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Coronavirus Pandemic

Dominance of specific lung bacteria over microbiota diversity in COVID-19 clinical trajectories

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Abstract

Introduction: This study investigates the impact of lung microbiota on COVID-19 outcomes.

Methodology: Clinical data and bronchoalveolar lavage fluid (BALF) data and bronchoalveolar lavage fluid (BALF) samples were retrospectively collected from 40 COVID-19 patients for Targeted Next-generation Sequencing (TNGS). Microbial diversity was then analyzed across different clinical severity groups. Additionally, biomarkers were identified using Linear Discriminant Analysis Effect Size (LEfSe) and evaluated by Receiver Operating Characteristic (ROC) - Area Under the Curve (AUC).

Results: The patients were classified by severity as mild (n = 3), moderate (n = 13), severe (n = 16), or critical (n = 8) symptoms. The α-diversity of respiratory flora showed no significant differences between groups ($p > 0.05$). While β-diversity analysis revealed significant compositional distinctions ($p < 0.05$). Critically ill patients had higher levels of *Pseudomonas aeruginosa* compared to other groups, ROC-plot AUC value of 0.856. Patients were then categorized into two outcome-based groups: Non-survivors (n = 5) and Survivors (n = 35). No significant differences in α-diversity of respiratory flora were observed between the two groups ($p > 0.05$), while β-diversity revealed distinct compositional differences ($p < 0.05$). Furthermore, the ROC curve for *Pseudomonas aeruginosa* (AUC = 0.846) indicated its predictive value for mortality.

Conclusions: This study has elucidated the characteristics of pulmonary microbiota across different COVID-19 severities, identifying bacteria associated with severe illness, mortality, and relevant clinical markers. The lung microbiota exhibits low diversity, making the pulmonary microecology more vulnerable to disruption. Therefore, invasive species may influence clinical outcomes in affected patients.

Key words: COVID-19; dysbiosis; biomarker; microbiota.

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Introduction

Since its emergence in late 2019, the coronavirus disease 2019 (COVID-19) has caused approximately 775 million infections and over 7.05 million deaths worldwide [1]. It continues to challenge global health systems and significantly affects various physiological systems [2]. As the primary organ of SARS-CoV-2 infection, the lungs are primarily affected by mild pneumonia in the majority of patients [3]. However, a minority of people develop severe pneumonia that may lead to death [4]. Recent research suggests that the lung microbiota may play a critical role in these disparate clinical outcomes [5]. Historically, the lungs were believed to be sterile due to their low microbial biomass —a view that persisted for decades [6]. The rapid development of high-throughput sequencing technologies has changed this view, allowing scientists to delve deeper into the complexities of the lung microbiome [7]. Recent findings indicate that respiratory flora significantly influences lung disease outcomes [8]. Changes in respiratory flora have been linked to the onset and progression of pneumonia, asthma, and chronic obstructive pulmonary disease (COPD) [9], and are also associated with adverse outcomes in critically ill patients [10].

Despite its importance, the role of the lung microbiota in lung diseases, including COVID-19, remains insufficiently understood. Key challenges include the relatively low biomass of the lung microbiota [11], interference from the host genome during sequencing, and the difficulty in obtaining samples that accurately reflect the lung microbiota. However, from an ecological perspective, in environments characterized by low-abundance species, greater emphasis should be placed on the impact of individual bacterial species [12]. Furthermore, research

substantiates that the composition of the lung microbiome influences various lung diseases. Pathogenic bacteria such as *Haemophilus* are increased in patients with asthma and COPD [13]. *Streptococci* and *staphylococci* were more abundant in lung cancer patients, whereas the opposite was true in non-cancer subjects [14]. In addition, it is hypothesized that the lung microbiota may have a significant impact on disease progression and could potentially serve as a prognostic indicator in critically ill patients [15]. The degree of bacterial diversity during infection with idiopathic pulmonary fibrosis (IPF) has been shown to predict patient survival [16]. Furthermore, Molyneaux and colleagues found an increased risk of death with increasing bacterial load in the lungs, suggesting that a single species within the respiratory tract can significantly influence disease progression. A cohort study of COPD patients showed that the absence of *Veillonella* in the lung microbiota increased the mortality risk thirteenfold, while the presence of *Staphylococcus* was associated with a sevenfold increase [17].

This study aims to investigate the characteristics of respiratory microbiota and to identify biomarkers in patients with varying COVID-19 severities and clinical outcomes. The present study retrospectively analyzed Targeted Next-generation Sequencing (TNGS) results from bronchoalveolar lavage fluid (BALF) samples and evaluated their diagnostic and prognostic significance.

Methodology

Study population

A total of 40 patients diagnosed with COVID-19 who attended the First Hospital of Shanxi Medical University between December 26, 2022, and February 17, 2023, were retrospectively enrolled in the study. Disease severity was categorized into four groups based on the Diagnostic and Treatment Protocol for Novel Coronavirus Infections (Trial Tenth Edition): (1) Mild, in which upper respiratory tract infections were the main manifestations, such as dry pharynx, sore pharynx, cough, and fever. (2) Moderate, with persistent high fever for > 3 days or cough, shortness of breath, etc., but respiratory rate (RR) < 30 breaths/minute and oxygen saturation > 93% on air intake at rest. Imaging shows characteristic neocoronavirus infection with pneumonia. (3) Severe, shortness of breath, RR ≥ 30 breaths/minute; 2. oxygen saturation ≤ 93% on in-spired air at rest; 3. partial pressure of arterial oxygen (PaO_2)/oxygen concentration (FiO_2) ≤ 300mmHg (1mmHg = 0.133kPa), and PaO_2/FiO_2 should be calibrated according to the following formula for high-altitude areas (altitude more than 1000m) Correction: PaO_2/FiO_2 × [760/atmospheric pressure (mmHg)]; 4. Progressive exacerbation of clinical symptoms, with lung imaging showing marked progression of > 50% of the lesion within 24 to 48 hours. (4) Critical, one of the following conditions: 1. Respiratory failure, and need mechanical ventilation; 2. Shock; 3. Combined with other organ failures requires ICU supervision and treatment.

Clinical data

The clinical data of enrolled patients comprised the following domains: (1) General information: age, gender, comorbidities (e.g., hypertension), and length of hospital stay; (2) Laboratory tests: white blood cells (WBC), red blood cells (RBC), neutrophils (NEUT), lymphocytes (LYM), platelets (PLT), procalcitonin (PCT), and C-reactive protein (CRP); (3) Prognosis: categorized as Non-Survivors or Survivors based on discharge outcomes.

Sample collection and processing

BALF samples were collected from 40 hospitalized patients diagnosed with COVID-19 under standardized clinical protocols. Following local oropharyngeal anesthesia, a fiberoptic bronchoscope was transorally introduced under direct visualization. Sterile saline (room temperature) was instilled into the subsegmental bronchus of the most affected lung lobe in sequential 20-60 mL aliquots, with immediate aspiration after each infusion. From the pooled lavage fluid, a 10 mL representative aliquot was separated; 2 mL was transferred to RNA preservation solution (RNAprotect, Sigma-Aldrich) for analysis, while the residual 8 mL was aliquoted into pre-chilled, sterile, nuclease-free DNA tubes. All samples underwent immediate flash-freezing in liquid nitrogen, followed by storage at -80 °C pending downstream analysis.

Sequencing and Bioinformatics Analysis

The TNGS technology was used to identify suspected pathogens in the samples, encompassing a panel of 418 clinically prevalent pathogenic microorganisms, including bacteria, fungi, viruses, and others. This design ensures effective coverage of > 95% of pathogens associated with clinical morbidity. In addition, the assay demonstrated high diagnostic performance, with sensitivity and specificity both exceeding 96%. The α-diversity was assessed using Chao, Shannon, Simpson, and Richness indices, while β-diversity was estimated by Bray-Curtis distance and

visualized by principal coordinate analysis (PCoA). For biomarker identification, different groups of differential bacteria were identified using linear discriminant analysis (LDA) and effect size (LEfSe) analysis. Candidate biomarkers with LDA > 3.5 were selected as final biomarkers. The discriminatory ability of the biomarkers was assessed by plotting the Receiver Operating Characteristic (ROC) curve and calculating the area under the ROC curve (AUC).

Statistical analyses

Continuous variables are reported as mean ± standard deviation and statistically compared using an independent t-test. Non-normally distributed variables were expressed as IQR and compared using the Mann-Whitney U test. Statistical analyses were performed using SPSS version 26.0 (SPSS Inc., Chicago, IL), and differences were considered significant at $p < 0.05$.

Results

Baseline data of the study population

The study population consisted of 40 patients diagnosed with COVID-19. The median patient age was 66.5 years, with males representing 65% (n = 26) of this study. Hypertension was the most prevalent comorbidity, identified in 42% (n = 16) of the study population. The majority of patients (n = 37) required hospitalization, exhibiting a mean hospitalization duration of 20.3 days. The remaining three patients (7.5%) were managed as outpatients (Table 1).

General characteristics of lung microecology.

The study revealed that each patient exhibited an average of four bacterial strains, with significant variation observed in the predominant strain among different individuals. The total number of species identified was 50, comprising 28 bacteria, 10 fungi, 9 viruses, and 3 mycoplasmas. The most detected pathogens were *Veillonella parvula* (40%), *Streptococcus mitis* (30%), *Enterococcus faecalis* (22.5%), and *Pseudomonas aeruginosa* (20%) in the patients (Figure 1).

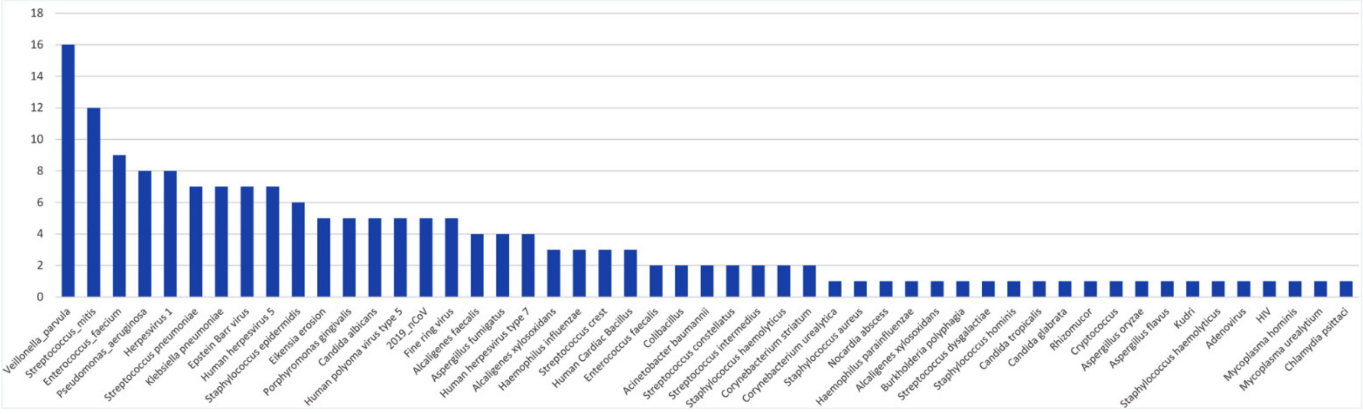
Table 1. Baseline data for 40 patients diagnosed with COVID-19.

Demographics	Total (n = 40)
Age, M (Q_1, Q_3)	66.50 (55.75, 72.75)
Genders, n (%)	
1	26 (65.00)
0	14 (35.00)
Hypertension, n (%)	16 (42.00)
Inflammatory index	-
WBC ($\times 10^9$/L), M (Q_1, Q_3)	9.30 (6.95, 11.85)
RBC ($\times 10^9$/L), M (Q_1, Q_3)	3.84 (3.40, 4.20)
NEUT ($\times 10^9$/L), M (Q_1, Q_3)	7.40 (4.70, 10.22)
LYM ($\times 10^9$/L), M (Q_1, Q_3)	1.10 (0.56, 1.44)
PLT ($\times 10^9$/L), M (Q_1, Q_3)	270.00 (149.50, 348.50)
CRP (mg/L), M (Q_1, Q_3)	64.89 (23.61, 102.17)
PCT (mg/L), M (Q_1, Q_3)	0.20 (0.06, 0.54)

COVID-19 Microbiological characteristics of the lungs of patients with different levels of severity.

The patients were stratified according to the COVID-19 Diagnostic and Treatment Programme (Trial 10th edition) as having mild (n = 3) (equivalent to mild COVID-19 recommended by WHO), moderate (n = 13), severe (n = 16) (equivalent to severe COVID-19 recommended by WHO), or critical (n = 8) (equivalent to fatal COVID-19 recommended by WHO) symptoms. A heatmap illustrates microbial abundance across conditions, with color intensity indicating relative abundance (Figure 2A). No significant differences in α-diversity were observed between

Figure 1. Microbial Distribution Identified in BALF Samples from COVID-19 Patients.



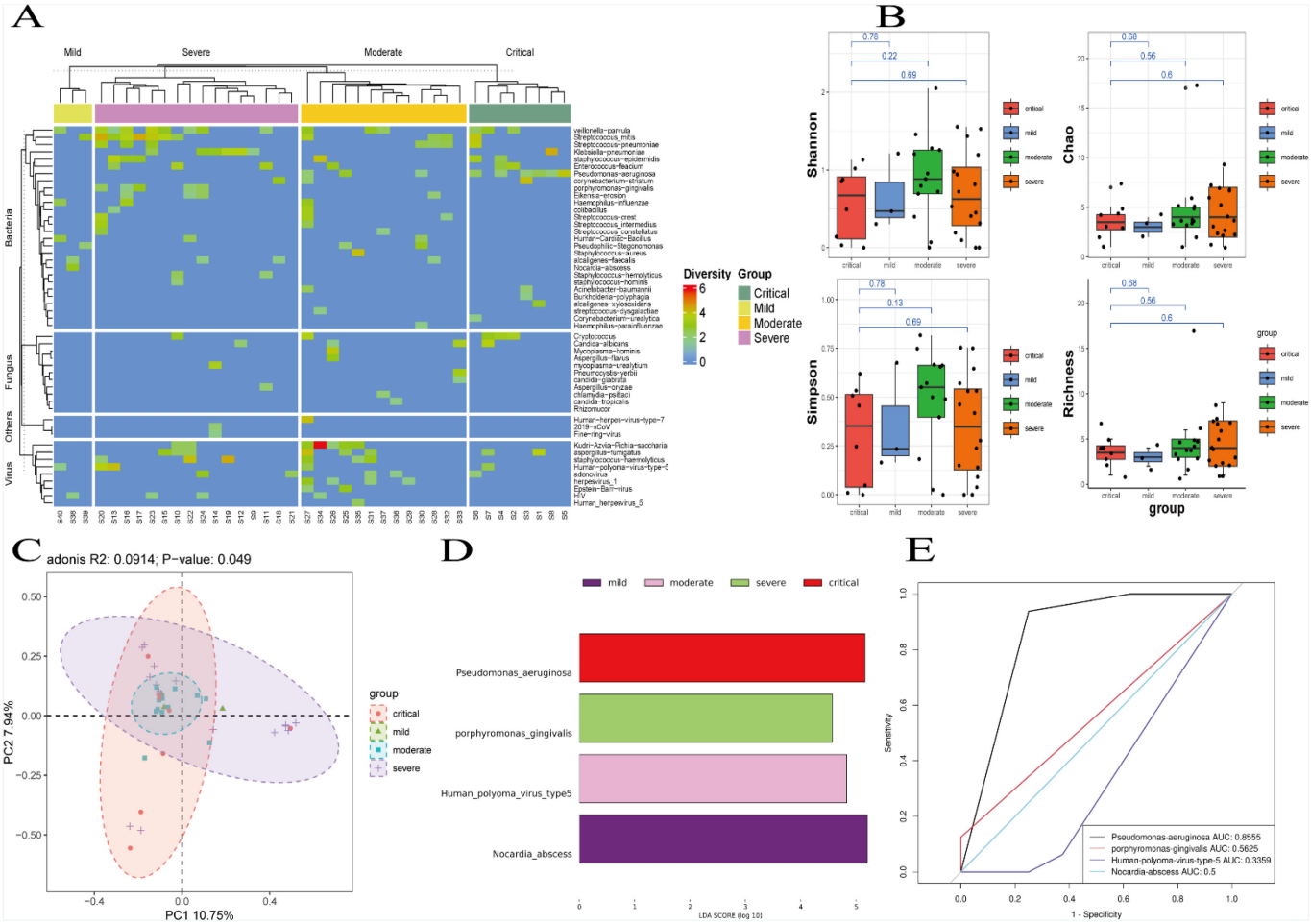
This bar chart shows the distribution and frequency of microbes detected in BALF samples collected from COVID-19 patients. Among the 50 microbial species identified, *Veillonella parvula* had the highest detection frequency (16 counts), followed by *Streptococcus mitis* (14 counts) and *Enterococcus faecalis* (10 counts). The chart highlights the microbial diversity present in the pulmonary microenvironment of COVID-19 patients.

severity groups (Figure 2B; $p > 0.05$). This null finding is likely attributable to the characteristically low baseline diversity of the pulmonary microbiome, which may obscure subtle α-diversity variations. In contrast, β-diversity reflects differences in microbial composition between groups, and such compositional variations are more apparent. β-diversity was detected between the groups ($p < 0.05$) (ANOSIM, R = 0.0914, $p < 0.05$) (Figure 2C). LEfSe analysis was used to determine and differentiate the composition of the four groups (LDA > 3.5). Critically ill patients exhibited significantly elevated relative abundances of *Pseudomonas aeruginosa* and *Candida albicans* compared to other severity groups ($p < 0.05$; Figure 2D). ROC analysis indicated that *Pseudomonas aeruginosa* abundance demonstrates good discriminatory power for critical illness (AUC = 0.856; Figure 2E), suggesting its potential utility as a predictive biomarker for severe COVID-19 outcomes.

Pulmonary microecological characteristics of COVID-19 patients with different outcomes.

The patients were stratified into two groups based on clinical outcomes: Non-Survivors (n = 5) and Survivors (n = 35). Microbial abundance differences between groups are visualized in a heatmap, wherein color intensity denotes relative abundance (Figure 3A). No significant differences in α-diversity of the respiratory microbiota were observed between the two groups ($p > 0.05$) (Figure 3B). However, β-diversity analysis revealed distinct compositional separation, as demonstrated by significant divergence in Bray-Curtis distances via PCoA (ANOSIM, R = 0.036, $p = 0.035$) (Figure 3C). LEfSe analysis was used to determine and

Figure 2. Patient Condition Severity and Microbial Diversity Heatmap and Analysis.



The patient conditions are as follows: 8 critical, 16 severe, 13 moderate, and 3 mild. Heatmap for different conditions, Different colors indicate abundance (A). The α-diversity, as measured by the Shannon, Chao, Simpson, and Richness index, showed no difference between the critical group and the other groups (B). The β-diversity was assessed using PCoA based on Bray-Curtis distances. Each point represented a sample, and there was a significant difference in β-diversity among the four groups ($p < 0.05$) (C). LEfSe was used to analyze the differences in microbial composition among the four groups (D). ROC curves were plotted for the differential bacteria. *Pseudomonas aeruginosa* (AUC:0.856) (E).

differentiate the composition of the two flora groups (LDA > 3.5). The non-survivor group exhibited significantly enriched abundances of *Pseudomonas aeruginosa*, *Candida albicans*, *Stenotrophomonas maltophilia*, and *Burkholderia multivorans* ($p < 0.05$) (Figure 3D). Additionally, ROC analysis confirmed *Pseudomonas aeruginosa* as a robust predictor of mortality (AUC = 0.846; Figure 3E).

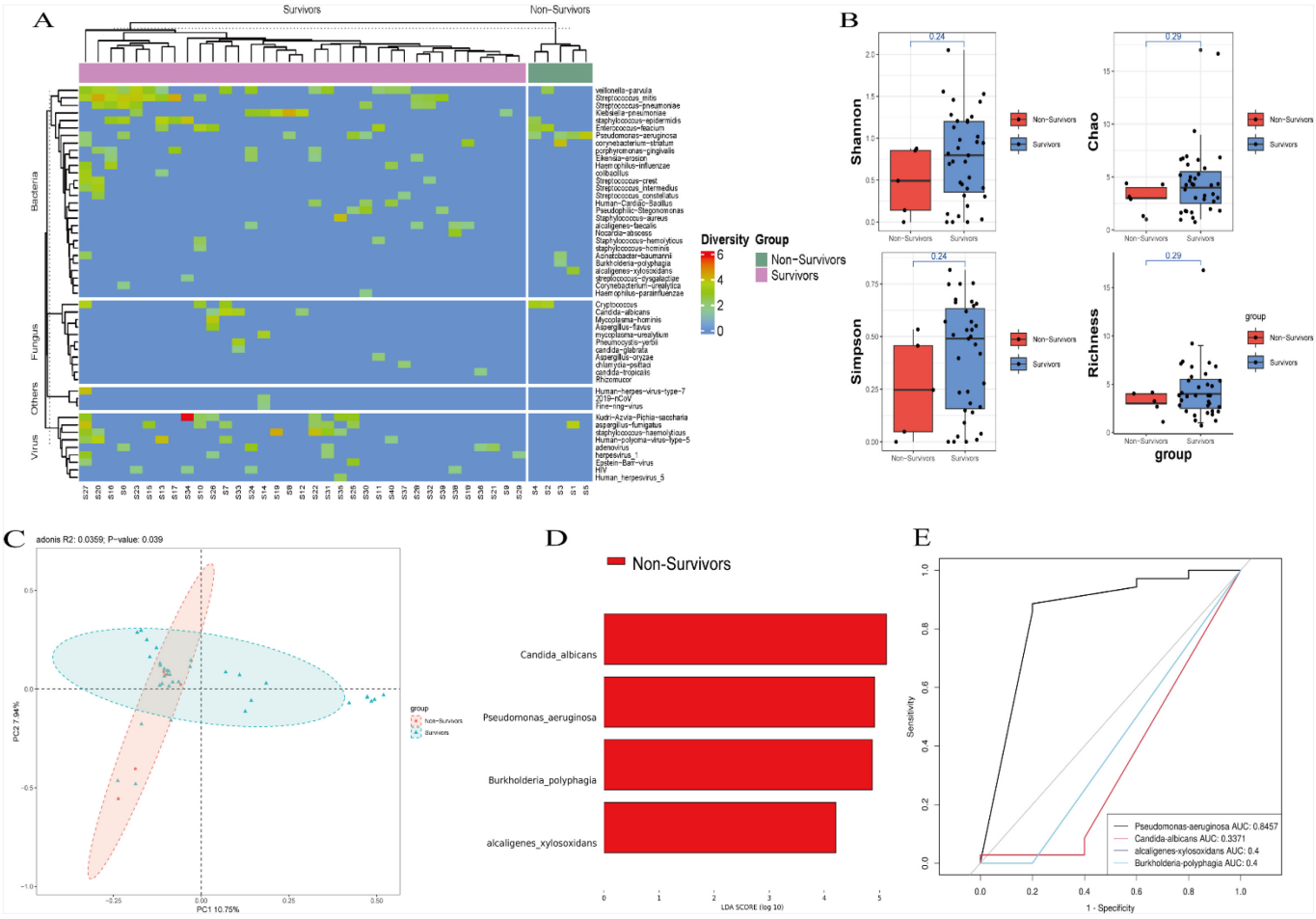
Comparative analysis of clinical indicators identified significantly reduced lymphocyte counts in non-survivors versus survivors ($p < 0.05$), aligning with established characteristics of COVID-19-associated lymphocytopenia [18]. PLT distinguishes the severity of disease in patients with COVID [19].

ROC analysis validating both LYM (AUC = 0.842) and PLT (AUC = 0.742) as individual prognostic markers (Figure 4A). Notably, a combined model integrating *Pseudomonas aeruginosa* abundance, LYM, and PLT achieved superior predictive accuracy (AUC = 0.966; Figure 4B), indicating synergistic value in stratifying COVID-19 severity.

Discussion

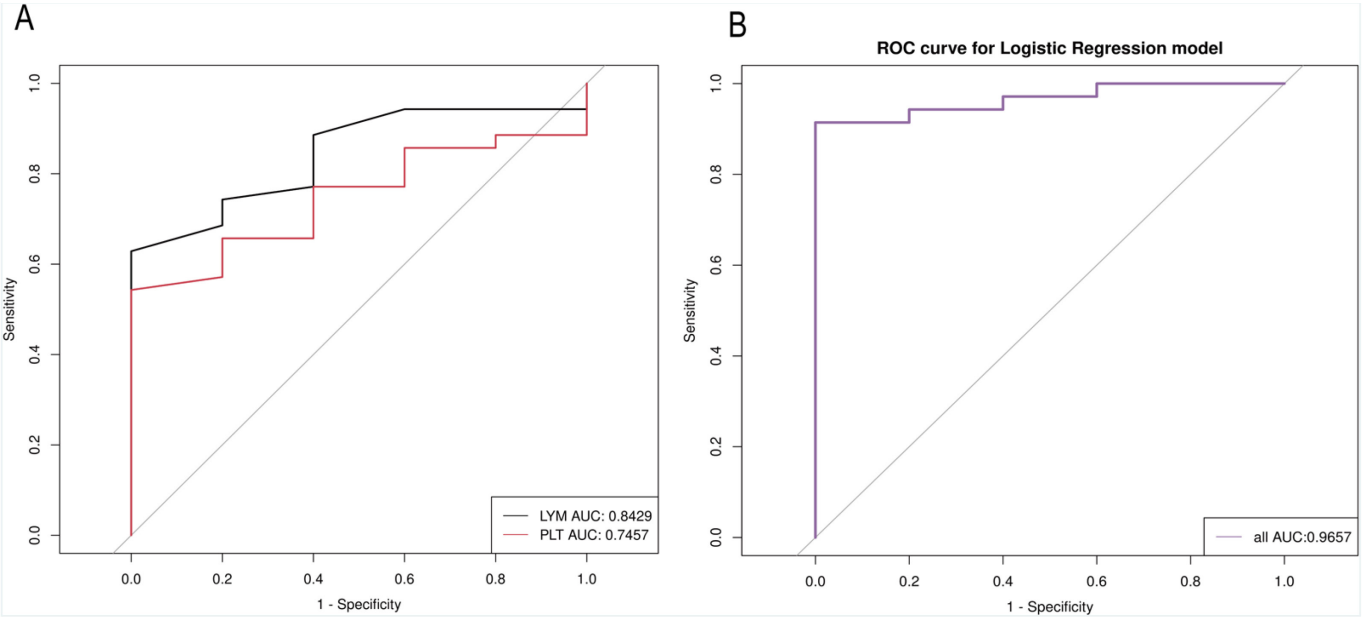
COVID-19 remains a critical global health challenge, necessitating deeper insights into the role of respiratory microbiota in disease progression. TNGS was employed to comprehensively characterize the lung microbiome using BALF samples from COVID-19 patients. Minimal differentiation in α-diversity was observed across disease severity strata or clinical outcomes. However, significant β-diversity disparities were identified, with *Pseudomonas aeruginosa*

Figure 3. Microbial Diversity and Composition Heatmap and Analysis Based on Patient Outcomes.



The patients were divided into two groups based on their outcomes: non-survivors (n = 5) and survivors (n = 35). Heatmap for different outcomes, Different colors indicate abundance (A). The α-diversity, as measured by the Shannon, Chao, Simpson, and Richness index, was not significantly different between the two groups (B). The β-diversity results were evaluated using principal coordinate analysis (PCoA) Bray-Curtis distances. Each point represented a sample, and a significant difference inβ-diversity was observed between the two groups ($p < 0.05$). LEfSe was used to analyze the difference in microbial composition between the two groups. Four bacteria were identified with an LDA threshold > 3.5: *Pseudomonas aeruginosa*, *Candida albicans*, *Alcaligenes xylosoxidans*, and *Burkholderia multivorans* (D). The ROC curve for *Pseudomonas aeruginosa* was plotted, and the area under the curve was 0.846(E).

Figure 4. ROC Analysis for Predicting COVID-19 Outcomes.



ROC curves for LYM and PLT in predicting COVID-19 outcomes. The AUC values were 0.8429 for LYM and 0.7457 for PLT, indicating their predictive performance (A). ROC curve for the logistic regression model combining *Pseudomonas aeruginosa*, LYM, and PLT. The combined model achieved an AUC of 0.9657, showing superior accuracy in predicting disease severity and outcomes (B).

demonstrating strong associations with critical disease manifestations ($p < 0.05$) and mortality. The lung microbiota exhibited characteristically reduced diversity, predisposing to ecosystem destabilization and pathogen proliferation.

BALF represents a clinically optimal specimen for analyzing immune cells, inflammatory profiles, cytological features, and microbial etiology at the alveolar level [20]. TNGS has become a staple of clinical practice due to several key advantages. Firstly, it exhibits sensitivity that is independent of the human genome and background microorganisms. Secondly, the cost of testing is lower. Thirdly, it requires a smaller sample size. Fourthly, the workflow can be standardized with ease. Finally, TNGS facilitates the simultaneous detection of DNA and RNA pathogens [21].

The respiratory flora α-diversity between groups was not found in different conditions. However, β-diversity was observed between the groups. Ling *et al.* conducted a study in which nasopharyngeal and throat swabs (NPSTSs) were collected from patients with COVID-19 for 16S rRNA gene amplicon sequencing. Their findings revealed that, after excluding samples influenced by antibiotic use, the alpha diversity of the upper respiratory microbiota was similar across different COVID-19 severity groups. However, beta diversity remained distinct, indicating differences in microbial community composition among the severity groups [22].

This study has demonstrated that a high abundance of *Pseudomonas aeruginosa* is found in both the critically ill and dead groups. These findings suggest that *Pseudomonas aeruginosa* can be used as a predictor of death, critical illness in cases of COVID-19. *Pseudomonas aeruginosa* is a common Gram-negative environmental microorganism that may be an important causative agent of serious infections in humans. This microbe is notable for its intrinsic antibiotic resistance to a wide range of antibiotics and its ability to form biofilms [23]. Infection with *Pseudomonas aeruginosa* can therefore cause serious therapeutic problems, and the pathogen is resistant to a wide range of antibiotics [24].

This finding also revealed that lymphocytes, platelets can be used as predictors of clinical outcome in COVID-19. Previous findings suggest that COVID-19 is characterized by pneumonia, lymphopenia, lymphocyte depletion, and cytokine storms [18]. Lymphopenia may be an important factor in the deterioration of COVID-19 patients [25]. Moreover, platelet count may be a simple, economical, rapid, and universally available laboratory parameter that can directly differentiate the severity of disease in patients with COVID-19 [19]. In patients diagnosed with COVID-19, the mechanism in thrombocytopenic patients may be multifactorial. Since the lungs may be the site of platelet release from fully mature

megakaryocytes, reduction or morphological changes in the pulmonary capillary bed may lead to platelet fragmentation disorders [26]. Moreover, when *Pseudomonas aeruginosa*, LYM, and PLT are combined, it has stronger accuracy and predictive value for the diagnosis of COVID-19 disease severity. In clinical practice, we can assess the severity of the disease in patients and take necessary interventions promptly.

However, there are limitations to this study. Firstly, our patient numbers were small and all recruited from the same hospital, and therefore may not be representative of the general population with COVID-19. Secondly, our study lacked a healthy control group, which is because BALF from a healthy population is not easily available. Third, although the changes observed in our study were significant and reproducible, the mechanism between COVID-19 and microecological changes in the lungs has not been determined.

The findings reveal a diminished diversity of respiratory flora in COVID-19, with no significant diversity differences among various groups but marked differences in composition. These compositional differences significantly correlate with the disease's severity and clinical outcomes. The absence of significant variation in α-diversity may be attributed to the low diversity of the pulmonary microbiome. A key discovery of this study is the predictive capability of *Pseudomonas aeruginosa* for mortality and severe illness in COVID-19 patients. In conclusion, this study has elucidated the characteristics of pulmonary microbiota across a spectrum of COVID-19 trajectories and clinical conditions, pinpointing specific bacteria correlated with severe illness and mortality, as well as pertinent clinical markers. The lung microbiota is characterized by low diversity, which leads to a limited role in disease progression. Furthermore, it renders the lung microecology more susceptible to disruption. Therefore, the presence of invasive species has the potential to modify clinical outcomes for patients. Nonetheless, the applicability of these findings across broader populations requires additional validation in more extensive patient cohorts.

Conclusions

In conclusion, this study has characterized the pulmonary microbiota across a spectrum of COVID-19 severities and clinical conditions, identifying specific bacterial taxa associated with severe illness and mortality. The lung microbiota exhibits low diversity, which may limit its regulatory role in disease progression. Moreover, this reduced diversity increases the susceptibility of the pulmonary microecology to disruption. As a result, the presence of invasive species has the potential to influence clinical outcomes. However, the generalizability of these findings to broader populations warrants further validation in larger and more diverse patient cohorts. Given these complexities, it is essential to conduct in-depth investigations into the role of the lung microbiome in various respiratory diseases. Such research is expected to provide deeper insights into the microbial contributions to lung pathophysiology.

Ethics statement

The studies involving humans were approved by the Ethics Committee of the First Hospital of Shanxi Medical University on Mar 27, 2023 (Approval Number: KYLL-2023-091).

Consent to participate

Informed consent was obtained from all individual participants included in the study.

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Author Contributions

Conceptualization: X.Y., Y.W.S.; Methodology: X.Y., Q.Y.L., Y.W.S.; Formal analysis and investigation: Q.Y.L., J.J.L., J.F.Z., T.X.; Writing - original draft preparation: Q.Y.L., J.J.L., J.F.Z., T.X.; Writing - review and editing: X.Y., Q.Y.L.; Funding acquisition: X.Y.

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Conflict of interest

No conflict of interest is declared.

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