative factors are remained poorly understood (4).

The human microbiome is one of the factors, which has an important role in maintaining health and regulating various inflammatory responses (5). The human microbiome can be described as the sum of all forms of microorganisms and their genomes, residing in an individual, at a given time (6). Microbiota moderates the dynamic process of development and regulation of the immune system that lies beneath the pathogenesis of COPD (7). Bacteria excrete extracellular membrane vesicles (EVs), also called outer membrane vesicles (OMVs), to communicate with other cells. EVs are nano-sized particles, 20–200 nm in diameter, excreted by Archaea, bacteria to eukaryotic cells and they contain various bioactive molecules which play an important role in intercellular communication (8).

Previous studies showed that bacteria-derived EVs can promote airway inflammation and induce emphysema in animal models (9, 10). Evidence suggests that EVs circulate systemically and secreted to urine. Jang and colleagues demonstrated in mice experiment that intraperitoneally injected EVs were rapidly distributed throughout the body with accumulation in the liver, lung, spleen, and kidney (11). We hypothesized that urinary derived EVs would reflect bodily microbiome of a host since EVs can be detected in various bodily fluids and is very stable (12).

We conducted a retrospective cohort study to investigate the microbiome that is distinctively found in COPD which may have a role in the pathogenesis of the disease. We identified microbiota from extracted the bacteria-derived EVs in urine samples of COPD and healthy subjects and compared the frequency of each microbiota between COPD and healthy subjects.

Methods

We conducted a retrospective cohort study to evaluate the difference in urine microbiome composition between the patients with COPD and healthy population.

Study population and design

The study cohort is included in an ongoing nationwide cohort study, The Korean Health and Genome Study (KoGES), which started in 2001 (13). The aim of the KoGES is to discover the genetic and environmental etiology of common chronic diseases in Koreans and to reduce the burden of chronic diseases. As part of this project, a population-based cohort was built in Ansan, an industrialized city with a population of 710,000. The baseline study was conducted from June 25, 2001, to January 29, 2003. Participants of the study cohort consisted of 5,015 male and female Korean aged 40 to 69 years, and they had a comprehensive health examination including routine anthropometric measurements, blood and urine samples, and pre- and post-bronchodilator spirometry and on-site interviews at Korea University Ansan Hospital at enrollment (14). The participants have been followed up biennially with structured questionnaires and tests including spirometry.

We retrieved data from 3,879 participants with a 14-year follow-up period, including baseline age, sex, body-mass index (BMI), smoking history, comorbidities, dietary patterns and pulmonary function tests at baseline and each follow-up visits. We used a baseline urine sample to identify urinary EVs excreted by the various microbiome. We excluded 11 subjects

with missing anthropometric data, 269 subjects with confirmed asthma or wheezing, and 13 patients with urinary tract infection. A total of 3,484 participants was eligible for analysis. During a 14-year follow-up period, a part of the healthy population developed COPD. We excluded those who developed COPD during the follow-up period from healthy control group.

We compared baseline characteristics, microbiome composition and difference of COPD patients with the healthy population.

Spirometry and diagnosis of COPD

Spirometry was conducted by a specially trained pulmonary technician abide by the 1994 ATS recommendations, using a spirometer (Vmax-229, Sensor-Medics, Yorba Linda, CA, USA) for all subjects (15). The predicted forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) were obtained using the method of Morris; the patients try forced expiratory maneuvers until three measurements met the ATS guideline specifications (16). Two doses of fenoterol hydrobromide (Berotec®, Boehringer Ingelheim, Ingelheim, Germany) 200 μg were administered 1–2 min apart. The forced expirations were repeated 15 min after the administration of the bronchodilator.

COPD was defined as a post-bronchodilator $FEV_1/FVC < 0.7$ according to the GOLD criteria (17). COPD prevalent cases were defined as subjects who had COPD at enrollment. COPD incident cases were defined as participants who developed COPD during follow-up visits which were excluded from the healthy population in statistical analysis.

Dietary Assessment

Dietary information was obtained using a semi-quantitative food frequency questionnaire (FFQ), which was developed and evaluated for validity by the Korea Centers for Disease Control and Prevention (Seoul, Korea) (18). For analysis deriving dietary patterns, the 103 food items from the FFQ were classified into 27 food groups similarly used in a previous study (19), which investigated dietary patterns among Koreans. The average frequency of consumption for a specific food group was calculated by adding up frequencies for all of the food items that belong to the food group. Food groups were used for factor analysis to generate major dietary patterns and factor loadings. Based on the factor loading scores (greater than 0.6), Factor 1 was characterized by high intake of noodles and flour products; factors 2 by high intake of milk and dairy products; factor 3 by high intake of red meats and chickens; factor 4 by high intake of grains; and factor 5 by high intake of vegetables.

Preparation of EVs and DNA extraction from urine samples

The differential centrifugal method was used for the isolation of bacteria-derived EVs from the urine samples as previously described (20). In brief, urine samples were centrifuged at 10,000 × g for 10 min at 4 ℃. The supernatant was filtered through a 0.22-μm membrane. Isolated EVs were dissolved in 100 μl PBS based on the protein amount. The DNA extraction process was described previously (21, 22). Briefly, isolated EVs were boiled at 100℃ for 15 min, centrifuged at 10,000 g for 20 min, and the supernatants were collected. For collected samples, a DNA extraction kit (PowerSoil DNA Isolation Kit, MO BIO,

Carlsbad, CA, USA) was used to extract bacterial DNA. Isolated DNA was quantified by using the QIAxpert system (QIAGEN, Hilden, Germany).

16S rRNA gene-based metagenomic sequencing and assignment of taxonomic unit

The method for bacterial DNA preparation is described previously (22). DNA was used for PCR amplification of the V3-V4 hypervariable regions in the 16S ribosomal RNA genes using the primer set of 16S_V3_F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACA GCCTACGGGNGGCWGCAG-3′) and 16S_V4_R (5′-GTCTCGTGGGCTCGGAGATGTG TATAAGAGACAGGACTACHVGGGTATCTAATCC-3′). 16S rDNA gene libraries were constructed using the PCR products that were used for the construction of following the MiSeq System guidelines (Illumina Inc., San Diego, CA, USA). The 16S rRNA gene libraries for each sample were measured using QIAxpert (QIAGEN, Hilden, Germany) and the equimolar amount was used for pyrosequencing with the MiSeq System (Illumina Inc., San Diego, CA, USA) following manufacturer's recommendations.

Raw pyrosequencing reads were filtered on the basis of the primer sequences using MiSeq Control Software version 1.1.1 (Illumina Inc., San Diego, CA, USA). The taxonomical assignment of the sequence reads was done using the MDx-Pro ver.1 profiling program (MD Healthcare Inc., Seoul, Korea). Operational taxonomic units (OTUs) were identified using UCLUST (23) and assigned using QIIME (24) against the 16S rRNA sequence database, the GreenGenes 8.15.13 (25). Taxonomic assignments were achieved based on the sequence similarities at the following levels: genus, >94% similarity; family, >90% similarity; order,

>85% similarity; class, >80% similarity; and phylum, >75% similarity. In cases where clustering was not possible at the genus level due to a lack of sequence information at the database or redundant sequences, the taxon was named based on the higher-level taxonomy with brackets.

Statistical analyses

Statistical analyses for baseline characteristics were performed by SPSS statistics version 21. Analysis of categorical variables was done using the chi-square test and Fisher's exact test and continuous variables were analyzed by independent t-test and Mann-Whitney U test. A p-value of < 0.05 was considered statistically significant.

Major dietary patterns were generated from food groups with the Varimax rotation method. After evaluation of eigenvalues (greater than 1.0) and the Scree test, five factors, which were labeled based on the nature of food groups loading highly on a factor, were determined and factor scores for each factor were calculated for each individual.

The alpha diversity of samples was estimated using the Shannon index and Simpson index (26). The beta-diversity of the samples was analyzed using distance matrices generated using the Jaccard index for community membership (27). Principal component analysis (PCA) was used to visualize the similarities between groups. Multiple logistic regression analysis was used to analyze the difference in microbiome between COPD patients and healthy population. Microbiome with detection rate under 2 % were excluded for analysis.

Results

A total of 3,484 subjects were eligible for analysis and 105 (3.0 %) participants had COPD at the time of enrollment. Among whose spirometry at enrollment was normal, 144 (4.2 %) persons developed COPD after a 14-year follow-up period.

Baseline characteristics of COPD and healthy subjects

We compared baseline characteristics of subjects who had COPD at enrollment and subjects who stayed normal throughout the follow-up period, excluding COPD incident cases (Table 1). The patients with COPD was older and male predominant than the normal subjects. They had more current and ex-smokers than the normal population. Dietary patterns and BMI were not significantly different between the two groups. FVC was higher in COPD patients than the normal population, but FEV_1, FEV_1/FVC was lower in COPD patients than the normal population.

COPD occurred in 144 patients during follow-up. The overall incidence of COPD among the study population was 5.0 %. We compared baseline characteristics of COPD incident cases and healthy subjects (Table 2). The patients who developed COPD were older and 77.1 % of them were male. They had lower BMI and had more current and ex-smokers than healthy subjects. Dietary patterns were not significantly different between the two groups. Spirometry results at the time of enrollment were compared between COPD incident cases

and healthy subjects. FVC, percent achieved of the predicted value of FEV_1, and FEV_1/FVC were lower in COPD incident cases than healthy subjects, even they did not have COPD at enrollment.

We selected 105 healthy subjects using propensity score matching. Table 3 shows a comparison of the baseline characteristics of the COPD and propensity score-matched healthy subjects.

Biodiversity and cluster analysis of samples

The alpha diversity measured using Shannon index and Simpson index did not show statistical difference among healthy subjects, COPD prevalent and incident cases (Figure 1, A and C). However, alpha diversity indices were significantly lower in propensity score match healthy subjects than COPD prevalent cases, indicating less diverse bacterial community in COPD prevalent cases (Figure 1, B and D).

The Jaccard index of healthy subjects, COPD prevalent and incident cases were not significantly different (Figure 2, A). COPD prevalent cases showed significantly lower Jaccard index than propensity score-matched population, meaning more dissimilar composition of bacterial community from that of the normal population.

The result of PCA is shown in Figure 3. PCA did not reveal distinct clusters of bacterial communities in COPD patients.

Comparison of microbiome between COPD and healthy subjects

We found 129 taxa that are more frequently detected in COPD prevalent cases than healthy

population which is shown in Table 4. A total of 73 Genera were more frequently found in COPD than in healthy subjects. When classified into the phylum they belong, Proteobacteria was the most abundant phylum followed by Actinobacteria, Firmicutes, and Bacteroidetes. They showed an extreme odds ratio and the taxa identified were microbiomes that are commonly found in environment.

We found 35 taxa that are more frequently detected in COPD patients than in propensity score-matched healthy population. There were 23 genera that more frequently found in COPD patients than healthy subjects, including genus Cellulomonas, Rothia, Lactobacillus, and family Enterobactericeae.

Figure 4 shows more frequently detected taxa in COPD patients compared to healthy subjects which were classified by the phylum they belong to. There were 5 major identified phyla; Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, and Acidobacteria. Compared with the propensity score-matched cohort, the phylum Actinobacteria was the most abundant phylum in COPD followed by Firmicutes and Bacteroidetes.

The result of relative abundance analysis of phylum Firmicutes and Bacteroidetes are shown in Table 6. We found that the relative abundance of phylum Firmicutes was increased and Firmicutes/Bacteroidetes ratio was decreased in COPD patients compared to propensity score-matched healthy cohort.

Discussion

To our knowledge, this is the first study that investigated the difference in microbiome between COPD and healthy subjects using bacteria-derived EV in the urine. We evaluated the difference of urine microbiome between COPD patients and healthy subjects. We found several taxa more commonly detected in COPD than in healthy subjects.

We used urine samples of the subjects based on the hypothesis that bacteria-derived urinary EVs reflects the total burden of the host microbiome, which are mainly abundant in the gastrointestinal tract (GIT). Previous studies showed that EVs in indoor dust induce neutrophilic pulmonary inflammation and COPD patients were 8 times more likely to sensitized by anti-EV IgG (28, 29). In addition, bacteria-derived EVs can induce lung inflammation and even emphysema in lung tissue, in a dose-dependent manner (30, 31). This pro-inflammatory effect of EVs is maintained when delivered into the abdomen. Jang et al. demonstrated in a mouse model that intraperitoneal injection of bacterial EVs can induce inflammation in the lungs (11). They also showed a dynamic distribution of EVs in mice, particularly, a significant portion of injected EVs was found in the kidneys. This suggests that microbiota at a certain location could affect or be affected by microorganisms or their immune response located at distant sites. Interactions of microbiota and host that mediated

by EVs may have the potential to shed a light on the insight of immune response and pathogenesis of chronic airway disease (32). For instance, Samra and colleagues investigated urinary EVs of children with allergic airway disease and found that they had a distinct composition (33).

Previous research on lung microbiome assessed the relationship between smoking and the disease state. Sze et al. showed that there are substantial differences in microbiota between patients with COPD compared with healthy smokers and never smokers by identifying bacterial DNA from lung tissue (34). In their analysis, the phylum Firmicutes was significantly associated with COPD lungs and Lactobacillus was the main genus associated with the increase in the Firmicutes. Kim et al. showed similar results using EVs extracted from lung tissue (35). They found that the diversity of the microbial community was decreased in COPD and healthy smokers and the prevalence of the phylum Firmicutes was higher in the COPD lung EVs. These results are consistent with our findings. The diversity of the bacterial community was significantly decreased in COPD when compared to that of propensity score-matched healthy cohort. We also observed one-third of frequently found genera in COPD belongs to phylum Firmicutes and genus lactobacillus was 2.437 times more frequently found in COPD patients than in propensity matched healthy subjects.

Meanwhile, the evidence support that there is a substantial difference in the microbiome of gut microbiota between healthy smokers and nonsmokers. A longitudinal study conducted by Beiderman et al. showed the composition and diversity of gut microbiome changed after

smoking cessation (36). Lee et al. observed an increased proportion of phylum Bacteroidetes with decreased Firmicutes and Proteobacteria in smokers than in never and former smokers using stool samples (37). Savin and colleagues found that the phyla of Proteobacteria and Bacteroidetes were increased in smokers, as well as the genera Clostridium, Bacteroides and Prevotella (38). However, phyla Actinobacteria and Firmicutes, as well as the genera of Bifidobacteria and Lactococcus, were decreased. It is known that chronic lung disease and chronic GIT diseases often occur together. One-third of the patients with irritable bowel syndrome and half of the patients with inflammatory bowel syndrome have pulmonary inflammation or impaired lung function (39, 40). However, there is no research available so far that assessed the changes in the gut microbiome in COPD patients compared with healthy subjects (41). We analyzed the difference in the microbiome composition of COPD and healthy subjects from bacteria-derived urinary EVs and found similar results. The Proteobacteria phylum was the most commonly found phylum in COPD patients which is consistent with previous findings (38). We observed increased relative abundance of the Firmicutes and decreased Firmicutes/Bacteroidetes ratio in COPD, which is consistent with the results of the previously mentioned study (37). Using bacteria-derived urinary EVs, our results are consistent with prior studies that used lung or fecal samples.

There are several merits in this study. This is the first analysis performed using bacteria-derived urinary EVs comparing COPD patients with a healthy population from a large community-based cohort with a follow-up period of 14 years. We used urine samples that are

easy to obtain and not invasive thus easily replicable. We analyzed bacteria-derived EVs, not microbiome itself because EVs may have a more crucial role in the relation between microbiota and chronic airway disease than microbiome itself since EVs have an important role in the elimination of competing bacteria and modulation of host immune responses. Also, we have our data adjusted for dietary patterns which could be a major confounding factor. We used a standardized questionnaire for dietary information and used for factor analysis. It is well known that consumption of particular types of food changes in host bacterial genera (42).

However, our study also has some limitations. Urine samples can be changed by some conditions such as urinary tract infection so we excluded patients with urinary tract infection. While we assumed bacteria-derived urinary EVs would reflect on the host microbiome, little is known about whether urinary EVs could represent the whole microbiome in a host. Moreover, is possible that the normal flora of urinary tract in COPD patients may differ from those of healthy subjects. We have only observational data and the metagenomics sequencing of urine samples was only carried out in the first visit. We assumed the microbiome would be stable throughout the follow-up period, as the previous study showed microbiome composition in a host remains stable even for decades (43). A longitudinal follow up of urine samples would have given more information on the effect of urine microbiome in COPD patients. Without well-structured longitudinal or interventional studies, the causal relationship between the host microbiome and lung disease cannot be determined.

Conclusion

The microbial composition of COPD assessed with bacteria-derived urinary EVs was different from those of the healthy population. The difference in bodily microbiota composition may affect morbidity of COPD. However, to elucidate the role of microbiota in many sites of the host on respiratory diseases, further longitudinal studies and improved interventional experiments will be required.

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Table 1. Baseline characteristics of the patients with or without COPD.

	Total	COPD	Normal	
No. (%)	3340 (100)	105 (3.1)	3235 (96.9)	P-value†
Age, years	48.4 ± 7.5	55.2 ± 8.4	48.2 ± 7.4	<0.0001
Male sex	1567 (46.9)	90 (85.7)	1477 (45.7)	<0.001
BMI, kg/m^2	24.74 ± 2.97	24.35 ± 2.89	24.75 ± 2.97	0.1753
Smoking				<0.001
Never	2069 (62)	29 (27.6)	2040 (63.1)	
Ex-smoker.	632 (18.9)	31 (29.5)	601 (18.6)	
Current smoker	539 (19.1)	45(42.9)	594 (18.3)	
Amount of smoking	7.3 ± 13.1	20.6 ± 20.7	6.9 ± 12.6	<0.0001
Dietary pattern				
Carbohydrate (flour)	-0.01 ± 0.58	-0.06 ± 0.52	-0.01 ± 0.58	0.408
Dairy	0.21 ± 0.88	0.2 ± 1.00	0.21 ± 0.87	0.8806
Protein	0.06 ± 0.84	0.05 ± 0.71	0.06 ± 0.84	0.8854
Carbohydrate (rice)	0.13 ± 0.95	0.08 ± 0.95	0.14 ± 0.95	0.5423
Vegetables	-0.18 ± 0.76	-0.13 ± 0.74	-0.18 ± 0.76	0.4931
Baseline PFT				
FVC (L)	3.75 ± 0.82	4.22 ± 0.86	3.74 ± 0.82	<0.0001
FEV1 (L)	3.09 ± 0.66	2.76 ± 0.62	3.1 ± 0.65	<0.0001

FEV1 (%pred)	113.22 ± 14.73	95.15 ± 14.65	113.81 ± 14.35	<0.0001
FEV1/FVC	82.59 ± 5.96	65.1 ± 4.08	83.16 ± 5.09	<0.0001

†Statistical analysis was done using the independent t-test for age and BMI, the chi square test and Fisher's exact test for other variables.

*Abbreviations: BMI = body-mass index; COPD = chronic obstructive pulmonary disease; FEV1 = forced expiratory volume in 1 second; FVC = forced vital capacity; No. = number; %pred = percent achieved of predicted value.

Table 2. Baseline characteristics of the COPD incident cases and the healthy subjects.

	Total	COPD	Normal	
No. (%)	2857 (100)	144 (5.0)	2713 (95.0)	P-value†
Age, years	48.1 ± 7.2	51.9 ± 7.3	47.9 ± 7.2	<0.0001
Male sex	1380 (48.3)	111 (77.1)	1269 (46.8)	<0.001
BMI, kg/m^2	24.72 ± 2.89	23.98 ± 2.37	24.76 ± 2.91	0.0002
Smoking				<0.001
Never	1753 (61.4)	46 (31.9)	1707 (62.9)	
Ex-smoker.	561 (19.6)	36 (25.0)	525 (19.4)	
Current smoker	543 (19.0)	62 (43.1)	481 (17.7)	
Amount of smoking	7.2 ± 12.8	14.6 ± 16.1	6.8 ± 12.5	<0.0001
Dietary pattern				
Carbohydrate (flour)	-0.01 ± 0.58	-0.03 ± 0.49	-0.01 ± 0.58	0.5951
Dairy	0.23 ± 0.88	0.14 ± 0.87	0.23 ± 0.88	0.2433
Protein	0.06 ± 0.85	-0.03 ± 0.68	0.06 ± 0.86	0.1243
Carbohydrate (rice)	0.13 ± 0.95	0.11 ± 0.98	0.14 ± 0.95	0.7877
Vegetables	-0.18 ± 0.76	-0.24 ± 0.87	-0.18 ± 0.76	0.3981
Baseline PFT				
FVC (L)	3.78 ± 0.81	4.19 ± 0.84	3.76 ± 0.8	<0.0001
FEV1 (L)	3.12 ± 0.64	3.13 ± 0.62	3.12 ± 0.64	0.8013
FEV1 (%pred)	113.24 ± 14.07	106.56 ± 13.11	113.59 ± 14.03	<0.0001
FEV1/FVC	82.8 ± 5.28	75.05 ± 4.15	83.21 ± 5.01	<0.0001

†Statistical analysis was done using the independent t-test for age and BMI, the chi-square

test and Fisher's exact test for other variables.

*Abbreviations: BMI = body-mass index; COPD = chronic obstructive pulmonary disease; FEV1 = forced expiratory volume in 1 second; FVC = forced vital capacity; No. = number; %pred = percent achieved of predicted value.

Table 3. Baseline characteristics of the patients with or without COPD using propensity score matching.

	Total	COPD	Normal	
No. (%)	210 (100)	105 (50.0)	105 (50.0)	SMD
Age, years	55.7 ± 8.3	55.2 ± 8.4	56.2 ± 8.2	-0.116
Male sex	393 (81.0)	90 (85.7)	84 (80.0)	0.1521
BMI, kg/m^2	24.45 ± 2.98	24.35 ± 2.89	24.54 ± 3.08	0.1521
Smoking				0.1748
Never	146 (30.0)	29 (27.6)	36 (34.3)	
Ex-smoker.	137 (28.2)	31 (29.5)	24 (22.9)	
Current smoker	203 (41.8)	45 (42.9)	45 (42.9)	
Amount of smoking	18.4 ± 19.0	20.6 ± 20.7	16.1 ± 16.8	
Dietary intake				
Carbohydrate (flour)	-0.07 ± 0.56	-0.06 ± 0.52	-0.07 ± 0.59	0.0215
Dairy	0.2 ± 1.02	0.2 ± 1.00	0.2 ± 1.03	-0.0069
Protein	0.13 ± 1.42	0.05 ± 0.71	0.21 ± 1.88	-0.1137
Carbohydrate (rice)	0.06 ± 0.97	0.08 ± 0.95	0.05 ± 0.99	0.0333
Vegetables	-0.16 ± 0.78	-0.13 ± 0.74	-0.19 ± 0.81t	0.0855
Baseline PFT				
FVC (L)	4.11 ± 0.79	4.22 ± 0.86	3.99 ± 0.71	
FEV1 (L)	2.99 ± 0.64	2.76 ± 0.62	3.23 ± 0.57	
FEV1 (%pred)	105.6 ± 18.19	95.15 ± 14.65	116.05 ± 15.17	
FEV1/FVC	72.95 ± 9.08	65.1 ± 4.08	80.81 ± 4.94	

*Abbreviations: BMI = body-mass index; COPD = chronic obstructive pulmonary disease; FEV1 = forced expiratory volume in 1 second; FVC = forced vital capacity; No. = number; %pred = percent achieved of predicted value; SMD = standardized mean difference.

Table 4. Taxon more frequently found in COPD patients than in the healthy population.

Taxon[†]	Odds ratio	95% confidence interval	P-value[‡]
Mesonia	57.481	7.164~461.199	0.0001
Phaeobacter	44.362	5.733~343.267	0.0003
Salinibacter	37.082	5.184~265.242	0.0003
Succinivibrio	25.844	1.779~375.489	0.0172
Lacibacter	22.223	2.965~166.559	0.0025
Gracilibacillus	21.661	4.463~105.135	0.0001
Caldanaerocella	21.239	3.464~130.205	0.001
Streptosporangium	20.28	3.821~107.632	0.0004
Acidaminococcus	20.144	3.539~114.658	0.0007
Saccharospirillum	19.446	2.797~135.203	0.0027
Winogradskyella	18.191	2.034~162.724	0.0095
Isobaculum	17.62	2.927~106.074	0.0017
Crenothrix	16.892	2.551~111.87	0.0034
Micromonospora	16.421	1.501~179.631	0.0219
Kibdelosporangium	15.933	1.695~149.781	0.0155
Methylovorus	15.717	2.484~99.463	0.0034
Sulfurospirillum	15.172	1.177~195.599	0.0371
Corallococcus	15.147	1.574~145.775	0.0186
Parapedobacter	14.561	1.24~170.932	0.0331
Ignatzschineria	13.019	1.392~121.773	0.0245
Actinopolymorpha	13.016	2.458~68.912	0.0025

Acetobacterium	12.798	2.25~72.789	0.004
Thermovum	12.254	1.308~114.83	0.0282
OR-59	11.933	1.226~116.16	0.0327
Fusibacter	11.47	2.274~57.856	0.0031
Aliivibrio	11.336	1.111~115.699	0.0405
Nodosilinea	11.175	1.895~65.908	0.0077
Lampropedia	10.861	2.128~55.435	0.0041
Rikenella	10.704	2.434~47.072	0.0017
Acaryochloris	10.391	1.936~55.777	0.0063
Serpens	10.298	1.191~89.013	0.0341
Azotobacter	10.065	1.065~95.131	0.0439
Helicobacter	9.991	1.993~50.099	0.0051
BSV43	9.929	1.058~93.208	0.0445
Fulvimarina	9.804	1.731~55.521	0.0099

Table 4. (continued).

Taxon[†]	Odds ratio	95% confidence interval	P-value[‡]
Kytococcus	9.267	2.392~35.902	0.0013
Listeria	9.241	1.035~82.49	0.0465
Marinimicrobium	9.181	1.561~54	0.0142
Desulfomonile	8.69	1.151~65.621	0.0361
RS62	8.178	1.624~41.183	0.0108
Leadbetterella	8.079	1.607~40.626	0.0112
Promicromonospora	7.918	2.287~27.413	0.0011
Gelidibacter	7.597	1.022~56.452	0.0475
Oligella	7.063	1.193~41.819	0.0312
Gallibacterium	7.008	1.174~41.84	0.0327
Methylophaga	6.926	1.343~35.721	0.0208
Pandoraea	6.598	1.638~26.574	0.0079
Herbaspirillum	6.511	1.259~33.673	0.0254
Edaphobacter	5.599	1.114~28.137	0.0365
Nostoc	5.221	1.365~19.973	0.0158
Psychrilyobacter	5.076	1.001~25.738	0.0498
Streptacidiphilus	4.585	1.189~17.679	0.027
Myxococcus	4.378	1.383~13.866	0.012
Geobacter	4.048	1.273~12.876	0.0179
Thermoanaerobacterium	3.866	1.232~12.132	0.0205
Nitrospira	3.812	1.676~8.672	0.0014

Abiotrophia	3.7	1.9~7.207	0.0001
Gillisia	3.618	1.307~10.017	0.0133
Oceanobacillus	3.549	1.382~9.114	0.0085
Pelosinus	3.509	1.084~11.363	0.0363
Schwartzia	3.454	1.24~9.624	0.0177
Alicyclobacillus	3.237	1.507~6.952	0.0026
Kineococcus	2.291	1.032~5.086	0.0416
Agrococcus	2.076	1.016~4.24	0.0451
Planomicrobium	1.933	1.085~3.443	0.0252
Amaricoccus	1.865	1.015~3.425	0.0446
Mycobacterium	1.768	1.182~2.645	0.0056
Actinobaculum	1.725	1.151~2.585	0.0082
Enterococcus	1.638	1.087~2.469	0.0184
Citrobacter	1.627	1.077~2.456	0.0207

Table 4. (continued).

Taxon[†]	Odds ratio	95% confidence interval	P-value[‡]
Rubellimicrobium	1.626	1.049~2.522	0.0297
Anoxybacillus	1.614	1.021~2.552	0.0404
Deinococcus	1.521	1.002~2.307	0.0487
Ruaniaceae (f)	44.172	5.946~328.173	0.0002
Dehalobacteriaceae (f)	26.323	4.276~162.026	0.0004
Halobacteriaceae (f)	20.452	1.585~263.881	0.0207
AKIW659 (f)	13.417	1.382~130.279	0.0252
Chlamydomonadaceae (f)	9.814	2.476~38.895	0.0012
0319-6A21 (f)	9.23	2.791~30.524	0.0003
Ruaniaceae (f)	7.622	1.192~48.748	0.0319
Oceanospirillaceae (f)	7.545	1.973~28.848	0.0031
Listeriaceae (f)	7.116	1.315~38.524	0.0228
Rivulariaceae (f)	6.405	1.125~36.458	0.0363
Jonesiaceae (f)	6.323	1.179~33.918	0.0314
Gordoniaceae (f)	5.995	1.546~23.248	0.0096
Helicobacteraceae (f)	5.715	1.528~21.38	0.0096
PAUC26f (f)	4.86	1.306~18.092	0.0184
Nocardiopsaceae (f)	4.567	1.401~14.889	0.0118
Iamiaceae (f)	4.395	1.388~13.911	0.0118
Rhodospirillaceae (f)	4.351	1.178~16.064	0.0274
Koribacteraceae (f)	3.709	1.151~11.949	0.0281

Syntrophobacteraceae (f)	3.151	1.571~6.323	0.0012
Gemmataceae (f)	3.133	1.236~7.941	0.0161
Acidobacteriaceae (f)	2.421	1.083~5.41	0.0311
Hyphomicrobiaceae (f)	1.923	1.001~3.693	0.0495
Staphylococcaceae (f)	1.849	1.162~2.941	0.0095
Dietziaceae (f)	1.763	1.055~2.947	0.0305
Nocardioidaceae (f)	1.661	1.104~2.5	0.0149
Coriobacteriaceae (f)	1.633	1.091~2.445	0.0172
Aeromonadaceae (f)	1.541	1.031~2.301	0.0348
Dermabacteraceae (f)	0.405	0.18~0.912	0.0291
S1198 (o)	28.982	4.594~182.849	0.0003
Cytophagales (o)	22.265	2.469~200.776	0.0057
Methylococcales (o)	21.704	3.036~155.138	0.0022
PL-11B10 (o)	18.593	1.188~290.972	0.0373

Table 4. (continued).

Taxon[†]	Odds ratio	95% confidence interval	P-value[‡]
MBNT15 (o)	13.403	2.604~68.992	0.0019
Chlamydiales (o)	11.641	2.194~61.779	0.0039
Myxococcales (o)	11.625	3.179~42.508	0.0002
MBA08 (o)	10.625	1.799~62.742	0.0091
Campylobacterales (o)	10.159	1.086~95.063	0.0421
Chroococcales (o)	9.904	2.048~47.892	0.0044
Thiohalorhabdales (o)	8.085	1.616~40.462	0.011
NB1-j (o)	7.05	1.282~38.771	0.0247
HOC36 (o)	6.622	1.268~34.577	0.025
CCM11a (o)	5.412	1.09~26.867	0.0389
Ellin6067 (o)	2.545	1.309~4.948	0.0059
Chlorophyta (o)	1.995	1.06~3.755	0.0322
MB-A2-108 (c)	26.808	3.795~189.368	0.001
Endomicrobia (c)	23.733	4.213~133.703	0.0003
028H05-P-BN-P5 (c)	22.275	2.257~219.875	0.0079
ABS-6 (c)	19.028	4.176~86.708	0.0001
3BR-5F (c)	8.627	1.688~44.096	0.0096
Deltaproteobacteria (c)	8.285	1.006~68.245	0.0494
ABY1 (c)	5.546	1.491~20.628	0.0106
TM7-1 (c)	2.21	1.348~3.623	0.0017
OctSpA1-106 (p)	18.395	1.755~192.76	0.0151

Gemmatimonadete (p)	15.633	1.643~148.767	0.0168
Chlorobi (p)	4.837	1.56~14.998	0.0063
FBP (p)	3.135	1.134~8.663	0.0276

†The taxa are shown at the genus level; those lacked genus name was annotated by "f" (=family), "o" (=order), "c" (=class), or "p" (=phylum).

‡Correlated two-part model for semicontiguous data was used for analysis.

*Abbreviations: COPD = chronic obstructive pulmonary disease.

Table 5. Taxon more frequently found in COPD patients than in the healthy population using propensity score matching.

Taxon†	Odds ratio	95% confidence interval	P-value‡
Mycetocola	10	1.28~78.117	0.0281
Dyadobacter	7	1.591~30.8	0.01
Pedobacter	6	1.343~26.808	0.019
Cellulomonas	4	1.129~14.175	0.0317
Cloacibacterium	4	1.129~14.175	0.0317
Rathayibacter	3.667	1.023~13.143	0.0461
Terracoccus	3.571	1.545~8.257	0.0029
Anaerococcus	3.4	1.254~9.216	0.0162
Oribacterium	2.909	1.466~5.771	0.0022
Curtobacterium	2.818	1.417~5.607	0.0032
Dermatophilus	2.467	1.354~4.494	0.0032
Actinoplanes	2.455	1.218~4.948	0.0121
Lactobacillus	2.437	1.362~4.362	0.0027
Chitinophaga	2.429	1.007~5.856	0.0482
[Ruminococcus]	2.3	1.095~4.832	0.0279
Gallicola	2.187	1.211~3.952	0.0095
Clavibacter	2	1.028~3.892	0.0413
Rothia	1.9	1.106~3.265	0.0202

Sphingobacterium	1.895	1.087~3.303	0.0242
Epulopiscium	1.889	1.067~3.344	0.0291
Sanguibacter	1.824	1.009~3.295	0.0465
Alkalibacterium	1.714	1.001~2.936	0.0497
Brevibacterium	1.667	1.005~2.765	0.0479
Paenibacillaceae (f)	5.5	1.219~24.813	0.0266
Micromonosporaceae (f)	3.444	1.64~7.235	0.0011
Sphingobacteriaceae (f)	3.25	1.06~9.967	0.0393
Planococcaceae (f)	3.25	1.06~9.967	0.0393
[Weeksellaceae] (f)	3	1.411~6.379	0.0043
Frankiaceae (f)	2.562	1.438~4.566	0.0014
Staphylococcaceae (f)	2.231	1.16~4.291	0.0162
Geodermatophilaceae (f)	2.091	1.019~4.289	0.0442
Actinomycetales (o)	3.125	1.41~6.928	0.005

Table 5. (continued).

Taxon[†]	Odds ratio	95% confidence interval	P-value[‡]
Sphingobacteriales (o)	3	1.348~6.678	0.0071
Gemm-5 (c)	2.182	1.069~4.454	0.0321
Gitt-GS-136 (c)	1.714	1.001~2.936	0.0497

[†]The taxa are shown at the genus level; those lacked genus name was annotated by “f” (=family), “o” (=order), "c" (=class), or "p" (=phylum).

[‡] Correlated two-part model for semicontiguous data was used for analysis.

*Abbreviations: COPD = chronic obstructive pulmonary disease.

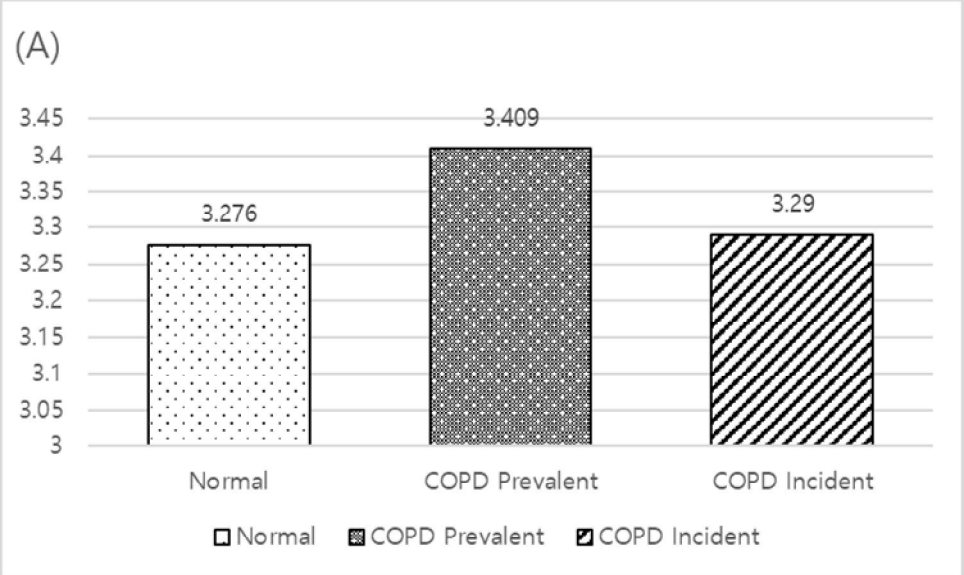
Table 6. The relative abundance analysis of phylum Bacteroidetes and Firmicutes in COPD patients.

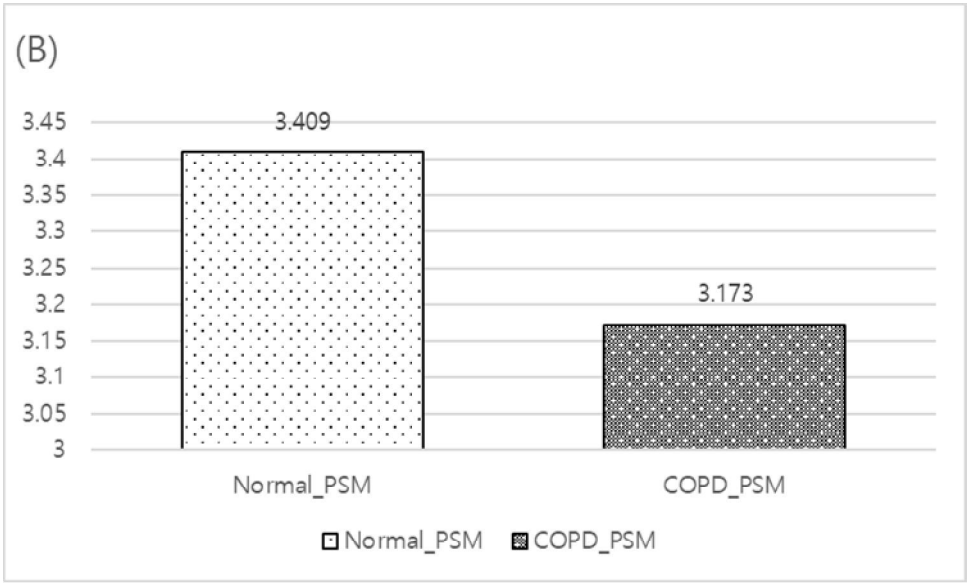
		Normal		COPD		
		Mean	SE	Mean	SE	P-value
Total cohort	p__Bacteroidetes	3.9215	0.0655	4.1671	0.3333	0.4712
	p__Firmicutes	19.8052	0.2229	19.2451	1.0565	0.605
	F/B ratio	117.1826	13.6198	72.7103	31.2863	0.1946
	F/B, median/IQR	5.3469	7.6631	4.5965	6.3646	0.1471
Propensity mated cohort	p__Bacteroidetes	4.0021	0.6068	4.1671	0.3333	0.8118
	p__Firmicutes	17.7117	1.0498	19.2451	1.0565	0.3044
	F/B ratio	272.7074	107.5604	72.7103	31.2863	0.0768
	F/B, median/IQR	6.6068	10.9201	4.5965	6.3646	0.0355

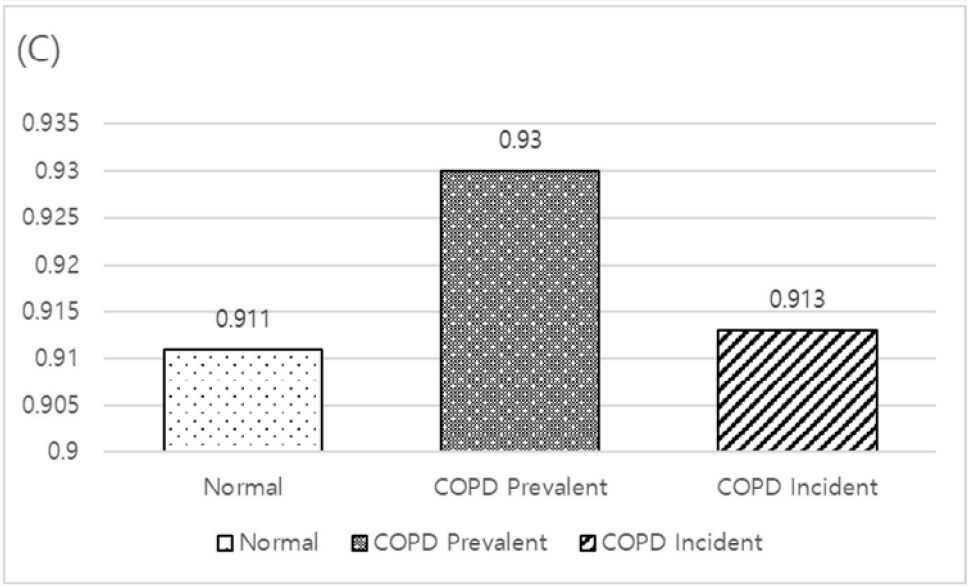
*Abbreviations: COPD = chronic obstructive pulmonary disease; F/B = Firmicutes/Bacteroidetes; IQR = interquartile range; p__ = phylum; SE = standard error.

Figure 1. Shannon and Simpson indices. (A) Shannon index of normal, COPD prevalent, COPD incident cases. (B) Shannon index of propensity score-matched normal cohort with COPD prevalent cases. There was significant difference in Shannon index of normal cohort with COPD prevalent cases (P-value = 0.0013). (C) Simpson index of normal, COPD prevalent, COPD incident cases. (D) Simpson index of propensity score-matched normal cohort with COPD prevalent cases. There was significant difference in Simpson index of normal cohort with COPD prevalent cases (P-value = 0.0046).

*Abbreviations: COPD = chronic obstructive pulmonary disease; PSM = propensity score-matched cohort.




(B)
3.45
3.4
3.35
3.3
3.25
3.2
3.15
3.1
3.05
3
3.409
3.173
Normal_PSM
COPD_PSM
Normal_PSM
COPD_PSM


(C)
0.935
0.93
0.925
0.92
0.915
0.91
0.905
0.9
0.911
0.93
0.913
Normal
COPD Prevalent
COPD Incident
Normal
COPD Prevalent
COPD Incident

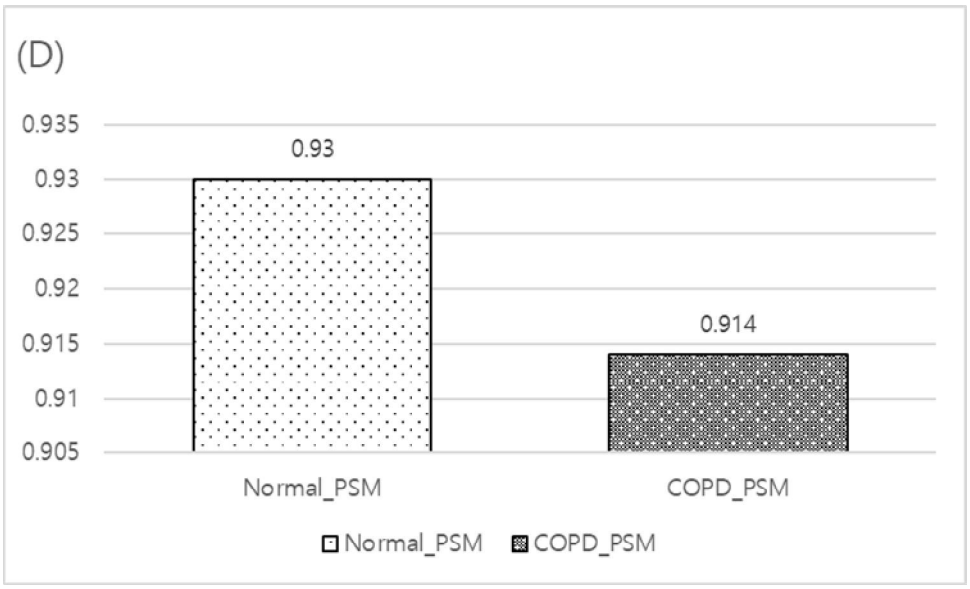


Figure 2. Beta diversity assessed with Jaccard index. (A) Jaccard index of normal, COPD prevalent, COPD incident cases. (B) Jaccard index of propensity score-matched normal cohort with COPD prevalent cases. There was significant difference in Shannon index of normal cohort with COPD prevalent cases (P-value = 0.0295).

*Abbreviations: COPD = chronic obstructive pulmonary disease; PSM = propensity score matched cohort.

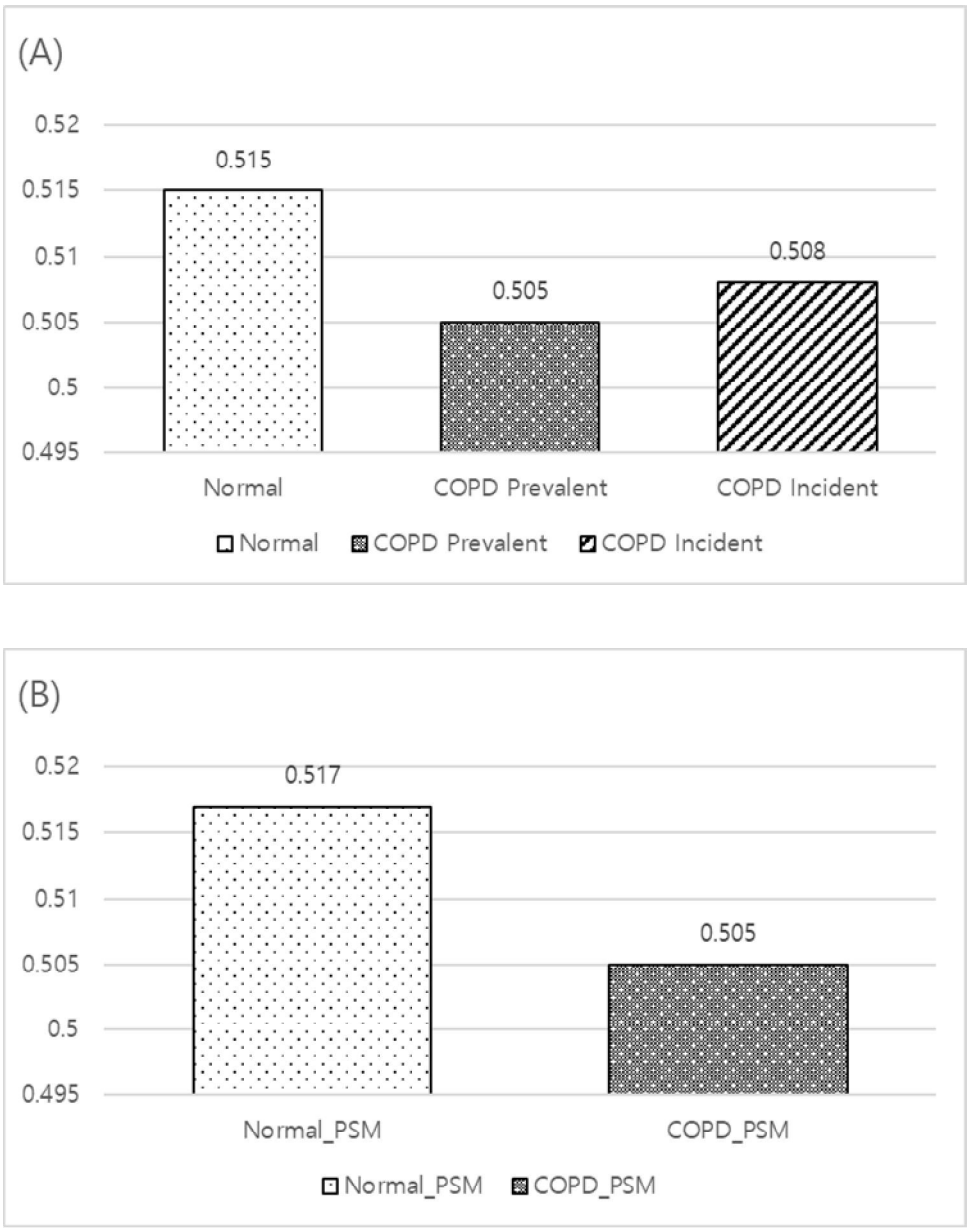
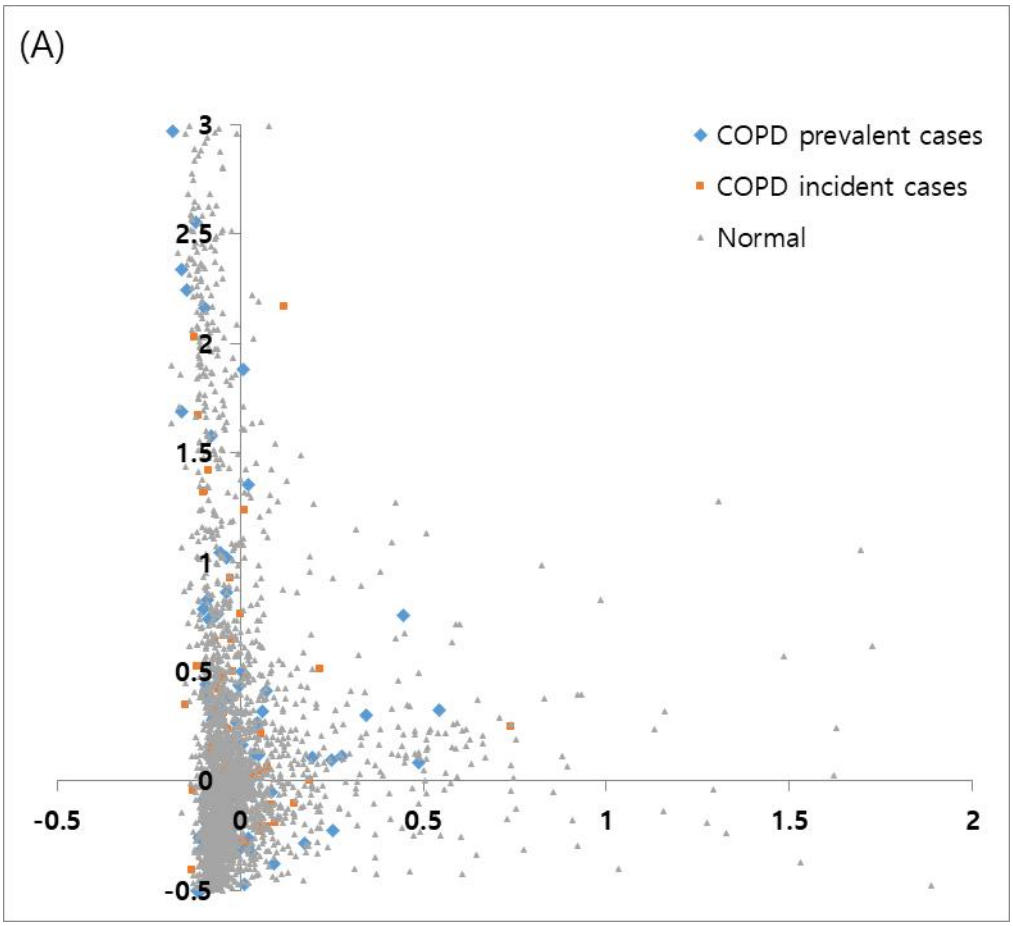


Figure 3. The result of principal component analysis (PCA). (A) PCA plot of total study subjects. (B) PCA plot of COPD and propensity score mated cohort of healthy subjects.

*Abbreviations: COPD = chronic obstructive pulmonary disease; PSM = propensity score matched cohort.


(A)
COPD prevalent cases
COPD incident cases
Normal
3
2.5
2
1.5
1
0.5
0
-0.5
-0.5
0
0.5
1
1.5
2

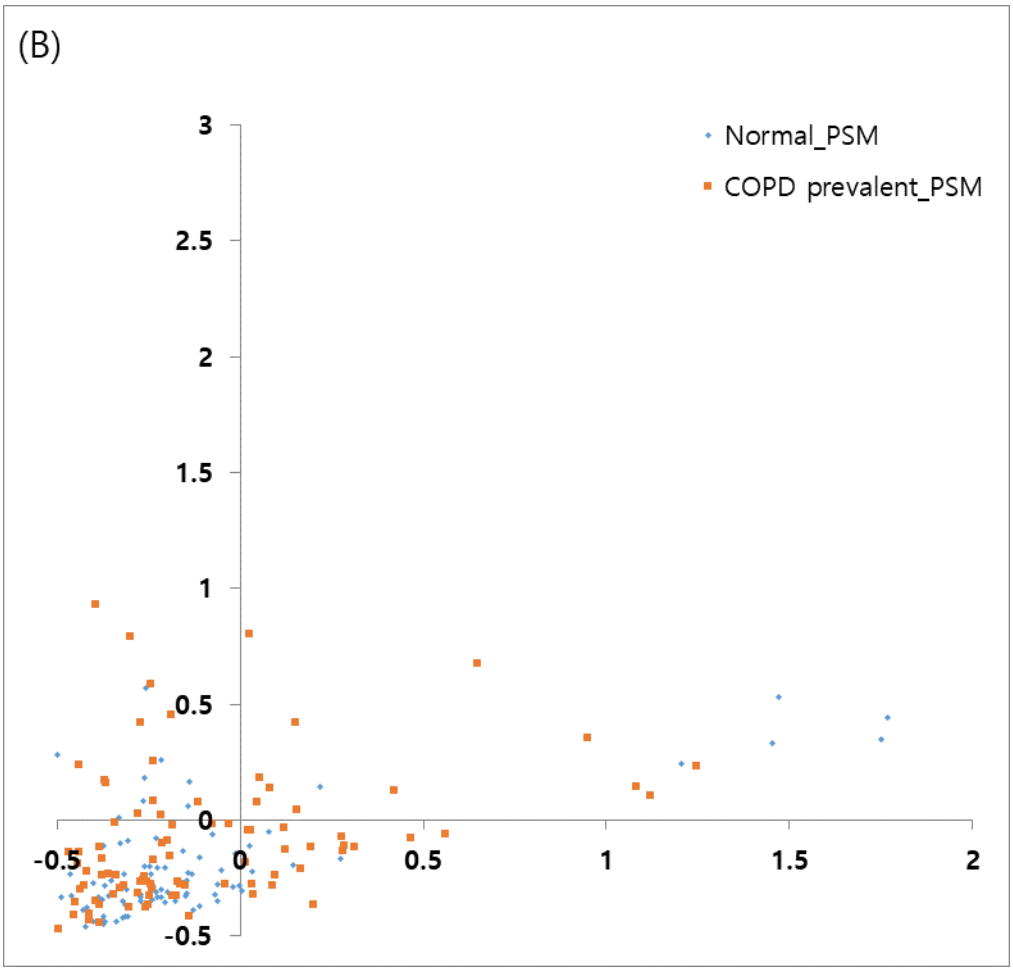

(B)
Normal_PSM
COPD prevalent_PSM
3
2.5
2
1.5
1
0.5
0
-0.5
-0.5
0
0.5
1
1.5
2

Figure 4. Comparison of frequently identified microbiome classified by the phyla in COPD patients compared to the healthy control group and propensity score matched healthy cohort. *Abbreviations: COPD = chronic obstructive pulmonary disease; PSM = propensity score matched cohort.