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Research Article

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Enrichment of oral-associated microbiota in bronchoalveolar lavage fluid is associated with altered immune markers in aspiration pneumonia, with *Gemella* linked to in-hospital mortality

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ABSTRACT

Background: Aspiration pneumonia (AP) is common and life-threatening among hospitalized patients, particularly older adults. However, the lower respiratory tract microbiome in AP and its clinical relevance remain incompletely understood.

Methods: We conducted a single-center retrospective study of intensive care unit patients and profiled bronchoalveolar lavage fluid (BALF) microbiota using metagenomic next-generation sequencing (mNGS). BALF samples were obtained from 49 patients with AP and 23 patients with community-acquired pneumonia (CAP). Alpha diversity (Shannon/Simpson) and beta diversity (Bray–Curtis; PERMANOVA) were compared between groups. Differentially abundant taxa were identified and correlated with inflammatory and immune markers using Spearman correlation with false discovery rate (FDR) correction. Within AP, associations between *Gemella* detection and in-hospital mortality were assessed using logistic regression adjusted for age, APACHE II score, and CD3⁺ percentage.

Results: Alpha diversity was significantly higher in AP than in CAP (Shannon $p = 0.008$; Simpson $p = 0.0068$). In contrast, the overall community structure showed no significant difference between the groups (PERMANOVA $R^2 = 0.022$, $p = 0.066$). AP samples were enriched in oral-associated genera including *Streptococcus* and *Rothia* and depleted in *Acinetobacter* and *Stenotrophomonas*. *Streptococcus* abundance correlated positively with lactate, procalcitonin, and NK cell percentage, and negatively with CD3⁺ and CD8⁺ T-cell proportions. Within AP, overall microbiome profiles were broadly similar between survivors and non-survivors; however, non-survivors had higher *Gemella* abundance and a higher detection rate (30.8% vs 4.3%, Fisher's exact $p = 0.026$). *Gemella* detection was associated with in-hospital mortality in univariate analysis and showed a non-significant trend after multivariable adjustment (adjusted OR 6.81, 95% CI 0.67–69.58; $p = 0.106$).

Conclusions: AP was characterized by enrichment of oral-associated taxa in BALF that were associated with inflammatory and cellular immune alterations. *Gemella* abundance and detection may be linked to in-hospital mortality in AP, warranting validation in larger prospective cohorts.

Keywords: aspiration pneumonia; bronchoalveolar lavage fluid; metagenomic next-generation

sequencing; immune markers; prognosis

1. Background

Aspiration Pneumonia (AP) is primarily defined as a lower respiratory tract infection caused by the inhalation of oropharyngeal or gastric contents, with a high incidence among elderly individuals, patients with dysphagia, or those with neurological disorders[1, 2]. The occurrence rate of AP accounts for 5%-15% of all pneumonia cases in hospitalized patients, while this proportion may rise to as high as 40% in bedridden or hospitalized elderly patients[3-5]. Furthermore, AP is closely associated with elevated in-hospital mortality and an increased risk of complications[6, 7]. Notably, previous studies have found the in-hospital mortality rate for AP can reach 20–30%, substantially exceeding that of other pneumonia subtypes[8]. The high incidence and mortality rates of AP mean that it has become an important public health issue on a global scale[1, 3].

It was traditionally believed that healthy lungs were sterile; however, mounting evidence has demonstrated the presence of complex microbial communities even in healthy lungs[9]. In recent years, multiple studies have revealed that alterations in the diversity and composition of the lower respiratory tract microbiota play a significant role in the pathogenesis and progression of respiratory diseases. For instance, in patients with community-acquired pneumonia (CAP) and ventilator-associated pneumonia (VAP), a marked reduction in respiratory microbiota diversity has been observed, which is closely associated with disease severity and clinical outcomes[10, 11]. In diseases such as chronic obstructive pulmonary disease (COPD) and acute respiratory distress syndrome (ARDS), the predominant colonization of specific pathogenic bacteria and microbial dysbiosis have been demonstrated to be associated with exacerbated inflammatory responses and worsened clinical outcomes[12, 13]. These findings suggest that the lower respiratory tract microbiome may serve not only as a potential biomarker for evaluating respiratory diseases but also as a novel therapeutic target for personalized treatment and prognostic assessment.

Although previous studies have employed 16S rRNA sequencing to untangle alterations in respiratory tract microbiota and diversity among patients with aspiration pneumonia (AP)[13, 14], large-scale investigations focusing on lung microbiota particularly using bronchoalveolar lavage fluid (BALF) in AP patients remain limited. Furthermore, the correlation between microbial community patterns and clinical indicators as well as patients' clinical outcomes has not yet been fully established. There is an urgent need to further elucidate the biodiversity and compositional changes of pulmonary microbiota in AP patients to facilitate risk stratification, personalized treatment, and targeted intervention.

In recent years, metagenomic next-generation sequencing (mNGS) has gained increasing preference among researchers due to its high throughput, unbiased nature, and comprehensive detection capabilities. Compared with 16S rRNA sequencing, mNGS provides broader microbial coverage and higher taxonomic resolution while circumventing amplification biases inherent in primer-specific methods, making it a pivotal tool for investigating lower respiratory tract microbiota[15-18].

This study employed next-generation sequencing (NGS) technology to analyze the microbial diversity and community structure in bronchoalveolar lavage (BAL) fluid samples obtained from patients with aspiration pneumonia (AP). By integrating clinical data with microbiome profiling, we aimed to enhance the understanding of the pathophysiological mechanisms underlying AP. The findings are expected to further elucidate the role of microbial dysbiosis in disease initiation and progression, building upon previous research. Additionally, this investigation may provide optimized clinical management strategies for affected patients.

2. Methods

2.1. Ethics statement and subjects

The study was approved by the Ethics Committee of Jinshan Hospital of Fudan University (approval number: JIEC 2024-S99). We retrospectively enrolled 72 ICU patients admitted to Jinshan Hospital of Fudan University between 27 September 2021 and 23 August 2024, including 49 patients with AP and 23 patients with CAP.

AP is diagnosed following confirmation of inflammatory findings in the lungs and overt aspiration (apparent aspiration), a condition in which aspiration is strongly suspected, or the existence of dysphagia is confirmed [19]. Patients with CAP were enrolled according to the Diagnosis and treatment of community-acquired pneumonia guidelines of the American Thoracic Society and Infectious Diseases Society of America [20]. Given the retrospective ICU-based design, CAP was defined according to the treating physicians' diagnosis at ICU admission based on ATS/IDSA guidelines [20]. Because the timing of infection onset and prior healthcare exposure could not be fully reconstructed for all patients, some misclassification with healthcare-associated pneumonia cannot be excluded.

Exclusion criteria were: (1) age < 18 years; (2) concurrent infections in other organ systems; (3) congenital immunodeficiency or long-term use of systemic glucocorticoids or immunosuppressants; and (4) prolonged antibiotic therapy (≥ 7 days) prior to BALF sampling.

2.2. Specimen collection

BALF was obtained from 72 participants. All subjects signed informed consent forms. Prior to bronchoscopy, the subjects' medical histories were collected and routine preoperative examinations were performed. BALF samples were collected from patients according to standard procedures [21]. Following topical anesthesia of the patient's throat with lidocaine, a fiberoptic bronchoscope was introduced. Through the bronchoscope, the lungs were repeatedly lavaged with sterile saline at room temperature (20 mL per aliquot, totaling 100 mL). The recovery rate of BALF samples exceeded 40%. The lavage fluid was collected via negative-pressure suction, and a 5 mL aliquot was extracted from the recovered solution and transferred into a nuclease-free DNA sampling tube. The BALF sample was transported within 24 hours to the sequencing company (Dinfectome Inc., Nanjing, China) for mNGS examination under refrigeration (4 – 8°C).

2.3. Data collection

The demographic characteristics and laboratory test results of the subjects were collected on the day of BALF sample acquisition. The demographic information included age, sex, and underlying diseases. Laboratory data encompassed complete blood count, inflammatory markers, blood gas analysis, and lymphocyte subsets. Clinical characteristics and patient information are presented in Table 1.

2.4. Nucleic Acid Extraction

Samples of BALF were collected from patients according to standard procedures. DNA was extracted using the TIANamp Magnetic DNA Kit (Tiangen) according to the manufacturer's protocols. The quantity and quality of DNA was assessed using the Qubit (Thermo Fisher Scientific) and NanoDrop (Thermo Fisher Scientific), respectively.

2.5. Library Preparation and sequencing

DNA libraries were prepared using the Hieff NGS C130P2 OnePot II DNA Library Prep Kit for MGI (Yeast Biotechnology) according to the manufacturer's protocols. Agilent 2100 was used for quality control and DNA libraries were 50bp single-end sequenced on DIFSEQ-200.

2.6. Bioinformatics Analysis

We used an in-house bioinformatics pipeline for taxonomic profiling and pathogen identification.

Quality control was performed with fastp to remove adapter sequences, duplicate reads, and low-quality/short reads (< 36 bp). Host-derived sequences were filtered by alignment to the human reference genome (hs37d5) using Bowtie2 (v2.4.2). The remaining non-human reads were then analyzed using Kraken2 (v2.1.1) for taxonomic classification against a custom microbial genome database (downloaded from GenBank release 238), followed by abundance estimation with Bracken (v2.6.2). The database included bacterial, fungal, and viral genomes (ftp://ftp.ncbi.nlm.nih.gov/genomes/genbank/).

Alpha diversity was estimated by the Shannon index and Simpson index, based on the taxonomic profile of each sample. Beta diversity was assessed by the Bray-Curtis measure, and compared between different groups by using Wilcoxon rank sum test, and was subsequently visualized by principal coordinate analysis (PCoA) plot. PERMANOVA was performed by the R package “vegan” to analyze Bray-Curtis distance in different groups. Differences in relative abundance of bacterial genera between groups were tested using Mann–Whitney U test implemented. The species with mean relative abundances greater than 0.1% and penetrance greater than 10% among all samples were compared. Spearman’s correlations between clinical features and the relative abundances of genera were calculated by the R package “cor.test”, and FDR correction was adopted to adjust all p values.

2.7. Statistical analysis

Categorical variables were expressed as percentages (%), and statistical comparisons were performed using the chi-square test or Fisher’s exact test, as appropriate. Continuous variables were reported as mean $\pm$ standard deviation (SD) or median (interquartile range, IQR), and compared using the Mann–Whitney U test. All statistical analyses were conducted using SPSS 27.0 statistical software (SPSS Inc.). A two-sided $p < 0.05$ was considered statistically significant.

Because *Gemella* abundance contained a high proportion of zero values, *Gemella* was modeled as a binary variable (detected vs. not detected; detected defined as abundance > 0). Multivariable logistic regression was performed to evaluate the association between *Gemella* detection and in-hospital mortality among AP patients, adjusting for age, APACHE II score, and CD3⁺ percentage. In-hospital mortality was coded as 1 (Non-Survival) and 0 (Survival).

3. Results

3.1. Participants and general characteristics

This study enrolled 49 patients with AP and 23 patients with CAP. Serological analysis was performed for each patient, including complete blood count, C-reactive protein (CRP), procalcitonin (PCT), monocyte count, and lymphocyte count. Blood gas analysis parameters such as partial pressure of oxygen (PaO₂), partial pressure of carbon dioxide (PaCO₂), and lactate levels were measured using standard methods. Additionally, lymphocyte subsets were classified using flow cytometry. The clinical characteristics and Baseline demographics data are presented in Table 1.

Table 1. Basic characteristics and clinic parameters of the AP and CAP group.

Data are presented as n (%) for categorical variables and as median (IQR) or mean $\pm$ SD for continuous variables, as appropriate. P values were calculated using the chi-square test or Fisher’s exact test for categorical variables and the Mann–Whitney U test for continuous variables. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Items	AP(n=49)	CAP(n=23)	P-value
Age	77.76(9.29)	75.83(8.35)	0.54
Gender, n, (% male)	39(79.59)	17(73.91)	0.76
Comorbidities			
Hypertension, n (%)	30(61.22)	18(78.26)	0.19

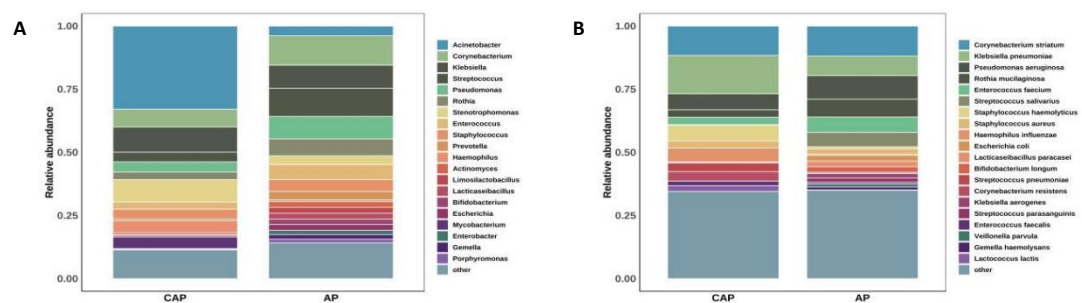
Items	AP(n=49)	CAP(n=23)	P-value
Diabetes, n (%)	11(22.45)	5(21.74)	1.00
Coronary heart disease, n (%)	9(18.37)	4(17.39)	1.00
Cerebral infarction, n(%)	16(32.65)	4(17.39)	0.26
Parkinson's disease, n(%)	4(8.16)	2(8.70)	1.00
Atrial fibrillation, n(%)	6(12.24)	3(13.04)	1.00
Chronic obstructive pulmonary disease, n(%)	6(12.24)	2(8.70)	1.00
Laboratory findings			
WBC ($\times 10^9/L$)	10.3(6.2,14.4)	8.4(6.6,13.3)	0.646
Neutrophils ($\times 10^9/L$)	8.5(5.2,13.1)	7.5(5.5,11.3)	0.583
Monocyte ($\times 10^9/L$)	0.4(0.2,0.7)	0.5(0.1,0.8)	0.957
Lymphocytes ($\times 10^9/L$)	0.6(0.4,1.0)	0.6(0.2,1.0)	0.349
CRP (mg/L)	49.8(20.9,112.2)	97.4(39.5,176.3)	0.085
PCT (mg/L)	1.1(0.5,3.8)	2.0(0.2,9.0)	0.966
pH	7.3(7.2,7.4)	7.4(7.3,7.5)	0.05
PaO ₂ (kpa)	14.0(10.1,18.9)	10.8(7.9,19.4)	0.19
PaCO ₂ (kpa)	5.8(4.8,8.2)	5.4(4.1,6.8)	0.251
Lactate (mmol/L)	2.1(1.4,3.0)	2.3(1.4,3.1)	0.668
CD3 ⁺ %	57.4(56.7,58.7)	58.7(57.9,61.7)	0.023*
CD4 ⁺ %	31.8(30.1,32.2)	21.9(5.5,26.8)	0.001**
CD8 ⁺ %	23.0(17.6,23.0)	23.1(21.7,24.1)	0.056
CD19 ⁺ %	17.9(13.7,17.9)	18.4(12.3,23.8)	0.048*
NK%	23.7(16.0,23.7)	19.4(10.6,19.4)	0.019*

Comparative analysis of baseline characteristics revealed no significant differences in age, gender, distribution of underlying diseases, complete blood count, C-reactive protein(CRP), Procalcitonin(PCT), or arterial blood gas analysis between the two groups. However, the aspiration pneumonia group exhibited significantly higher ratios of CD4 and NK cells compared to the CAP group ($P = 0.001, P = 0.019$), while the CD3 cell ratio was significantly lower in the aspiration pneumonia group ($P = 0.023$)(Table 1).

3.2. The Composition of the Lower Respiratory Tract Microbiota in AP and CAP

We first characterized the overall composition of the lower respiratory tract microbiota in both aspiration pneumonia (AP) and community-acquired pneumonia (CAP) patients. Differences emerged at the genus and species levels (Figure 1A, B).

Figure 1. Microbial composition at genus and species level in CAP and AP groups. (A) Stacked bar chart showing the top 20 genera by relative abundance in the two groups. (B) Stacked bar chart showing the top 20 species by relative abundance in the two groups.



At the genus level, the CAP group exhibited a community strongly dominated by *Acinetobacter*, which accounted for an average relative abundance of 32.88%, followed by *Klebsiella* (9.96%) and *Stenotrophomonas* (8.89%). In stark contrast, the AP group showed a more evenly distributed profile of

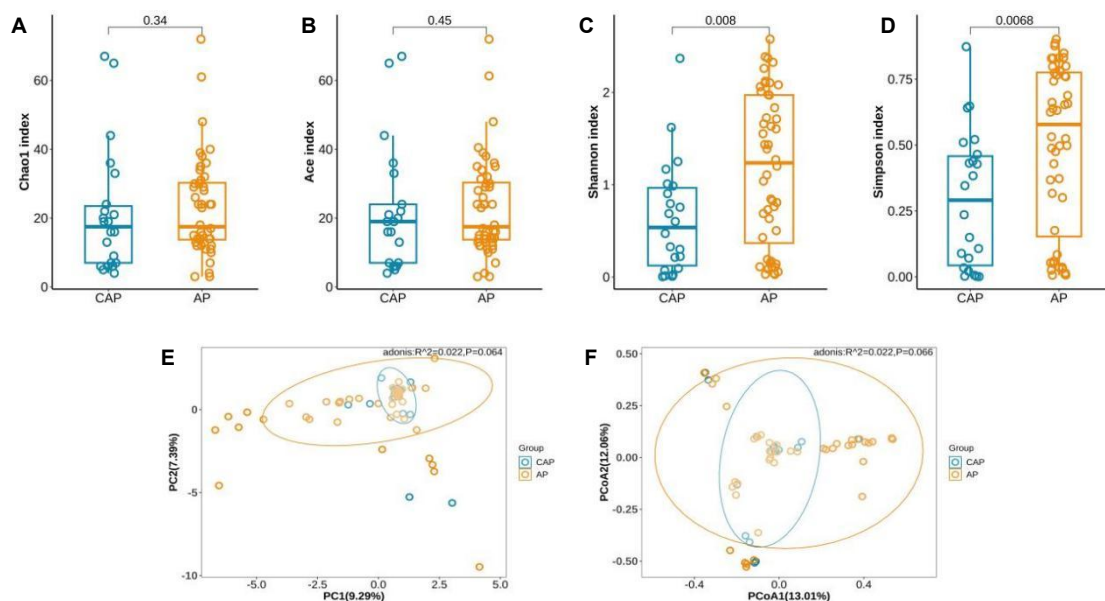
dominant genera. *Corynebacterium* was the most abundant genus in AP patients (11.65%), followed by *Klebsiella* (9.08%), *Pseudomonas* (8.90%), and the oral-associated bacterium *Rothia* (6.72%). Notably, the dominant genus in CAP, *Acinetobacter*, constituted only 3.85% of the microbiota in the AP group. These distinct patterns were also evident at the species level. In the AP group, the top five most abundant species were *Corynebacterium striatum* (11.92%), *Pseudomonas aeruginosa* (9.32%), *Klebsiella pneumoniae* (7.70%), *Rothia mucilaginosa* (7.08%), and *Enterococcus faecium* (6.12%). In the CAP group, the profile was led by *Klebsiella pneumoniae* (15.14%) and *Corynebacterium striatum* (11.67%), followed by *Pseudomonas aeruginosa* (6.31%) and *Staphylococcus haemolyticus* (6.24%). To further assess the community overlap, we performed a Venn diagram analysis. At the genus level, only 27 genera were shared between the two groups. The CAP group harbored a larger number of unique genera (90) compared to the AP group (51) (Supplementary Figure 1A). At the species level, 77 species were shared, while the AP group contained more unique species (178) than the CAP group (161) (Supplementary Figure 1B).

3.3. Microbiota differences between AP and CAP

To characterize the microbial distinctions between AP and CAP, we analyzed the diversity and composition of the lower respiratory tract microbiome in both patient cohorts.

First, we assessed the within-sample microbial richness and diversity using alpha diversity indices. Although no significant differences were observed in the Chao1 and Ace indices (Figures 2A, 2B), the Shannon index ($p = 0.008$) and Simpson index ($p = 0.0068$) revealed that the diversity of the lower respiratory tract microbial community in the AP group was significantly higher than that in the CAP group (Figures 2C, 2D). Subsequently, beta diversity was utilized to compare the overall community structures between the two groups through principal component analysis (PCA) and principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity. The PCoA plot indicated a tendency for separation between the AP and CAP microbiomes, although the difference did not reach statistical significance (PERMANOVA, $R^2 = 0.022$, $p = 0.066$). (Figure 2E, 2F). These findings suggest that despite partial overlap, distinct types of pneumonia exhibit unique microbial community structural features.

Figure 2. Diversity of lower respiratory tract microbiome among CAP and AP groups. (A) Alpha diversity analysis based on Chao1 index. (B) Alpha diversity analysis based on ACE index. (C) Alpha diversity analysis based on Shannon index. (D) Alpha diversity analysis based on Simpson index. Beta diversity of lower respiratory tract microbiome in Balf samples from AP and CAP groups. (E) Principal component analysis (PCA) based on the original data matrix. (F) Principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity.

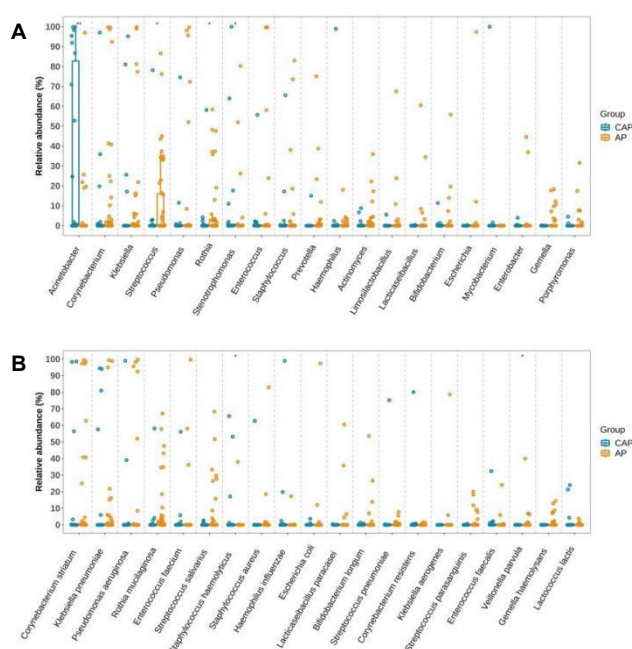


To identify the specific taxa responsible for these differences, we performed a differential abundance analysis. At the genus level, the AP group was characterized by a significantly higher relative abundance of *Streptococcus* ($p < 0.05$) and *Rothia* ($p < 0.05$), which are commonly associated with the oral cavity. Conversely, the abundances of *Acinetobacter* ($p < 0.01$) and *Stenotrophomonas* ($p < 0.05$) were significantly lower in the AP group compared to the CAP group (Figure 3A).

At the species level, the analysis revealed that *Veillonella parvula*, another oral commensal, was significantly enriched in AP patients. In contrast, the relative abundance of *Staphylococcus haemolyticus* was significantly lower in the AP group compared to the CAP group (Figure 3B).

Figure 3. Comparison of relative microbial abundance of bacteria between CAP and AP groups.

(A) Differential relative abundance of the top 20 most abundant genera. (B) Differential relative abundance of the top 20 most abundant species. Statistical significance was evaluated using the Wilcoxon rank-sum test with a p-value threshold of 0.05. *p-value < 0.05; ** p-value < 0.01.



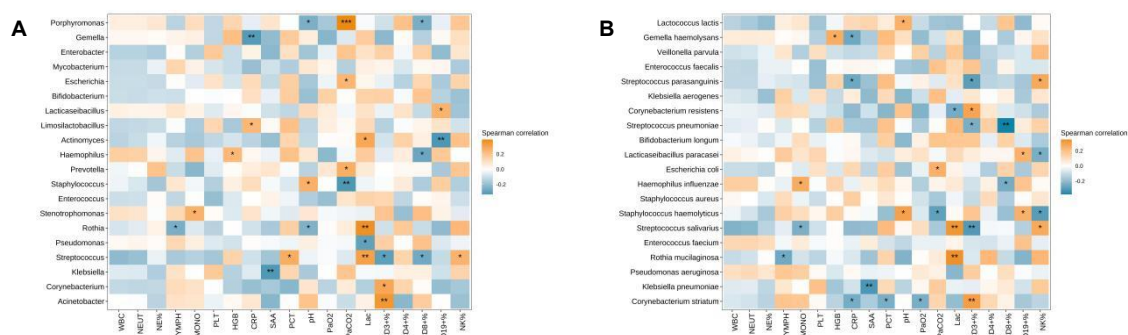
3.4. Correlation of Differential Microbiota with Clinical and Immunological Indices

To investigate the clinical relevance of the observed microbial alterations, Spearman correlation analysis was performed to evaluate the associations between the lower respiratory tract microbiota and clinical parameters in patients, with a focus on taxa showing significantly different relative abundances between the AP and CAP groups.

At the genus level, we found that *Streptococcus* and *Rothia*, which were enriched in the AP group, showed significant associations with selected inflammatory and immune markers. Specifically, the abundance of *Streptococcus* showed a positive correlation with lactate ($p < 0.01$), PCT ($p < 0.05$), and NK% ($p < 0.05$), but was negatively correlated with CD3+% and CD8+%. Similarly, the abundance of *Rothia* was positively correlated with lactate ($p < 0.01$), yet negatively correlated with LYMPH and blood pH ($p < 0.05$). In contrast, genera with reduced abundance in the AP group also demonstrated significant correlations. The abundance of *Acinetobacter* was positively correlated with CD3+% ($p < 0.01$), while *Stenotrophomonas* showed a positive correlation with MONO ($p < 0.05$) (Figure 4A).

At the species level, *Staphylococcus haemolyticus*, which was significantly depleted in AP patients, showed a positive correlation with blood pH ($p < 0.05$) and the CD19+% ($p < 0.05$). Conversely, its abundance was negatively correlated with NK% ($p < 0.05$) and blood PaCO₂ ($p < 0.05$). Interestingly, *Veillonella parvula*, despite being significantly enriched in the AP group, did not show any significant correlation with the clinical and immunological indices included in our analysis (Figure 4B).

Figure 4. Associations between clinical indicators and bacteria relative abundances in CAP and AP groups. (A) Heatmap illustrating correlation coefficients between the top 20 most abundant genera and clinical indicators. (B) Heatmap illustrating correlation coefficients between the top 20 most abundant species and clinical indicators. Statistical significance was evaluated using Spearman correlation analysis with the R package "cor.test", and p-values were adjusted using false discovery rate (FDR) correction. * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001.



3.5. Microbiome characteristics in survival vs. non-survival AP patients

To further explore the relationship between microbial features and patient outcomes, a detailed analysis of clinical parameters and microbiota was conducted between deceased and surviving patients within the AP group.

Comparative analysis of baseline characteristics revealed significant differences between the survival and deceased groups in terms of age and lymphocyte counts (Table 2). Patients in the deceased group were significantly older than those in the survival group ($p < 0.05$), suggesting that advanced age may be a risk factor for mortality in AP patients. Furthermore, compared with the survival group, the deceased group exhibited significantly lower percentages of multiple lymphocyte subsets, including CD3+% ($p < 0.05$), CD4+% ($p < 0.05$), CD8+% ($p < 0.01$), and NK% ($p < 0.01$), while the CD19+% was markedly elevated ($p < 0.0001$). These findings indicate that advanced age and reduced

lymphocyte counts may serve as predictive markers for poor prognosis in AP patients.

Table 2. Basic characteristics and clinic parameters of the Non-Survival and Survival group in AP. Data are presented as n (%) for categorical variables and as median (IQR) or mean $\pm$ SD for continuous variables, as appropriate. P values were calculated using the chi-square test or Fisher's exact test for categorical variables and the Mann–Whitney U test for continuous variables. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Items	Non-Survival(n=26)	Survival(n=23)	P-value
Age	80.65(9.04)	74.48(8.63)	0.03*
Gender, n, (% male)	21(80.77)	18(78.26)	1
Comorbidities			
Hypertension, n (%)	17(65.38)	13(56.52)	0.57
Diabetes, n (%)	4(15.38)	7(30.43)	0.30
Coronary heart disease, n (%)	6(23.08)	3(13.04)	0.47
Cerebral infarction, n(%)	8(30.77)	8(34.78)	1.00
Parkinson's disease, n(%)	3(11.54)	1(4.35)	0.61
Atrial fibrillation, n(%)	4(15.38)	2(8.70)	0.67
Chronic obstructive pulmonary disease, n(%)	1(3.85)	5(21.74)	0.09
Laboratory findings			
WBC ($\times 10^9/L$)	11.0(6.0,16.5)	8.4(6.2,11.8)	0.155
Neutrophils ($\times 10^9/L$)	10.1(4.7,14.6)	7.8(5.3,10.3)	0.176
Monocyte ($\times 10^9/L$)	0.5(0.2,0.9)	0.4(0.1,0.6)	0.078
Lymphocytes ($\times 10^9/L$)	0.7(0.4,1.1)	0.4(0.3,0.7)	0.118
CRP (mg/L)	43.6(17.3,86.1)	53.3(38.1,130.4)	0.452
PCT (mg/L)	2.2(0.5,6.3)	1.0(0.5,2.5)	0.249
pH	7.3(7.2,7.4)	7.3(7.3,7.4)	0.609
PaO ₂ (kpa)	10.9(9.2,17.6)	15.8(12.0,19.5)	0.029*
PaCO ₂ (kpa)	5.8(4.8,8.1)	5.5(4.8,8.3)	0.928
Lactate (mmol/L)	2.4(1.5,3.5)	1.8(1.3,2.9)	0.189
CD3 ⁺ %	56.4(53.8,61.3)	59.0(54.2,63.0)	0.044*
CD4 ⁺ %	31.0(28.8,33.2)	33.1(31.1,37.1)	0.027*
CD8 ⁺ %	22.4(13.3,22.4)	24.0(23.4,27.3)	0.002**
CD19 ⁺ %	21.8(16.6,22.2)	11.7(10.7,14.4)	0.000**
NK%	20.7(14.5,21.0)	28.4(20.6,30.2)	0.003**

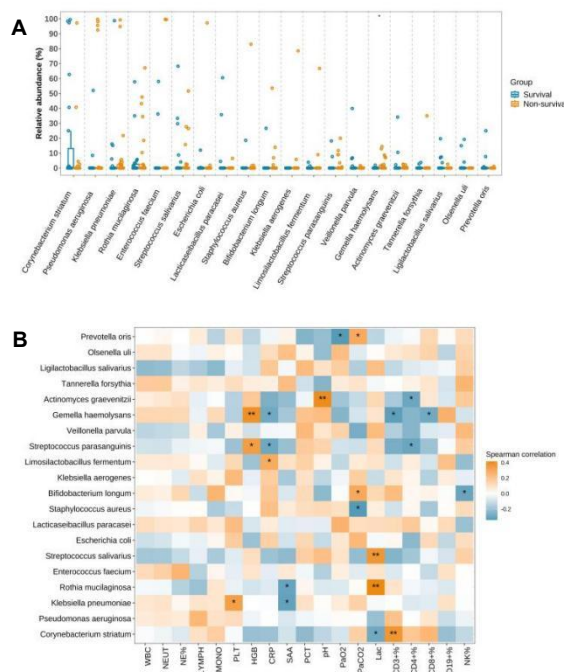
Although no significant differences were observed in alpha or beta diversity between non-survivors and survivors (Supplementary Figure 2), notable differences were observed for *Gemella*. In BALF samples from non-survivors, *Gemella* showed a higher relative abundance than in survivors ($p < 0.05$; Figure 5A). Because *Gemella* abundance was zero-inflated, *Gemella* was analyzed both as relative abundance and as detection status (detected vs not detected; detected defined as relative abundance > 0). Within the AP cohort ($n = 49$), *Gemella* was detected in 8 of 26 non-survivors (30.8%) and in 1 of 23 survivors (4.3%); the detection rate was significantly higher in non-survivors (Fisher's exact test, $p = 0.026$; Supplementary Table S1). Spearman correlation analysis further suggested that *Gemella* abundance was negatively correlated with CD3⁺% and positively correlated with CD19⁺% ($p < 0.05$; Figure 5B).

To evaluate whether *Gemella* detection was independently associated with in-hospital mortality, multivariable logistic regression was performed including age, APACHE II score, CD3+%, and *Gemella* detection status (death = 1). Older age was independently associated with higher odds of in-hospital mortality (adjusted OR 1.087 per year, 95% CI 1.003–1.179; $p = 0.042$). *Gemella* detection showed a positive but non-significant association with mortality after adjustment (adjusted OR 6.809, 95% CI 0.666–69.584; $p = 0.106$), with the wide confidence interval likely reflecting the limited number of *Gemella*-positive cases. APACHE II score and CD3+% were not statistically significant in this model ($p = 0.098$ and $p = 0.788$, respectively) (Table 3).

Table 3. Multivariable logistic regression for in-hospital mortality among patients with AP (n = 49). Outcome was in-hospital mortality (coded as 1 = death and 0 = survival). *Gemella* detection was defined as abundance > 0 (coded as 1 = detected and 0 = not detected). The model included age, APACHE II score, CD3+ percentage, and *Gemella* detection status.

Predictor	Adjusted OR (95% CI)	P value
Age (per 1-year increase)	1.087 (1.003–1.179)	0.042*
CD3+% (per 1% increase)	0.993 (0.943–1.046)	0.788
APACHE II score (per 1-point increase)	1.091 (0.984–1.210)	0.098
<i>Gemella</i> detected (yes vs no)	6.809 (0.666–69.584)	0.106

Figure 5. Comparison of microbial relative abundance in subgroups and correlation with clinical indicators. (A) Differential relative abundance of top 20 most abundant species between Survival and Non-survival groups. (B) Heatmap of Spearman correlation between top 20 most abundant species and clinical indicators. * p -value < 0.05; ** p -value < 0.01.



4. Discussion

In this study, mNGS was employed to systematically analyze the composition and diversity of the lower respiratory tract microbiome in BALF samples from patients with AP and CAP. Furthermore, the associations between differentially abundant microbiota and patients' immune phenotypes as well as clinical outcomes were investigated.

We first compared the diversity and composition of the lower respiratory tract microbiota between the two patient groups. It was found that patients with AP exhibited significantly higher alpha diversity (Shannon index and Simpson index) compared to those with CAP. This observation appears to contradict the prevailing view in previous studies that respiratory microbial diversity is generally reduced in AP[22, 23]. The discrepancy observed in the present study may be attributed to the following reasons: First, AP is often caused by a single, substantial introduction of oropharyngeal or gastroesophageal reflux contents, which inherently carry a large number of anaerobic commensal bacteria[24]. This explanation is supported by the significant enrichment of oral-origin microbiota, such as *Streptococcus* and *Rothia*, detected in the AP group in this study. In contrast, CAP is typically acquired via microaspiration or aerosol transmission, with a pathogen spectrum concentrated in a limited number of facultative anaerobes such as *Streptococcus pneumoniae* and *Haemophilus influenzae*, resulting in inherently lower initial diversity[25]. Secondly, differences in the timing of antibiotic exposure may have amplified the observed disparities. Patients with CAP typically seek medical attention early, and all CAP patients included in this study were admitted to the ICU and had already been subjected to broad-spectrum antibiotic therapy prior to sample collection. In contrast, AP patients often experience recurrent microaspiration during hospitalization, leading to disease progression and a relatively delayed initiation of antibiotic treatment[26]. Currently, there is no unified clinical diagnostic criterion for aspiration pneumonia[27], and variations in sampling methods and detection techniques across previous studies may also influence the results of the Shannon and Simpson indices[28].

Although no significant separation in beta diversity was observed between the AP group and the CAP group ($p > 0.05$) in this study, suggesting that the overall microbial structure in both groups still belongs to a "pneumonia-type" community. However, the genus and species levels differences identified by the Kruskal–Wallis test were sufficient to serve as microbial markers for clinical phenotype differentiation.

First, *Streptococcus* and *Rothia*, which were significantly enriched in the lower respiratory tract of AP patients, are both core commensals of the oropharyngeal microbiota. This finding strongly aligns with the pathological model of “overt aspiration,” wherein oropharyngeal microorganisms are directly disseminated into the lower airways. In a study by Akata et al. involving 16S rRNA sequencing of BALF from 77 pneumonia patients, the detection rate of oral streptococci in BALF was significantly higher in patients with high aspiration risk than in those without aspiration risk (31.0% vs. 14.7%, $p = 0.009$) [29]. And another study analyzing lower respiratory tract specimens from 17 patients with aspiration pneumonia also identified a high relative abundance of *Streptococcus* and *Rothia* genera by both culture and 16S rRNA sequencing[30], supporting the pathway linking “oropharyngeal-aspiration” to enrichment of *Streptococcus* and *Rothia*. Furthermore, compared with the AP group, the CAP group exhibited significant enrichment of hospital-acquired multidrug-resistant bacteria such as *Acinetobacter* and *Stenotrophomonas*, which commonly colonize the lungs of patients subjected to broad-spectrum antibiotic therapy[31]. Their relative enrichment in the CAP group is consistent with the timeline of community-acquired infection onset and the clinical characteristics of early antibiotic exposure prior to ICU admission. These results reproduced genus-level differences in an independent cohort, further confirming the stability of these two genera as microbial biomarkers for AP. Notably, the CAP cohort in this study consisted of critically ill ICU patients, in whom prior antibiotic exposure and the ICU microbial ecology may shift the respiratory microbiome toward healthcare-associated taxa.

Spearman’s correlation analysis was further performed to explore specific relationships between

dominant bacterial microbiome in different groups and the immune microenvironment of the lower respiratory tract. This study focused on the correlation between differentially abundant bacteria and significantly altered immune parameters. The results revealed that the relative abundance of *Streptococcus* was negatively correlated with CD3⁺% in peripheral blood ($p < 0.05$) and significantly positively correlated with NK% ($p < 0.05$). Consistent with these findings, enrichment of *Streptococcus* in the AP group (Figure 2A), along with a significant decrease in CD3⁺% and a marked increase in NK% (Table 1), was observed. This phenomenon may be attributed to the potential rapid proliferation of orally derived commensal bacteria in the lower respiratory tract following substantial aspiration. Previous studies have shown that bacterial cell wall components such as lipoteichoic acid (LTA) and peptidoglycan can activate TLR2/4, inducing substantial release of IL-6 and TNF- α , which promotes sustained T cell activation and eventual exhaustion, while NK cells may be compensatorily activated to maintain early antibacterial defense[32-34]. In the CAP group, the relative abundance of the enriched *Acinetobacter* genus positively correlated with CD3⁺% levels($p < 0.01$) but showed no significant correlation with NK% levels (Figure 2A). Previous clinical data indicated that patients in the CAP group exhibited higher CD3⁺% levels and lower NK% levels($p < 0.05$)(Table 1). This phenomenon may be attributed to the fact that *Acinetobacter* infection in the lower respiratory tract stimulates the production of interleukins, thereby promoting the generation and migration of naïve T cells[35, 36]. Additionally, *Acinetobacter* is obligately aerobic microorganisms, and their infection sites are characterized by a hyperoxic, low-interferon microenvironment, which may lack a virus-like IFN- α storm[37, 38]. This could explain the relatively small variation in NK% levels observed in CAP patients with enriched *Acinetobacter*. At the species level, correlation analysis revealed that the normal oral bacterium *Veillonella parvula*, which was enriched in the AP group did not correlate well with the included immunological indicators. In contrast, *Staphylococcus haemolyticus*, which was enriched in the CAP group showed a significant negative correlation with NK%. *Staphylococcus haemolyticus* has traditionally been considered a colonizing bacterium of the respiratory tract mucosa. However, recent studies have revealed an increasing incidence of *Staphylococcus haemolyticus* as a causative pathogen of CAP [39]. Unlike other coagulase-negative staphylococci, *Staphylococcus haemolyticus* has been shown to evade host immune clearance (such as NK cells) through biofilm formation. Additionally, by altering surface protein expression, it reduces the likelihood of phagocytosis by neutrophils and macrophages[40].

To further investigate the correlation between the lower respiratory tract microbiota and prognosis in patients with AP, we specifically compared clinical indicators and lower respiratory tract microbiota changes between the non-survival group and survival group of AP patients. The study revealed no significant differences in community diversity (alpha diversity and beta diversity) between the non-survival and survival subgroups of AP patients, a finding that diverges from previous studies. A 2024 cohort study on pediatric acute lower respiratory tract infections reported no significant difference in alpha diversity ($p > 0.05$) but significant separation in beta diversity between survival and non-survival groups[41]. In contrast, another cohort study involving adults with severe pneumonia observed higher beta diversity in non-survivors[42]. These discrepancies may be attributed to differences in the diseases studied and cohort sizes. The lack of significant differences in microbial diversity between the non-survival and survival groups in the present study may be due to the early timing of sample collection, during which changes in microbial diversity might not yet have become apparent.

The non-survival group exhibited a significantly lower T-cell immune level, while the proportion of

CD19⁺ B cells involved in humoral immunity was markedly elevated ($p < 0.001$), suggesting that differences in immune response pathways may lead to divergent clinical outcomes in AP patients. Additionally, the relative abundance of the genus *Gemella* was found to be significantly higher in the non-survival group compared to the survival group. In the prognostic analysis of AP, *Gemella* was more frequently detected in non-survivors than in survivors (30.8% vs. 4.3%, Fisher's exact $p = 0.026$), suggesting a potential link between *Gemella* presence in the lower respiratory tract and poor outcome. However, after adjustment for age, APACHE II score, and CD3⁺ percentage, *Gemella* detection showed a positive but non-significant association with in-hospital mortality (adjusted OR 6.809, 95% CI 0.666–69.584; $p = 0.106$). Spearman correlation analysis further confirmed its association with immune markers, showing a significant negative correlation with CD3⁺% and a significant positive correlation with CD19⁺% ($p < 0.05$) (Figure 5B). *Gemella* is a genus of Gram-positive facultative anaerobic cocci commonly found in the normal microbiome of the human oral cavity, upper respiratory tract, and gastrointestinal tract[43]. The higher abundance of *Gemella* in the lower respiratory tract of deceased patients may be proportional to the severity or frequency of aspiration episodes. Although studies on *Gemella* in aspiration pneumonia are limited, recent research on the respiratory microbiota of hospitalized COVID-19 patients has identified *Gemella* as a potential biomarker for distinguishing disease severity[44]. Taken together, these findings support *Gemella* as a candidate microbial marker associated with adverse outcomes in AP, but its prognostic utility and biological role require confirmation in larger, prospectively collected cohorts.

This study has several limitations. First, as a single-center retrospective analysis, residual confounding such as antibiotic exposure, mechanical ventilation, and sampling timing cannot be fully excluded. Second, BALF is a low-biomass specimen and upper-airway carryover or reagent contamination may influence the detection of oral-associated taxa; future studies incorporating negative controls and standardized decontamination procedures are warranted. Third, the sample size limited the ability to build fully adjusted prognostic models and external validation is needed.

5. Conclusion

Our study suggests that, although AP and CAP shared broadly similar pneumonia-associated microbiome profiles, AP was characterized by enrichment of oral-associated taxa in BALF, which were associated with inflammatory and cellular immune markers. In addition, higher *Gemella* abundance was observed in non-survivors with AP, supporting its potential as a candidate prognostic marker that requires prospective validation.

List of abbreviations

AP = Aspiration Pneumonia

ARDS = Acute Respiratory Distress Syndrome

CAP = Community-Acquired Pneumonia

CD3⁺% = CD3 T-Lymphocyte Percentage

CD4⁺% = CD3CD4 T-Helper Cell Percentage

CD8⁺% = CD3CD8 Cytotoxic T Cell Percentage

CD19⁺% = CD19 B-Lymphocyte Percentage

COPD = Chronic Obstructive Pulmonary Disease

CRP = C-Reactive Protein

ICU = Intensive Care Unit

mNGS = Metagenomic Next-Generation Sequencing

NK% = Natural Killer Cell Percentage

PaCO₂ = Partial Pressure of Carbon Dioxide

PaO₂ = Partial Pressure of Oxygen

pH = Potential of Hydrogen

PCT = Procalcitonin

VAP = Ventilator-Associated Pneumonia

WBC = White Blood Cell Count

Declarations

Ethics approval and consent to participate

The study was approved by the respective ethics committees of the institutional review board of Center of Emergency and Critical Medicine, Jinshan Hospital of Fudan University (approval number: JIEC 2024-S99). The study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

Consent for publication

Not applicable

Availability of data and materials

The datasets presented in this study can be found in online repositories. The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2025) in National Genomics Data Center (Nucleic Acids Res 2025), China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA033525). The dataset is publicly available at: <https://ngdc.cncb.ac.cn/gsa/s/J0dy437q>

Competing interests

The authors declare that they have no competing interests. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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Authors' contributions

RW and YS contributed to study design, data analysis, and manuscript drafting. LZ and WM contributed to clinical data collection and interpretation. CZ contributed to sequencing and bioinformatics support. FZ and JS supervised the study and revised the manuscript. All authors read and approved the final manuscript.

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