

# Lung Microbiota is Associated with Bacterial Detection and with Clinical Improvements in Severe Community-Acquired Pneumonia Patients

**Sisi Du**

China-Japan Friendship Hospital

**Binbin Li**

China-Japan Friendship Hospital

**Yimin Wang**

China-Japan Friendship Hospital

**Yudi Xia**

China-Japan Friendship Hospital

**Lianhan Shang**

China-Japan Friendship Hospital

**Donghao Yu**

China-Japan Friendship Hospital

**Zhibo Liu**

China-Japan Friendship Hospital

**Chunlei Wang**

China-Japan Friendship Hospital

**Xinmeng Liu**

China-Japan Friendship Hospital

**Zhujia Xiong**

China-Japan Friendship Hospital

**Xiaohui Zou**

China-Japan Friendship Hospital

**Binghuai Lu**

China-Japan Friendship Hospital

**Yingmei Liu**

China-Japan Friendship Hospital

**Bin Cao** (✉ [caobin\\_ben@163.com](mailto:caobin_ben@163.com))

China-Japan Friendship Hospital

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# Abstract

**Background:** Few studies have described the key features of the lung microbiota in patients with severe community-acquired pneumonia (SCAP). We conducted the study to identify the association between the lung microbiota on admission and the clinical prognosis in SCAP patients.

**Methods:** The consecutive SCAP patients admitted from intensive care unit (ICU) were enrolled prospectively. 16S rRNA gene sequencing was applied to bronchoalveolar lavage fluid (BALF) collected within 48 hours. The clinical information was recorded during the stay of hospitalization. The primary endpoint was the rate of clinical improvements defined as a decrease of 2 categories and above on a 7-category ordinal scale within 14 days following bronchoscopy.

**Results:** Sixty-seven patients were included. Multivariable Permutational multivariate analysis of variance (PERMANOVA) found that bacterial detection had the strongest independent relationship with the lung microbiota ( $R^2=0.033$ ;  $p=0.018$ ), followed by acute kidney injury (AKI  $R^2=0.032$ ;  $p=0.011$ ) and plasma MIP-1beta level ( $R^2=0.027$ ;  $p=0.044$ ). Random forest identified that families *Prevotellaceae*, *Moraxellaceae*, *Staphylococcaceae* were the biomarkers related to bacterial detection. In the patients with positive bacteriology results, the mean relative abundance of families *Prevotellaceae* and *Actinomycetaceae* decreased while families *Moraxellaceae*, *Staphylococcaceae* and *Streptococcaceae* increased. Multivariable Cox regression showed that the increase in alpha-diversity and the abundance of families *Prevotellaceae* and *Actinomycetaceae* were associated with clinical improvements.

**Conclusions:** The bacterial detection and patients' intrinsic factors were associated with the lung microbiota. The increased alpha diversity and the enrichment of families *Prevotellaceae* and *Actinomycetaceae* in the lung microbiota were associated with clinical improvements.

## Background

Patients with severe community acquired pneumonia (SCAP) are usually complicated with hypoxemia, acute kidney injury (AKI), sepsis and required intensive care unit (ICU) care[1, 2]. Although the broad-spectrum antibiotic and advanced oxygen support are used commonly in the therapeutic regimen, the death in SCAP patients remains as high as 36%[3]. Next generation sequencing (NGS) technology has revealed that the human lung contains complex and dynamic microbiota community[4]. Recently, several studies have suggested that the lung microbiota is associated with the respiratory infection and disease outcomes. The murine model proves that the intact microbiota contributes to the protection against bacterial pathogens with GM-CSF signaling[5]. The sputum microbial composition is found to be associated with length of stay and ICU admission in the children hospitalized for CAP[6]. Besides, the enrichment of specific taxon in the airway microbiota, such as *Enterobacteriaceae*, might be related to high plasma inflammatory cytokine level and the development of acute respiratory distress syndrome (ARDS), and be predictive of fewer ventilator-free days in patients with critical illness[7, 8]. However, few studies have described the key features of the lung microbiota in patients with SCAP.

A range of factors may contribute to the complexity of the lung microbiota in SCAP patients. The overgrowth of the invasive pneumonia-associated pathogens in patients' lower respiratory tract may cause a decline in the lung microbiota diversity and the microbiota might even be dominated by single species[9]. Based on the new conceptual models of respiratory microbiology proposed by Robert P Dickson, the lung microbiota is determined by three factors: microbial immigration, elimination, and the relative reproduction rates of the members which are affected by the regional growth conditions such as oxygen tension, pH, alveolar ventilation and temperature in the lung[10, 11]. Critical illness alters the internal environment and pathophysiology of the respiratory tract of the patients. Laryngeal dysfunction, supine positioning, aspiration or confusion, the ICU management including antibiotic use, mechanical ventilation and vasopressors have impacts on the balance of the immigration and elimination of the airway microorganisms[12]. Collectively, these clinical parameters have the potential to change the lung microbial composition of the SCAP patients.

Therefore, we conducted the study to explore the clinical factors that may be associated with the lung microbiota in patients with SCAP and identify the important taxa which may predict the clinical prognosis.

## Methods

### Study populations

All consecutive severe pneumonia cases admitted from ICU between March 2018 and March 2019 were registered prospectively. The inclusion criteria were (1) age  $\geq$  18 years old; (2) diagnosed with SCAP according to the 2007 Infectious Disease Society of America/American Thoracic Society guidelines (Table S1)[13]; (3) The time from onset of illness  $\leq$  7 days. The patients whose time of disease less than 14 days while condition exacerbation within 7 days before admission to ICU were also included (Fig. 1). Patients were excluded if they had one of the following criteria: (1) a history of hospitalization within 14 days before illness onset; (2) bronchoscopy couldn't be performed within 48 hours after admission; (3) peripheral blood specimens were not available within 24 hours; (4) pregnancy or breastfeeding; (5) had an alternative diagnosis at the end of the study, including lung cancer, pulmonary tuberculosis or pulmonary embolism.

### Sample Collection

The bronchoscopy was performed at bedside within 48 hours after the patients admitted to ICU using the standard clinical protocol. The bronchoscope was inserted through the nose or orotracheal tube and the bronchoalveolar lavage fluid (BALF) samples were collected. About 15 ml specimen was immediately sent to the microbiology laboratory for routine bacterial, fungal and viral examinations, and the remaining BALF was stored at  $-80^{\circ}\text{C}$  until further processing. Blood samples were obtained within 24 hours of ICU arrival. They were centrifuged and the plasma stored at  $-80^{\circ}\text{C}$ .

# 16s Rrna Gene Sequencing

DNA was extracted from BALF and negative control using the Maxwell® RSC Whole Blood DNA Kit (Promega, USA). The V3-V4 hypervariable region of the 16S rRNA gene of all samples was amplified by polymerase chain reaction (PCR). The pooled library was sequenced on an Illumina Miseq platform (Illumina, San Diego, CA, United States) using pair-end sequencing (2 × 300 bp). Details were shown in Supplementary Material.

## Data Collection

The following data were collected using a standard case report form: demographic data, underlying diseases, the time of illness onset, clinical symptoms, laboratory findings, microbiology results, radiographic data, antimicrobial use, glucocorticoid use, mechanical ventilation use and so on. The included patients were followed up until they were discharged or died.

## Cytokine Measurement

The Plasma IL-4, IL-6, IL-8, MIP-1beta, VEGF-A and MMP-9 of the SCAP patients were detected using a magnetic bead-based multiplex immunoassay and read on a Bio-Plex 200 suspension array system (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

## Definition

The primary endpoint was the rate of clinical improvements, which were defined as a decrease of 2 categories and above on a 7-category ordinal scale within 14 days following bronchoscopy. The ordinal scales which were also used in our influenza and Covid-19 studies consisted of the following categories: 1, not hospitalized with resumption of normal activities; 2, not hospitalized, but unable to resume normal activities; 3, hospitalization, not requiring supplemental oxygen; 4, hospitalization, requiring supplemental oxygen; 5, hospitalization, requiring nasal high-flow oxygen therapy and/or noninvasive mechanical ventilation; 6, hospitalization, requiring extracorporeal membrane oxygenation and/or invasive mechanical ventilation; 7, death[14–16]. Immunocompromised status in our study was defined as the patients having a history of cancer with neutropenia (absolute neutrophil count < 0.5 × 10<sup>9</sup>/L), hematological malignancies, solid malignancies receiving chemotherapy during the previous 3 months, solid organ or bone-marrow transplant, active graft-versus-host disease, bronchiolitis obliterans, human immunodeficiency virus infection, immunoglobulin deficiency, using immunosuppressive agents, or current treatment with systemic corticosteroids (≥ 20 mg of prednisone per day or its equivalent) for > 30 continuous days before illness onset[17]. The presence of ARDS on admission were diagnosed according to the Berlin definition[18]. For the nonventilated subjects who had a history of acute respiratory failure within 7 days because of a known respiratory events and bilateral pulmonary infiltration on chest x-ray

with PaO<sub>2</sub>/FiO<sub>2</sub> below 300 mmHg, ARDS was also considered. Sepsis, septic shock and acute kidney injury were diagnosed based on the third international consensus and Kidney Disease Improving Global Guidelines (KDIGO) clinical practice guidelines, respectively[19, 20]. Acute cardiac insufficiency was diagnosed by cardiologists based on clinical vitals, laboratory findings and echocardiography. Pneumonia severity was assessed by CURB-65, APACHE-II and PSI risk class. The order of performing bronchoscopy is based the time sequence of signing the bronchoscopy informed consent.

## Pathogen Detection

All the specimens for the microbiology diagnosis were collected within 48 hours after admission. Viral etiology was considered positive if the respiratory virus was detected in sputum, endotracheal aspirates (ETA), BALF, or nasopharyngeal (NP) swabs by real-time PCR (Zhijiang, Shanghai, China), including respiratory syncytial virus, influenza virus types A and B, parainfluenza virus, rhinovirus, coronavirus, human metapneumovirus and adenovirus. Bacterial or atypical pathogen detection were considered positive if one of the following criteria was met: (1) positive bacterial culture from blood or pleural fluid; (2) positive urinary antigen for *Legionella pneumophila* (Binax Now; Trinity Biotech, Bray, Ireland) or *Streptococcus pneumoniae* (Binax Now; Emergo Europe, Amsterdam, The Netherlands); (3) detection of *Mycoplasma pneumoniae* or *Chlamydia pneumoniae* in sputum, BALF, ETA, or NP swabs by real-time PCR (Zhijiang, Shanghai, China); (4) detection of *L. pneumophila* in sputum, BALF, or ETA by real-time PCR (Zhijiang, Shanghai, China); (5) bacteria with moderate to heavy growth (> 3 + growth) in qualified sputum or ETA, or quantified culture in BALF of  $\geq 10^4$  CFU/mL[17]. The diagnosis of IFD was based on the revision and update of the consensus definitions of invasive fungal disease[21].

## Statistical analysis

The software VSEARCH (version 2.7.1) and USEARCH version (10.0) were used to process the sequencing data. Reads were denoised into Zero-radius Operational Taxonomic Units (ZOTUs) with UNOISE3. After removal of ZOTUs identified as contaminants with decontam package or observed in the controls (Table S2) and whose relative abundance was less than 0.01%, a total of 490 ZOTUs were analyzed. Statistical analysis was performed in R version 3.6.2 via the Rstudio interface. PERMANOVA (vegan R-package) based on Bray-Curtis distance was performed to assess the association between the clinical factors and the lung microbiota. A random forest learning approach (randomForest R-package) was used to identify the clinical factors-associated taxon. Wilcoxon rank sum test and generalized linear models (GLM) were performed to compare the relative abundance of the species. Multivariable-adjusted Cox regression (adjusted for sample season, plasma IL-8 level, CURB-65, APACHEII scores, presence of shock at sampling, oxygen index on admission, creatinine level and microbiology results) was performed to assess the association between lung microbiota and clinical improvements.

## Results

# Patients' characteristics and bacterial profiles

A total of 67 patients were included (Table 1) and the flowchart was shown in Fig. 1. 67 BALF samples from the patients were analyzed. *Proteobacteria* was the most abundant phylum in the lung microbiota, accounting for 43.7% of the total filtered reads, followed by *Firmicutes* (30.94%), *Actinobacteria* (7.93%), *Bacteroidetes* (4.6%) and *Fusobacteria* (1.17%). At family level, the most abundant taxa were *Pseudomonadaceae* (23.22%), *Streptococcaceae* (13.71%), *Pasteurellaceae* (11.33%), *Corynebacteriaceae* (5.72%), *Moraxellaceae* (5.15%), *Staphylococcaceae* (4.94%), *Bacillaceae\_1* (4.5%), *Veillonellaceae* (2.98%), *Prevotellaceae* (2.11%) and *Enterobacteriaceae* (1.74%).

Table 1  
Baseline characteristics of the patients

Variable	N = 67
Age, years, median ± IQR	64 ± 22
Male, n (%)	50 (74.63)
Smoking status <sup>a</sup>	
Current smoker, n (%)	16 (23.88)
Former smoker, n (%)	19 (28.36)
Nonsmoker, n (%)	32 (47.76)
Drinking history, n (%)	13 (19.4)
Immunocompromised status, n (%)	10 (14.93)
Chronic respiratory disease, n (%)	11 (16.42)
Sample season, n (%)	
Spring	16 (23.88)
Summer	12 (17.91)
Autumn	12 (17.91)
Winter	27 (40.30)
Symptoms, n (%)	
Fever	53 (79.1)
Cough	50 (74.63)
Sputum	36 (53.73)
Dyspnea	54 (80.60)
Confusion/disorientation	15 (22.39)
Laboratory findings on admission	
PH, median ± IQR	7.443 ± 0.058
PaO <sub>2</sub> /FiO <sub>2</sub> , mmHg, mean ± sd	181.36 ± 78.62
Procalcitonin > 1 ng/mL, n (%)	36 (53.73)
Creatinine > 106 μmol/L, n (%)	28 (41.79)
Initial radiographic findings, n (%)	

<b>Variable</b>	<b>N = 67</b>
Diffuse bilateral pulmonary infiltration	56 (83.58)
Pleural effusion	25 (37.31)
Pathogen identified <sup>b</sup> , n (%)	
Viral detection only	26 (38.81)
Bacterial detection only	12 (17.91)
Viral-bacterial co-infection	6 (8.96)
Atypical pathogens <sup>c</sup>	9 (13.43)
Probable IFD	8 (11.94)
Complications at sampling, n (%)	
ARDS	29 (43.28)
Sepsis	65 (97.01)
Septic shock	21 (31.34)
AKI	23 (34.33)
Acute cardiac insufficiency	15 (22.39)
Antibiotic used before sampling <sup>d</sup> , n (%)	67 (100)
Carbapenems	22 (32.84)
Beta-lactams plus fluoroquinolones only	37 (55.22)
Beta-lactams only	5 (7.46)
Fluoroquinolones only	3 (4.48)
Vancomycin <sup>e</sup>	11 (16.42)
Oxygen support before sampling, n (%)	
High-flow nasal cannula only	25 (37.31)
Mechanical ventilation	41 (61.19)
Mechanical ventilation $\geq$ 2 days, n (%)	23 (34.33)
Severity variables at sampling	
APACHE-II, mean $\pm$ sd	12.78 $\pm$ 5.65
PSI risk class IV-V, n (%)	41 (61.19)

Variable	N = 67
CURB-65 risk score 3–5, n (%)	26 (38.81)
7-category ordinal scale at sampling, n (%)	
5: Hospitalization, requiring HFNC and/or non-IMV	41 (61.19)
6: Hospitalization, requiring ECMO and/or IMV	25 (37.31)
Decrease of 2 categories with 14 days	21 (31.34)
Days from illness onset to admission, median ± IQR	5 ± 4
Hours from admission to sampling <sup>f</sup> , median ± IQR	21 ± 13
ICU outcomes	
ICU length of stay, days, median ± IQR	9 ± 9.5
Death in ICU	11 (16.41)
Day 14 mortality	7 (10.45)
Plasma Biomarker on admission, pg/ml, mean ± sd	
IL-4	5.64 ± 12.6
IL-6	336.30 ± 725.60
IL-8	61.50 ± 144.24
MIP-1beta	169.01 ± 498.12
VEGF-A	1343.97 ± 761.88
MMP-9	142.34 ± 156.14
a: Smoking status was grouped as nonsmokers, current smokers and former smokers (defined as the previous smokers who had quit at least 6 months before).	
b: Two patients had positive urinary antigen for <i>Streptococcus pneumoniae</i> with negative detection in bronchoalveolar lavage fluid (BALF). Other microbiology results were totally obtained from the detection in BALF collected within 48 hours.	
c: Atypical pathogens consisted of <i>Legionella pneumophila</i> and <i>Mycoplasma pneumoniae</i> .	
d: Only 6 patients (8.96%) had not received antibiotics before admitted to ICU. Among them, 2 patients received carbapenems and 4 patients received beta-lactams plus fluoroquinolones only.	
e: Consisting of vancomycin, teicoplanin and linezolid.	
f: The order of performing bronchoscopy is based the time sequence of signing the bronchoscopy informed consent.	
Abbreviation: IFD, invasive fungal disease; ARDS, acute respiratory distress syndrome; AKI, acute kidney injury; IQR, interquartile range; SD, standard deviation; ICU, intensive care unit.	

# Bacterial Detection Shaped The Lung Microbial Composition

Plasma MIP-1beta level (Bray-Curtis distance;  $R^2 = 0.029$ ;  $P = 0.03$ ), procalcitonin level (PCT  $R^2 = 0.029$ ;  $P = 0.026$ ), complicated with AKI ( $R^2 = 0.033$ ;  $P = 0.012$ ), bacterial detection ( $R^2 = 0.033$ ;  $P = 0.013$ ) and the time from admission to sampling ( $R^2 = 0.049$ ;  $P = 0.018$  Fig. 2a-e) were significantly associated with the lung microbiota by univariate PERMANOVA. Age, baseline chronic respiratory diseases, antibiotic use, complicated with ARDS and septic shock (Fig. S1, Table S3) were not significantly associated with the lung microbiota.

We performed a multivariable analysis to assess the independent effect of individual factor. Smoking status ( $R^2 = 0.036$ ;  $P = 0.185$ ), time on ventilator ( $R^2 = 0.04$ ;  $P = 0.097$ ) before sampling and sputum production ( $R^2 = 0.021$ ;  $P = 0.118$  Fig. 2f-h) whose  $P$  value less than 0.2 were also included. Kappa test didn't show a severe multicollinearity problem among the eight factors ( $K = 4.8$  Fig. 2f). Bacterial detection showed the strongest independent relationship to the lung microbiota composition (multivariable PERMANOVA,  $R^2 = 0.033$ ;  $p = 0.018$ ), followed by AKI ( $R^2 = 0.032$ ;  $p = 0.011$ ) and plasma MIP-1beta level ( $R^2 = 0.027$ ;  $p = 0.044$ ).

## Bacteriology Results Influenced Lung Microbial Taxa

*Prevotellaceae* family played the most important role in distinguishing bacterial detection positive from negative samples, followed by the pathogenic taxa, such as, families *Moraxellaceae*, *Staphylococcaceae* and *Streptococcaceae* (Fig. 3a). In the patients with positive bacteriology results, the mean relative abundance of families *Prevotellaceae*, *Microbacteriaceae* and *Actinomycetaceae* decreased while families *Moraxellaceae*, *Staphylococcaceae* and *Streptococcaceae* increased (Fig. 3a-b), which were in line with their high isolation rate of typical bacterial culture (Table 2). 16S rRNA gene sequencing was able to produce the same results as culture methods in 61% of patients (Table 2). However, there was no significant difference in the relative abundance of family *Pseudomonadaceae* between the two groups patients (Fig. 3a). The samples with bacteria detected had lower alpha diversity while the difference wasn't significant (Fig. 3d).

Table 2  
Comparison between bacterial detection and sequencing results

<b>MV <math>\geq</math> 2 days<sup>a</sup></b>	<b>Bacterial detection</b>	<b>The most abundant ZOTU in sequencing</b>
<b>Yes</b>	<i>Acinetobacter baumannii</i>	<b>ZOTU4_f_ Streptococcaceae (38.43%)<sup>c</sup></b>
Yes	<i>Acinetobacter baumannii</i>	ZOTU5_f_ <i>Moraxellaceae</i> (87.82%)
Yes	<i>Acinetobacter baumannii Klebsiella pneumoniae</i>	ZOTU5_f_ <i>Moraxellaceae</i> (37.71%)
Yes	<i>Pseudomonas aeruginosa</i>	ZOTU1_f_ <i>Pseudomonadaceae</i> (98.5%)
Yes	<i>Pseudomonas aeruginosa</i>	ZOTU1_f_ <i>Pseudomonadaceae</i> (83.84%)
<b>Yes</b>	<i>Staphylococcus aureus</i>	<b>ZOTU18_f_ Streptococcaceae (54.3%)</b>
Yes	<i>Staphylococcus aureus</i>	ZOTU10_f_ <i>Staphylococcaceae</i> (29.84%)
<b>No</b>	<i>Pseudomonas aeruginosa</i>	<b>ZOTU18_f_ Streptococcaceae (13.98%)</b>
No	<i>Pseudomonas aeruginosa</i>	ZOTU1_f_ <i>Pseudomonadaceae</i> (69.33%)
No	<i>Pseudomonas aeruginosa</i>	ZOTU1_f_ <i>Pseudomonadaceae</i> (83.92%)
No	<i>Pseudomonas aeruginosa</i>	ZOTU1_f_ <i>Pseudomonadaceae</i> (36.14%)
<b>No</b>	<i>Staphylococcus aureus</i>	<b>ZOTU13_f_ Corynebacteriaceae (15.91%)</b>
No	<i>Staphylococcus aureus</i>	ZOTU10_f_ <i>Staphylococcaceae</i> (59.17%)
No	<i>Streptococcus pneumoniae<sup>b</sup></i>	ZOTU4_f_ <i>Streptococcaceae</i> (98.02%)

a: The patients had more than 2 days on ventilator were defined as “Yes” and less than 2 days or those never received mechanical ventilation were defined as “No”.

b: Two patients had positive urinary antigen for *Streptococcus pneumoniae* with negative detection in bronchoalveolar lavage fluid (BALF).

c: The relative abundance of the most abundant ZOTU was shown.

The bold font represented that there were different results between bacterial detection and sequencing method.

Abbreviation: MV, mechanical ventilation.

MV $\geq$ 2 days <sup>a</sup>	Bacterial detection	The most abundant ZOTU in sequencing
No	<i>Streptococcus pneumoniae</i> <sup>b</sup>	ZOTU1_f_ Pseudomonadaceae (27.86%)
No	<i>Acinetobacter baumannii</i>	ZOTU5_f_ Moraxellaceae (50.41%)
<b>No</b>	<b>Klebsiella pneumoniae</b>	<b>ZOTU59_f_ Bacteroidaceae (12.28%)</b>
<b>No</b>	<b>Escherichia coli</b>	<b>ZOTU44_f_ Corynebacteriaceae (13.28%)</b>
a: The patients had more than 2 days on ventilator were defined as “Yes” and less than 2 days or those never received mechanical ventilation were defined as “No”.		
b: Two patients had positive urinary antigen for <i>Streptococcus pneumoniae</i> with negative detection in bronchoalveolar lavage fluid (BALF).		
c: The relative abundance of the most abundant ZOTU was shown.		
The bold font represented that there were different results between bacterial detection and sequencing method.		
Abbreviation: MV, mechanical ventilation.		

## Lung Microbiota Predicted Clinical Improvements

The lung microbial community composition of the patients on admission wasn't associated with the clinical improvement (PERMANOVA; Bray-Curtis distance;  $R^2 = 0.011$ ;  $P = 0.76$ ). Compared with the patients whose richness of baseline lung microbiota was higher than 200, the multivariable-adjusted Hazard Ratio (HR) for the favorable prognosis in the lower Richness group patients was 0.17 and 0.16, respectively (95% CI: 0.04–0.71,  $p = 0.02$ ; 95% CI: 0.03–0.88,  $p = 0.04$  Fig. 4a, Table 3). The increased Shannon index predicted a faster decrease of 2 categories which was analyzed continuously (adjusted HR 1.54, 95% CI: 1.08–2.20,  $p = 0.02$ ) or by organizing their value into ranges (adjusted HR 0.08, 95% CI: 0.01–0.51,  $p = 0.007$  Table 3, Fig. 4b, Table 3). Although the bacterial detection wasn't associated with clinical improvements, we found that every 1% increase in the relative abundance of families *Prevotellaceae* and *Actinomycetaceae* in the lung microbiota of the patients raised the probability for clinical improvements by 13% and 12% respectively (95% CI: 1.02–1.25,  $p = 0.02$ ; 95% CI: 1.03–1.21,  $p = 0.007$  Table 3, Fig. 4c-d). However, families *Moraxellaceae*, *Staphylococcaceae* and *Streptococcaceae* were not associated with clinical improvements (Table 3, Fig. 4c-d). The proportions of patients with ARDS, septic shock, CURB-65  $\geq$  3, PSI  $\geq$  IV were similar irrespective of the different level of alpha diversity or relative abundance of taxa (Table S4).

Table 3  
Association of lung microbiota with clinical improvements in multivariable analysis.

Variable	adjusted HR <sup>a</sup>	95% CI	P value
Pathogen identified <sup>b</sup>			
Bacterial detection only	reference		
Viral-bacterial co-infection	0.60	0.06–6.54	0.67
Viral detection only	1.82	0.48–6.92	0.38
Others	0.69	0.19–2.46	0.57
Richness (continuous)	1.01	1.00-1.02	0.06
Richness (categorical) <sup>c</sup>			
≥ 200	reference		
199 - 150	0.17	0.04–0.71	0.02
< 150	0.16	0.03–0.88	0.04
Shannon (continuous)	1.54	1.08–2.20	0.02
Shannon (categorical) <sup>c</sup>			
>4.5	reference		
4.5-2	0.38	0.11–1.28	0.12
<2	0.08	0.01–0.51	0.007
<i>Prevotellaceae</i> , 1% <sup>d</sup> (continuous)	1.13	1.02–1.25	0.02
<i>Prevotellaceae</i> , % (categorical) <sup>c</sup>			

a: Multiple cox regression analysis adjusted for sample season, plasma IL-8 level, CURB-65, APACHEII scores, presence of shock at sampling, oxygen index on admission, creatinine level and microbiology results.

b: Multiple cox regression analysis adjusted for sample season, plasma IL-8 level, CURB-65, APACHEII scores, presence of shock at sampling, oxygen index on admission, creatinine level.

c: We turned the continuous variables into categorical variables by organizing their value into ranges. Firstly, according to the value of the variable, 67 samples were put in order. Secondly, we divided them into 10 groups evenly. Finally, we combined the neighboring groups that had the similar probability for the event happened.

d: 1% referred to that the probability for clinical improvements of every 1% increase in the relative abundance of taxa.

Abbreviation: HR, hazard ratios; CI, confidence interval.

Variable	adjusted HR <sup>a</sup>	95% CI	P value
> 2.4	reference		
2.4 – 0.3	0.37	0.10–1.38	0.14
< 0.3	0.08	0.01–0.39	0.002
<i>Actinomycetaceae</i> ,1% (continuous)	1.12	1.03–1.21	0.007
<i>Actinomycetaceae</i> , % (categorical) <sup>c</sup>			
>0.5	reference		
0.5-0.005	0.37	0.11–1.21	0.10
<0.005	0.06	0.01–0.33	0.001
<i>Moraxellaceae</i> ,1% (continuous)	1.04	0.98–1.09	0.17
<i>Staphylococcaceae</i> ,1% (continuous)	0.95	0.89–1.02	0.19
<i>Streptococcaceae</i> ,1% (continuous)	1.01	0.98–1.04	0.62
<i>Pseudomonadaceae</i> ,1% (continuous)	0.99	0.97-1.00	0.09
<i>Enterobacteriaceae</i> ,1% (continuous)	0.95	0.82–1.10	0.49
a: Multiple cox regression analysis adjusted for sample season, plasma IL-8 level, CURB-65, APACHEII scores, presence of shock at sampling, oxygen index on admission, creatinine level and microbiology results.			
b: Multiple cox regression analysis adjusted for sample season, plasma IL-8 level, CURB-65, APACHEII scores, presence of shock at sampling, oxygen index on admission, creatinine level.			
c: We turned the continuous variables into categorical variables by organizing their value into ranges. Firstly, according to the value of the variable, 67 samples were put in order. Secondly, we divided them into 10 groups evenly. Finally, we combined the neighboring groups that had the similar probability for the event happened.			
d: 1% referred to that the probability for clinical improvements of every 1% increase in the relative abundance of taxa.			
Abbreviation: HR, hazard ratios; CI, confidence interval.			

## Discussion

The core findings of our study were that bacterial detection had the strongest independent relationship with the lung microbiota composition on patients' admission, followed by AKI and plasma MIP-1beta level. The increased alpha diversity and the enrichment of families *Prevotellaceae* and *Actinomycetaceae* in the lung microbiota were associated with clinical improvements.

In this study, the positive bacterial detection on admission was found to be the most important factor related to patients' lung microbiota. The bacteria that could be identified by culture-based methods might have a higher load in the lower respiratory tract[22]. Therefore, 16S rRNA gene sequencing revealed that the lung microbiota dominated by the corresponding taxa in the patients with positive bacteriology results (such as *Acinetobacter baumannii* and *Staphylococcus aureus*). Although the results of 16S rRNA and culture-based methods were consistent in terms of dominant bacteria, the results related to *Pseudomonas aeruginosa* differed. The relative abundance of family *Pseudomonadaceae* wasn't significantly different between the bacteriology-positive and bacteriology-negative groups despite the high isolation rate (33.33%) of *Pseudomonas aeruginosa*. *P. aeruginosa* is one of the main nosocomial pathogens in ICU. The risk factors for *P. aeruginosa* acquisition consisted of antibiotics pressure, mechanical ventilation use, length of hospitalization, cross-transmission among patients and medical staffs and environmental factors, such as contamination of water tap[23–25]. All of our patients had received antimicrobial treatment and more than half were exposed to mechanical ventilation before bronchoscopy. They might have similar opportunity to obtain *P. aeruginosa* colonization under the ICU status. Although 16S rRNA gene sequencing had a low accuracy for pathogen identification, it could capture all the bacterial information, including fastidious bacteria in the respiratory tract, which might explain the different results between culture-dependent and culture-independent analysis in our study[26, 27].

After adjusted for the microbiology results and severity of illness, multivariate analysis showed that clinical improvements were associated with increased relative abundance of family *Prevotellaceae* and *Actinomycetaceae*. It was found that relative abundance of *Prevotella* was inversely associated with airway inflammation in cystic fibrosis patients[28]. An increase in the abundance of *Prevotella* in nose/throat microbiota was related to lower susceptibility to influenza A infection[29]. Another set of researches showed that the enrichment of lung microbiota with *Prevotella* enhanced the level of BALF inflammatory cytokines, and was associated with the development of asthma in children and ARDS in severe patients[7, 30, 31]. The conclusions appeared to be inconsistent. However, the reduction in abundance of *Prevotella* was observed at the initial stage of chronic pulmonary inflammation in mice[32]. The causal relationship between *Prevotella* and the diseases couldn't be clearly inferred from these association studies[33]. Besides, families *Prevotellaceae* and *Actinomycetaceae* usually resided in healthy the respiratory tract or oral cavity[30, 34, 35]. Earlier study reported that the commensal bacteria (commensal *Prevotella* spp and *Actinomyces* spp) could induce weak activation of human dendritic cells compared with pathogenic species (*Haemophilus* spp. and *Moraxella* spp)[36]. While co-culture experiments observed that *Prevotella* spp. were able to inhibit *Haemophilus influenzae*-induced IL-12p70 in dendritic cells[36]. Conclusively, we thought that *Prevotellaceae* and *Actinomycetaceae* served as commensal colonization and might promote the recovery of the lung from infection in our study.

The antibiotic use was also proved to decrease the airway microbial diversity in COPD patients[37]. However, the type of antibiotic use before sampling was not associated with the lung microbiota in our study, consistent with the result of a study in intubation patients[9]. As all of our patients had received antibiotics before bronchoscopy, we couldn't exclude the possibility of an association between the

antibiotics and the lung microbiota. Besides, the lung microbiota of our patients wasn't clustered by the presence of ARDS or septic shock at sampling. Unlike the report in two previous published studies, the relative abundance of family *Enterobacteriaceae* had no association with the severity of pneumonia or clinical outcomes[7, 8]. In fact, those studies primarily focused on the patients with ARDS caused by severe trauma, cardiac arrest or cerebral vascular events. In Robert P. Dickson et al.'s study, the pneumonia or sepsis patients merely accounted for 13% (12/91) while in Ariane R. Panzer's study, the ARDS in all patients was caused by trauma[7, 8]. Thus, the conclusions of the studies might not be applied to the SCAP patients.

Our study has several limitations. Firstly, the lung microbiota analysis was only performed on ICU admission. The variation of the lung microbiota during the patients' hospitalization and the association between that with the clinical improvements could not be analyzed. Secondly, the sample size of the patients was small. Finally, we couldn't explain that the relative abundance of Families *Prevotellaceae* and *Actinomycetaceae* or the alpha diversity was not associated with the severity of pneumonia. It seemed that high diversity and the presence of commensal colonization in the lung only suggested the ability to recover from infection. Further studies are needed to explore whether the lung microbiota play a role in the pathogenesis of pneumonia.

## Conclusions

Conclusively, we found that the bacterial detection and patients' intrinsic factors were associated with the lung microbiota. The lung microbiota on admission was predictive of clinical improvements.

## Abbreviations

SCAP, severe community-acquired pneumonia; ICU, intensive care unit;

BALF, bronchoalveolar lavage fluid; PERMANOVA, Permutational multivariate analysis of variance; AKI, acute kidney injury; ARDS, acute respiratory distress syndrome; HR, hazard ratios; CI, confidence interval.

## Declarations

### Ethics approval and consent to participate

Our study was conducted in China-Japan Friendship Hospital, Beijing, China and approved by the China-Japan Friendship Hospital Ethics Committee (Approval No. 2018-20-K15). It was conducted in accordance with the amended Declaration of Helsinki. All subjects provided written consents.

### Consent for publication

Not applicable.

### Availability of data and materials

The sequence data have been deposited in the NCBI Sequence Read Archive under accession PRJNA616057. (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA616057>). Feature, taxonomy, metadata tables and a reproducible workflow of the analysis are available for download at [https://github.com/Zoey-Du/SCAP\\_microbiota](https://github.com/Zoey-Du/SCAP_microbiota).

### Competing interests

The authors declare that they have no competing interests.

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### Authors' contributions

B.C., Y.L. and B.L. conceived the idea. S.D., B.L., X.Z. and B.C. finished the protocol. S.D., The samples were collected by B.L., Z.X., D.Y., Z.L., and Y.D. S.D., L.S., C.W., X.L. and X.Z. performed the laboratory testing and data analysis. S.D., L.S. and B.C. contributed to the interpretation of the data. S.D., B.L., L.S. and B.C. drafted the manuscript. Y.L., B.L. and B.C. revised the manuscripts. All authors approved the manuscript.

### Acknowledgements

Not applicable.

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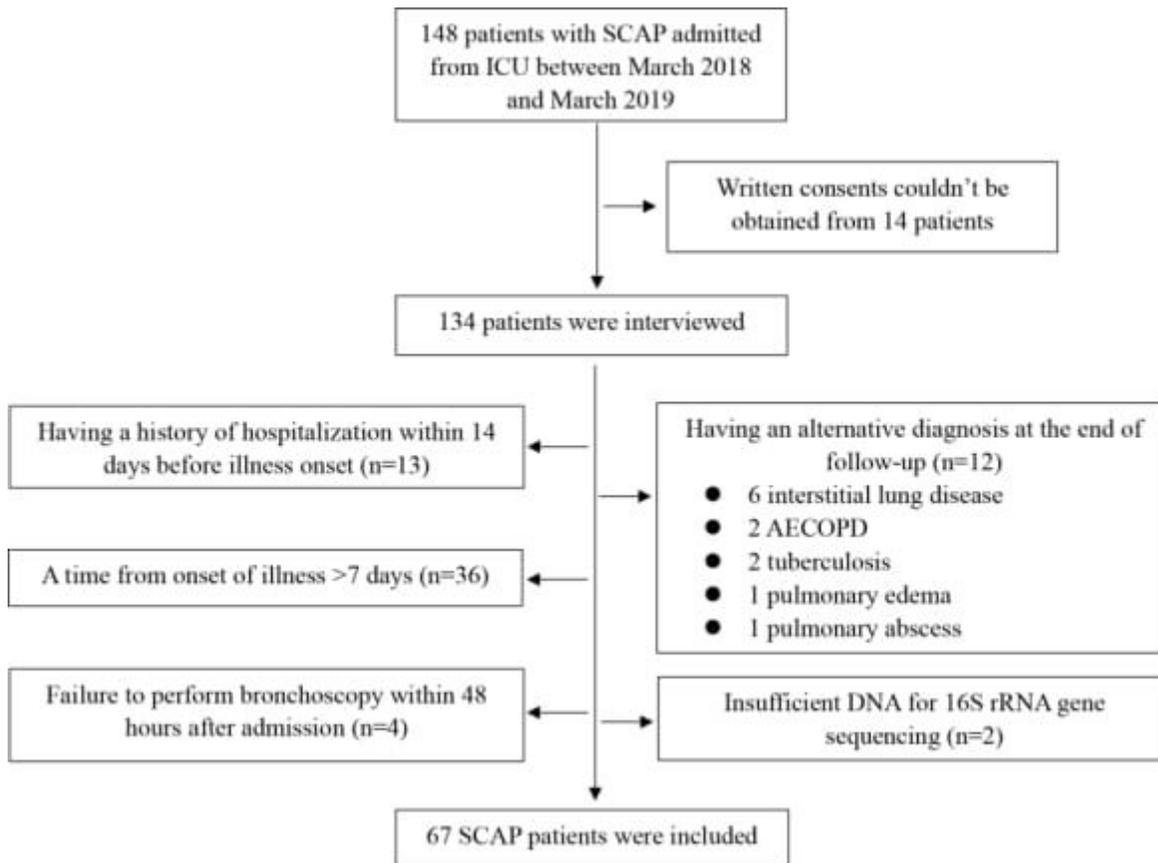
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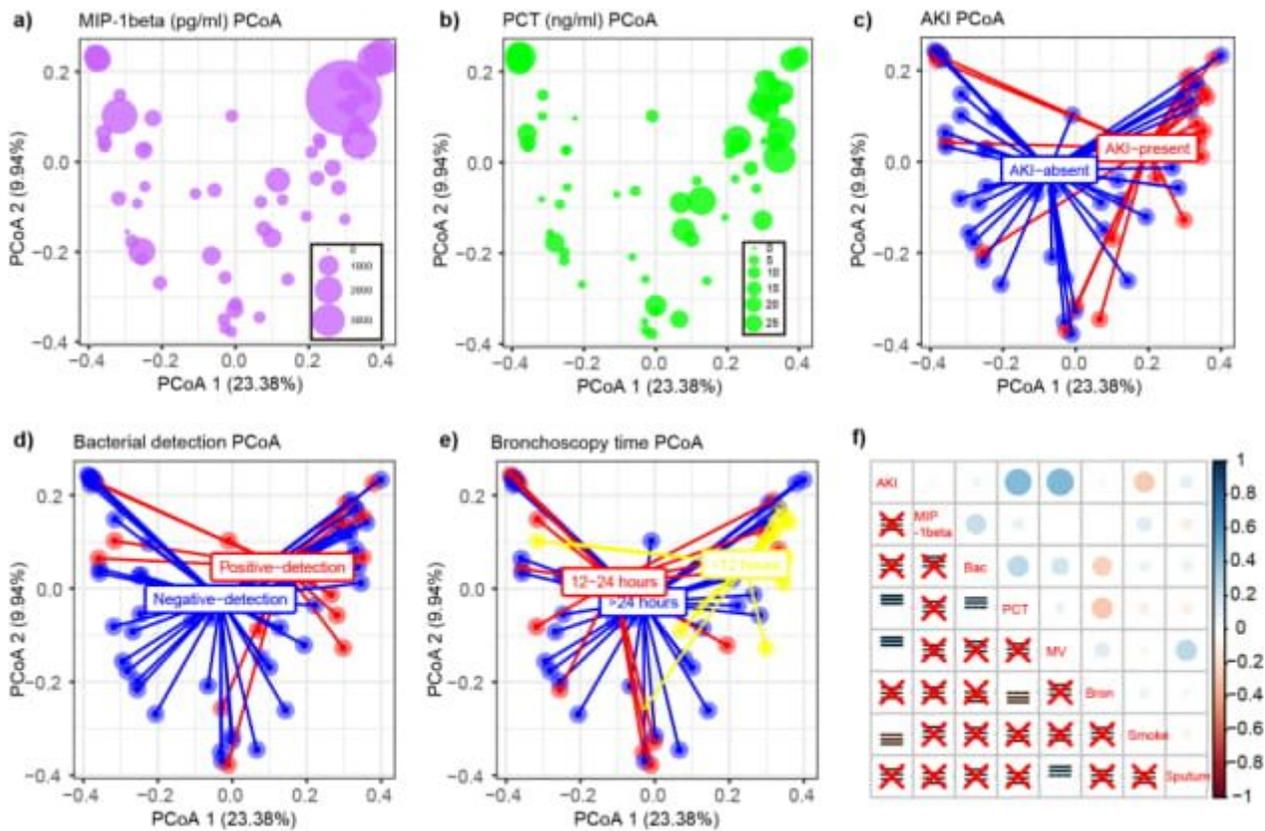
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## Figures



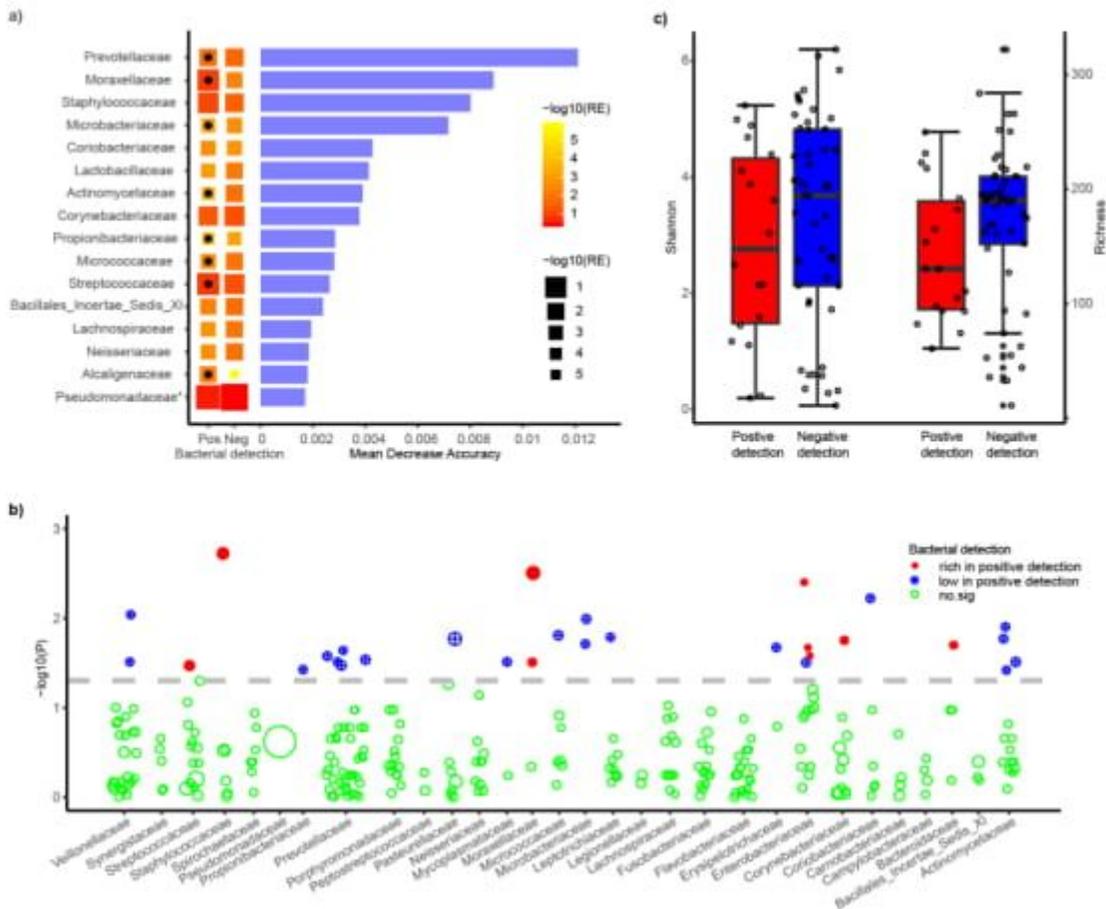
**Figure 1**

Study flow chart \*The patients whose time of disease less than 14 days while condition exacerbation within 7 days before admission to ICU were also included. Abbreviation: SCAP, severe community acquired pneumonia; ICU, intensive care unit; AECOPD, acute exacerbation of chronic obstructive pulmonary disease.



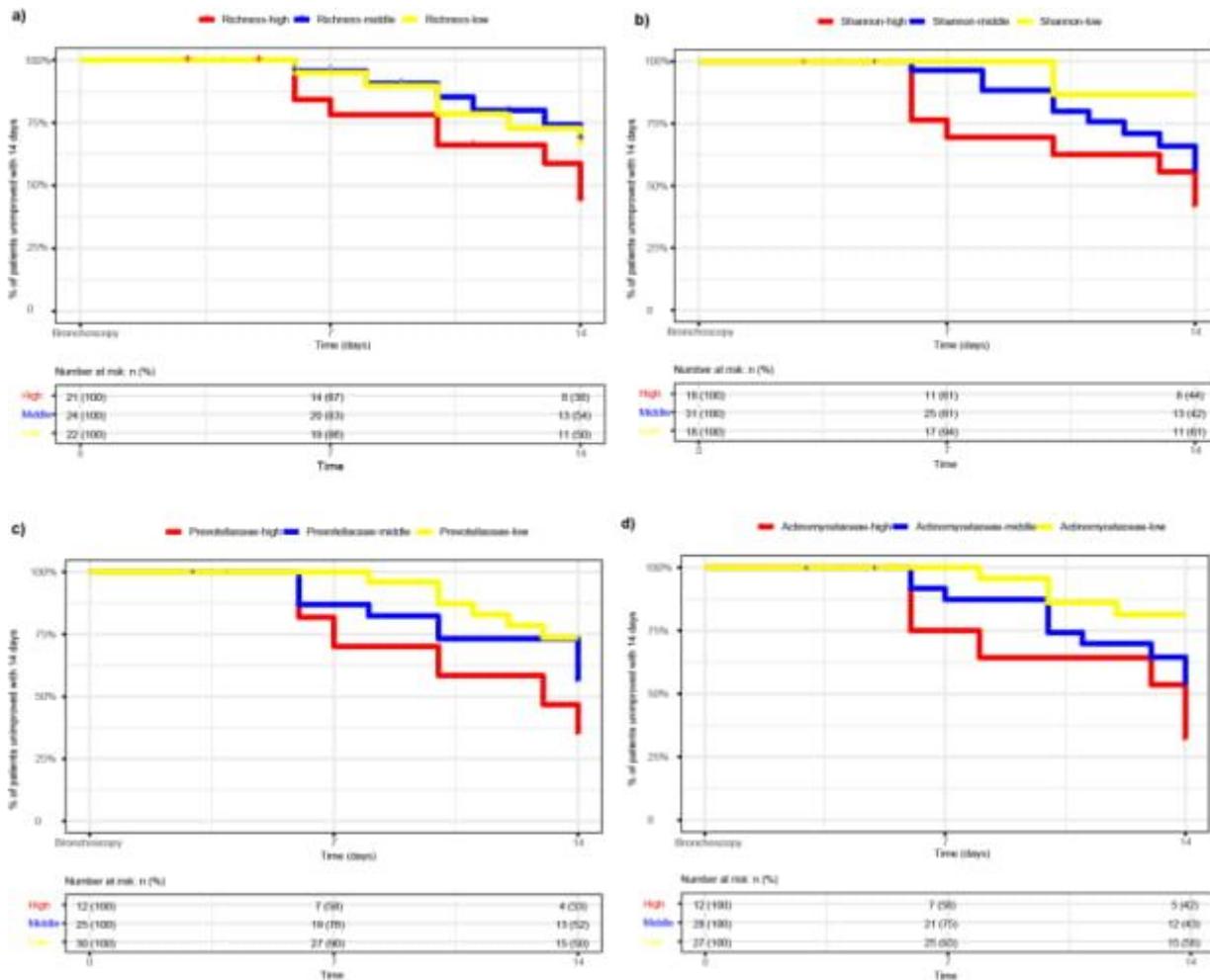
**Figure 2**

Factors associated with the lung microbiota Principal Coordinate Analysis (PCoA) plot based on Bray-Curtis distance revealed that the lung microbial community composition was significantly associated with the concentration of MIP-1beta (a) and procalcitonin (PCT b). The lung microbiota was associated with the presence of acute kidney injury (AKI) on admission (c), bacterial detection (Bac d) and the time from admission to sampling (Bron e). The co-association between the 8 clinical factors was calculated by Pearson correlation analysis (f). The circles were proportional to the absolute value of the correlation coefficients. The squares represented the P values. "X" represented the P values were larger than 0.05.



**Figure 3**

The influence of bacteriology results on lung microbiota. The top 15 biomarker taxa associated with bacterial detection (a). The black points represented there were significantly different in the relative abundance of the species between the two groups. The color and size of the squares were proportional to the value of  $-\log_{10}(\text{mean relative abundance})$ . \*: The variation of the relative abundance in family Pseudomonadaceae that was not included in the top 15 was shown. (b) The positions of the ZOTUs were determined by the value of  $-\log_{10}(P)$  value from the comparison of the mean relative abundance in ZOTUs between the groups). The size of circles was proportional to the relative abundance of the ZOTUs in microbiota. (c) The boxplots represent the diversity measures (Shannon index and Richness) for the patients (center line, median; box limits,  $\pm$ interquartile range; whisker limits, maximum/minimum).



**Figure 4**

Lung microbiota predicting clinical improvements Survival curves were plotted using the Kaplan-Meier method. The primary endpoint was the rate of clinical improvements (defined as a decrease of 2 categories and above on a 7-category ordinal scale) within 14 days following bronchoscopy. Richness (a), Shannon index (b), the relative abundance of families Prevotellaceae (c) and Flavobacteriaceae (d) were protective of the clinical improvements after adjusted for sample season, plasma IL-8 level, CURB-65, APACHEII scores, presence of shock at sampling, oxygen index on admission, creatinine level and microbiology results. The categorical cut-offs were consistent with those in Table 3

## Supplementary Files

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