의학박사 학위논문

농흉 환자의 흉강 및 구강 내 미생물 군집 분석 및 메타유전체 연구

A metagenomic analysis of pleural fluid microbiome in patients with pleural empyema, and its association with oral cavity microbiome

울 산 대 학 교 대 학 원

의 학 과

오 지 미

농흉 환자의 흉강 및 구강 내 미생물 군집 분석 및 메타유전체 연구

지 도 교 수　　나 승 원

이 논문을 의학박사 학위 논문으로 제출함

2024 년 02 월

울 산 대 학 교　대 학 원

의　　학　　과

오 지 미

오지미의 의학박사학위 논문을 인준함

심사위원 권 중 근 (인)

심사위원 김 철 민 (인)

심사위원 나 승 원 (인)

심사위원 이 세 원 (인)

심사위원 제 갈 양 진 (인)

울 산 대 학 교 대 학 원

2024 년 02 월

감사의 글

많이 부족한 저이지만 여러 분들의 따뜻한 지지와 격려 속에서 박사 과정을 무사히 마무리할 수 있었습니다. 함께 해 주신 모든 분들에게 깊은 감사의 말씀을 드리고자 합니다.

먼저 이 연구의 방향을 제시해 주시고, 큰 가르침을 주신 나승원 교수님께 진심으로 감사 드립니다. 교수님의 직관과 지혜로움을 가까이에서 접하고 배울 수 있었던 박사 과정은 저의 앞날에 무엇보다 든든한 디딤돌이 될 것입니다. 다음으로 실험 및 분석 과정에 많은 도움을 주신 설옥주 교수님, 채정훈 박사님, 최혜원 선생님께 감사드립니다. 또한 바쁘신 와중에도 귀중한 시간을 할애하시어 학위논문 작성을 지도해 주신 김철민 교수님, 권중근 교수님, 이세원 교수님, 그리고 제갈양진 교수님께 감사 드립니다. 크고 작은 부분에 대하여 고견을 나누어 주신 여러 교수님들 덕분에 이 논문을 완성할 수 있었습니다. 이 자리를 빌어, 저를 참된 학문의 길로 이끌어 주신 교수님들께 깊은 존경과 감사의 인사를 올리고자 합니다. 훌륭하신 교수님들을 본받아 항상 최선을 다하는 모습을 보여드리도록 노력하겠습니다.

더불어 무사히 대학원 박사 과정에 진학하고 수료까지 할 수 있도록 도와주신 울산대학교병원 마취통증의학과 교수님들께도 감사의 인사를 드립니다.

언제나 내 곁에 머물며 믿음과 격려를 주시는 큰 영웅, 박철 에게 깊은 감사의 말씀과 사랑을 전합니다. 그리고 논문 작성에 대한 응원과 힘을 실어 주신 작은 영웅, 박주원 에게도 사랑을 듬뿍 담아 감사의 마음을 전하고자 합니다. 또한, 부족한 저에게 지원과 응원을 아끼지 않으시며 무한한 사랑을 보내주신 시부모님께도 감사의 인사를 드립니다. 가족 모두가 항상 건강하길 기원하며, 마지막으로 저를 세상에 있게 하시고, 끝없는 애정과 믿음으로 키워주신 부모님께 이 논문을 바칩니다.

Abstract

Background: Several studies have suggested that the oral microbiome is associated with various diseases. Although epidemiological studies on the oral microbiome in infectious diseases are increasing, no systemic studies exist on the oral microbiome of patients with pleural empyema. In this study, we present the microbiome characteristics of patients with pleural empyema and controls based on 16S ribosomal RNA gene sequencing.

Methods: Twenty individuals–10 with pleural empyema and 10 in the control group–were recruited for this study. Specimens of the oral cavity (saliva, gingival tissue, tongue, and buccal mucosa) and pleural fluid were collected from all participants. Oral and pleural microbiomes were analyzed using the Illumina MiSeq system, and the obtained sequence data were further analyzed using bioinformatics methods. The medical records of the enrolled patients were retrospectively reviewed.

Results: No significant differences were observed in alpha and beta diversities among the oral sample sites. We found a high abundance of *Acinetobacter, Staphylococcus*, and *Enterococcus* in the oral mucosa and saliva of patients with empyema. Our results showed significantly low alpha diversity and microbial dysbiosis in patients with empyema (Shannon index, 1.8 ± 1.1 vs. 2.8 ± 0.6, $p < 0.001$). Likewise, beta-diversity analysis revealed significant differences in the composition of microbiomes between the empyema and control groups ($p < 0.001$). In addition, the alpha diversity of the pleural microbiome of patients with empyema was lower than that of controls in terms of both diversity (Shannon index, 1.2 ± 1.1 vs. 3.6 ± 0.4, $p < 0.001$) and richness (Chao1 index, 49.2 ± 22.2 vs. 199.7 ± 98.4, *p*

= 0.001). Principal coordinate analysis indicated a significant difference in the pleural microbiome between empyema and control groups. Linear discriminant analysis of the functional potential of the oral microbiome demonstrated that two-component systems, amino acid metabolism, xenobiotic biodegradation, and metabolic pathways were overrepresented in patients with empyema relative to those in controls.

Conclusion: This is the first study to explore the relationship between the oral microbiome and pleural empyema. This demonstrates that the oral microbiome harbors unique bacterial communities and identifies potential taxonomic and functional biomarkers in patients with empyema. Further investigation of the potential mechanism of population dynamics in the oral microbiome during the progression of pleural empyema is required.

Keywords: Bacteria, Empyema, Pleural infection, Microbiota, Oral Health.

Contents

Tables

Figures

Introduction

Pleural infections are not recent medical issues. The Egyptian physician Imhotep (3000 BC) was first described. Hippocrates (460–377 BC) documented pleurisy and its potential progression to empyema when left untreated, and he was among the first to introduce a method for draining the intercostal space (1). Although many centuries have passed since, pleural infections persist as a common issue worldwide. The incidence of pleural infections continues to increase with unknown causes (2). Despite advancements in diagnosis and paradigm shifts in treatment, pleural infections are still associated with substantial morbidity and mortality, ranging from 15 to 20% (3). In older individuals with comorbidities, the mortality rate has increased to 30%.

Among patients with community-acquired pneumonia, 15–44% develop parapneumonic effusion and 6–10% develop pleural infections (4, 5). However, if appropriate antimicrobial therapy is initiated early, the fluid usually resolves. Pleural infections typically manifest as a progressive process that transforms from a freely flowing “simple” parapneumonic pleural effusion into a septated “complicated” parapneumonic effusion (typically caused by bacterial invasion of the pleura) or “empyema” (involving the presence of pleural pus). Pleural infections are characterized by bacterial or purulent effusions in the pleural cavity. Generally, the progression of pleural infection is categorized into three phases: i) simple exudate, ii) fibrinopurulent, and iii) organizing with a thick pleural rind. In the initial exudative phase, fluid enters the pleural space because of increased capillary vascular permeability (6). This is accompanied by the release of proinflammatory cytokines, including tumor necrosis factor-alpha (TNF-α) and interleukin 8 (IL-8). These cytokines induce changes in the pleural mesothelial cells, promoting fluid influx into the pleural cavity. These cytokines induce changes in the pleural mesothelial cells, promoting fluid influx into the pleural cavity. If left untreated, a simple parapneumonic effusion can advance to the fibrinopurulent stage as fluid

accumulates and bacteria invade the compromised endothelium. Bacterial invasion accelerates the immune response, leading to enhanced neutrophil migration and the activation of the coagulation cascade. This increases pro-coagulant activity and reduces fibrinolytic activity (7, 8). Neutrophil phagocytosis and bacterial death further intensify inflammation by releasing bacterial cell-wall fragments and proteases. These events collectively result in increased lactic acid and carbon dioxide production, causing a decrease in pleural fluid pH (9). Subsequently, an "organizing stage" ensues, which is marked by the growth of fibroblasts in the pleura, forming a thick pleural rind that prevents lung re-expansion. Progression through these stages of pleural infection can be influenced by various factors, such as triggering bacteria, host immunity, and comorbidities.

However, the pathogenesis of pleural infection is not fully understood. Various disease-causing factors have been suggested; however, these remain controversial. Recent research has suggested that pleural infections may not necessarily be related to lung parenchymal infections (10-12). The pleural cavity and lungs are significantly different in pH, oxygen content, and other parameters; thus, the bacterial spectrum of pleural infection is not the same as that of pneumonia. Previous studies have reported that the most common pathogens in community-acquired pleural infections are *Streptococcus*, followed by anaerobes and *Staphylococcus* (13). In patients with pneumonia, pathogens can surpass pulmonary defense mechanisms and spread directly from the respiratory alveoli to the pleural cavity. *Streptococcus pneumoniae* is known for its proficiency in this process (14). The representative anaerobic bacteria in pleural infections include *Streptococcus intermedius* and *Fusobacterium nucleatum*. We found no evidence of similar ability. Interestingly, these facultative and anaerobic bacteria have been recognized as part of the oral bacterial community. This suggests that aspiration of these bacteria or their dissemination through deoxygenated venous blood may contribute to the development of pleural infection (15). In a previous study, lung abscesses and empyema were successfully induced by inoculating the trachea of animals with human periodontal material

(16). Conditions, such as impaired mucociliary clearance and reduced oxygen levels, in consolidated or atelectatic lungs provide an environment conducive to the proliferation of oropharyngeal anaerobic bacteria in the lungs, potentially leading to their spread to the pleural space. Therefore, oral bacteria can affect the composition of pleural infections. Previous studies on pleural empyema have identified poor oral hygiene and periodontal disease as major risk factors (17). Poor oral hygiene is a major factor leading to an imbalance (dysbiosis) in the complex microbial communities of the oral cavity (18). The frequent association between oropharyngeal bacteria and poor oral hygiene in pleural infections contributes to the evidence that microaspiration is a critical cause of the development of pleural infections. Recent studies have indicated that the oral microbiome contributes to health and disease. In healthy individuals, the oral microbiome is vital for preserving mucosal barrier function and coordinating innate and adaptive immune responses, while actively inhibiting the establishment of oral pathogens (19). During illness, violation of the mucosal barrier allows commensal bacteria to act as persistent inflammatory triggers in the adjacent tissues (20). Therefore, defining microbial clusters in the oral cavity of patients with pleural infections is crucial for understanding the onset and progression of pleural infections.

The oral microbiome is the second largest microbial environment in the body. The oral microbiome is colonized within various microbial niches, including the gingiva, tongue, buccal mucosa, hard and soft palate, saliva, teeth, and subgingival and supragingival plaques, with bacterial communities differing across these sites. Within the oral microbiome, these subsites exhibit distinct compositions and function as unique microenvironments in which the microbiome can thrive and perform specialized functions (21). A recent study demonstrated that the gingiva and buccal mucosa share a similar microbiome, while the saliva, tongue, and supra- and subgingival plaques exhibit distinctive microbial communities (22). The finely tuned equilibrium of the oral microbiome ecosystem plays a critical role in the maintenance of oral health. Therefore, investigations on oral microbiomes are ongoing for an extended

period of time. Early microbiological studies have reported an association among anaerobic oral bacteria, such as *Peptostreptococcus, Prevotella, Bacteroides*, and *Fusobacterium* (23, 24). However, a significant portion of the microbiome identified within the human oral cavity is yet to be successfully cultured. This traditional culture method may also give rise to underestimate the "true" biodiversity of the oral microbiome. To date, high-throughput sequencing of 16S ribosomal RNA (rRNA) has provided a comprehensive understanding of microbial community composition. Microorganisms can be identified and classified using 16S rRNA gene variable and conserved regions. These sequences are predominantly used for microbial diversity analysis, and advances in high-throughput sequencing technologies have expedited their application. Thus, the concept of a sterile lower respiratory tract in healthy individuals has been revised, and genetic material from anaerobic oral bacteria, including *Prevotella* and *Veillonella*, has been identified in the lungs of healthy individuals. The correlation between oral microbiome dysbiosis and the progression of several respiratory pathologies, including pneumonia, lung cancer, cystic fibrosis, lung disease, chronic obstructive pulmonary disease, and asthma, has been elucidated (25). A recent report has demonstrated that specific oral microbial dysbiosis may be associated with stroke-related pneumonia (26). Another study using metagenomic 16S rRNA identified oral microbiomes, such as *Streptococcus* and *Fusobacterium,* as the predominant causative agents of pleural empyema in pleural fluid samples (27). However, little is known about the oral microbiome profile of patients with pleural empyema. Therefore, we hypothesized that there are distinct differences in the oral and pleural fluid microbiome profiles between patients with and without empyema. This study aimed to define the discriminating features of oral and pleural fluid microbiomes in patients with and without empyema.

Methods

Design and setting

This exploratory pilot study included prospectively enrolled adult patients' pleural fluid as well as specimens from the oral mucosa and saliva. This study was approved by the Institutional Review Board of Ulsan University Hospital (IRB File No. 2021-10-009), and written informed consent was obtained from all patients or their guardians prior to enrollment. This trial was registered with the Korea Clinical Research Information Service (KCT0007055; cris. nih. go. kr).

Study participants

Between March 2022 and August 2023, we prospectively recruited patients aged ≥18 years who underwent thoracentesis and pleural fluid analysis. In our study, the empyema group comprised patients with infectious pleural effusions who met the following inclusion criteria: The inclusion criteria were as follows.

- Pleural fluid with a pH <7.2, low glucose level <40 mg/dL, or lactate dehydrogenase concentration >1,000 IU/L.
- Gram staining or culture-positive pleural fluid.
- Macroscopically purulent pleural fluid following thoracentesis.

(Patients met the inclusion criteria if any of the above conditions were present.)

Exclusion criteria included:

- Inability to written agreement
- Uncooperative patient (inability to collect oral samples)
- Presence of tumors in the oral cavity
- Patients who received antibiotics 1 month prior to enrollment.

(Patients were excluded if any of the above conditions were present.)

Compared to infectious pleural effusion, autoimmune disease-associated pleural effusion, transudate pleural effusion, and tuberculous pleurisy are reported to have similar pleural fluid profiles to malignant pleural effusion (28). Other studies have suggested that autoimmune disease-associated pleural effusion and tuberculous pleural effusion may exhibit distinct pleural microbiome profiles (29, 30). Therefore, patients with autoimmune disease-associated pleural effusion and tuberculous pleurisy were excluded from the control group. Recent reports have indicated similarities in the oral microbiomes of patients with lung cancer and pneumonia (31, 32). Therefore, the control group comprised patients with non-infectious pleural effusions, including malignant pleural effusion from cancers other than lung cancer and transudate pleural effusion. Non-infectious pleural effusion was defined as effusion with neither clinical nor biochemical evidence of infection at the time of thoracentesis. All experimental procedures were conducted in accordance with relevant regulations and guidelines. Our study was performed in accordance with the flowchart shown in **Figure 1**. Demographic and clinical information, such as medical history and microbiological (sputum, blood, pleural fluid) and laboratory data, were collected from electronic medical records.

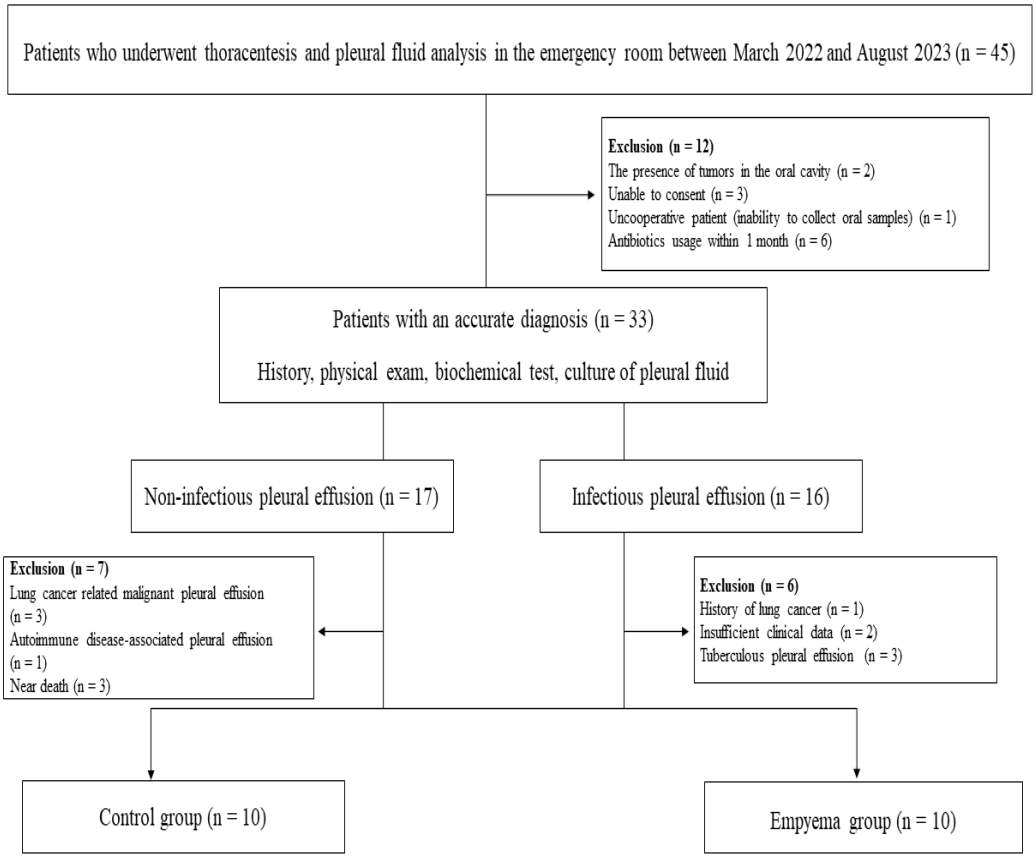

Figure 1. Flowchart of the study population.

Sample collection

Pleural fluid samples were collected immediately after thoracentesis using a standardized method under sterile conditions. Twenty-five milliliters of pleural fluid were collected using aseptic techniques and sent for tests per clinical indication. Five milliliters of pleural effusion were used for metagenomic NGS analysis and 20 mL was used for traditional culture. A total of 20 mL of pleural effusion (10 mL each for aerobic and 10 mL for the anaerobic bottle) was drawn for one set of routine culture tests. The remaining fluid was collected in sterile test tubes containing DNA-stabilizing solution. Initially, the pleural fluid samples were stored at a temperature of -20 °C, and within a week, they were moved to a -80 °C freezer in a refrigerator for further processing and analysis. Simultaneously, oral samples were collected using a method similar to that described in the Human Microbiome Project protocol (Manual of Procedures for Human Microbiome Project, Core Microbiome Sampling, Protocol A, HMP Protocol # 07–001, Version Number: 12.0, 29 Jul 2010). The patients were instructed to refrain from eating or drinking for at least 2 h before sample collection. The specimens included three types of mucosal tissue (tongue, buccal mucosa, and keratinized gingiva) and saliva. Although existing data indicate that subgingival and supragingival plaques may have distinct microbiomes, these sites were excluded because of the invasive procedures required for sampling. Prior to any intervention, oral mucosal tissues were sampled by swabbing for 40 s using sterile rayon swabs (Copan, Brescia, Italy). Sterile swabs were opened immediately before sample collection, with special care taken to avoid contact with or contamination of other parts of the oral cavity. Following the swabbing procedure, each swab was promptly introduced into a sterile 1.5-mL microtube, which had been preloaded with 0.5 mL of sterile phosphate-buffered saline (PBS). The sampled sites included the dorsum of the tongue (a 1 cm^2 section at the central part of the tongue, subjected to a 5-s rub), buccal mucosa (entire area on both sides, each subjected to a 10-s rub), and keratinized gingiva (the attached gingiva of the maxillary anterior region, rubbed for 10 s). Subsequently, the patients underwent oral

rinsing of saliva using 15 mL of sterile PBS for 1 min. Subsequently, they expectorated the oral contents into a 50-mL centrifuge tube. Each collected specimen was refrigerated (2–8 °C) and transported to the laboratory for analytical evaluation. All clinical samples were processed within a time frame of 30 min.

Culture of pleural effusion

Using the BD BactecTM FX400 detection system, aerobic and anaerobic pleural effusion cultures were incubated according to the manufacturer's instructions. Positive cultures were subsequently subcultured, and the microorganisms were identified using standard methods. The culture duration was 5 d.

DNA extraction for microbiome analysis

Bacterial DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) using the bead-beating method. The pleural fluid and oral samples were centrifuged at 20,000 × g at 4 ℃ for 15 min. After discarding the supernatant, the pellets were stored at -80 °C. Prior to analysis, the samples were thawed on ice and resuspended in 360 μL of Buffer ATL and autoclaved with 0.25 g sterile 0.1-mm-diameter zirconia beads (BioSpec Products, Bartlesville, USA) (33, 34). Bead-beating was performed using a bullet blender (Next Advance, Troy, USA) at the maximum speed for 45 s. To prevent sample loss due to bubbling during incubation, the samples were centrifuged at 20,000 g. After incubating the samples at 56 °C for 30 min, a second round of bead-beating was performed. Following the second bead-beating, 40 μL of Proteinase K was added (35). The tubes were incubated overnight at 56 °C and then vortexed for 15 s. After centrifugation at 20,000 × g for 1 min, the supernatants were transferred to new 1.5-mL tubes and the beads were discarded. Subsequently, 200 μL AL Buffer was added to the sample and mixed thoroughly via vortexing. Following a 1-h incubation at 56 °C, 200 μL of ethanol (96–100%) was added to the tube and vortexed again.

The mixture was transferred to a DNeasy Mini spin column and centrifuged for 1 min at 6,000 × g. The column was washed with AW1 and AW2 buffers, followed by elution with 30 μL AE Buffer. Purity and quantity were measured using a Nanodrop (Thermo Fisher Scientific, Wilmington, USA) and stored at -80 °C.

Polymerase chain reaction (PCR) amplification and sequencing

Using fusion primers, we performed PCR amplification targeting the V3–V4 regions of the 16S rRNA gene. These primers included 341F and 805R with specific sequences for bacterial DNA amplification. The amplification process consisted of an initial denaturation at 95 °C for 3 min, followed by 25 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The quality of the purified final product was assessed using a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA), and sequencing was performed using an Illumina MiSeq Sequencing system (Illumina, San Diego, CA, USA) at CJ Bioscience, Inc. (Seoul, Korea).

Internal validity and reagent control

Contamination remains a concern in microbiome research. To ensure internal validity and reagent control, 10 negative controls were used to identify contaminants related to the DNA extraction reagents. Sequencing of the 10 reagent control samples yielded less than 1 ng/μL DNA in each well.

Microbiome data analysis pipeline

Raw reads and microbiome data analyses were performed as previously described (36). Briefly, the initial processing of raw reads included quality assessment and filtering of low-quality reads (< Q25) using Trimmomatic version 0.32. After quality control validation, the paired-end sequence data were merged using the "fastq_mergepairs" command from VSEARCH

version 2.13.4 with default parameters. Primer sequences were removed using the alignment algorithm proposed by Myers and Miller3 with a similarity cutoff of 0.8. Subsequently, nonspecific amplicons lacking 16S rRNA were detected using the nhmmer4 module within the HMMER software package, version 3.2.1, utilizing mm profiles. Unique sequences were extracted, and redundant sequences were merged using the "derep_fullength" command in VSEARCH. Taxonomic assignment was performed using the EzBioCloud 16S rRNA database with the "usearch_global" command in VSEARCH. Chimeric reads with less than 97% similarity were removed using the UCHIME algorithm6 for reference-based chimeric detection. Subsequently, reads with less than 97% similarity, which could not be identified at the species level in the EzBioCloud database, were combined. The "cluster_fast" command was then used for de novo clustering, generating additional Operational Taxonomic Units (OTUs). Finally, the OTUs consisting of only a single read (singleton) were excluded from further analysis.

Bioinformatic analysis, visualization, and statistical analysis

Microbiome diversity is typically described in terms of within (i.e., alpha) and between samples (i.e., beta). The bioinformatics analyses in our study were assessed using an online platform (https://www.ezbiocloud.net) provided by CJ Bioscience, Inc. (Seoul, South Korea). Alpha diversity measures the microbial community diversity and abundance in a sample. It was assessed using three diversity indices (37): i) Chao1, Richness: Estimating the number of species present. ii) Shannon Index, Evenness: Used to represent diversity and is calculated as $H = -\sum (\boldsymbol{pi} * \ln(\boldsymbol{pi}))$, where $\boldsymbol{pi}$ is the proportion (n/N) of individuals of a particular species found (n) and s represents the number of species. iii) Phylogenetic diversity: An index that quantifies biological diversity by measuring the phylogenetic differences between species. It is calculated by summing the shortest distances between nodes in a phylogenetic tree, with higher values indicating greater diversity. Higher richness indicates a more diverse or complex

species composition, while higher evenness suggests a more uniform microbial community composition within the cluster. Alpha diversity was visualized using R package software version 3.2.5 (R Foundation for Statistical Computing, Vienna, Austria).

Beta diversity is a measure that compares diversity between samples and is calculated by assessing the dissimilarity in features, which results in a distance matrix between all pairs of samples (38, 39). Beta diversity analysis employs various diversity metrics, which can be categorized as quantitative (using sequence abundance, e.g., Bray–Curtis or weighted UniFrac) or qualitative (considering only the presence or absence of OTU, e.g., binary Jaccard or unweighted UniFrac). To visualize beta diversity data, ordination techniques, such as principal coordinate analysis (PCoA) and principal component analysis (PCA) were used in R (version 3.3.1). These techniques reduce complex distance matrices into two- or three-dimensional scatterplots for a straightforward visual interpretation. We used permutational multivariate analysis of variance (PERMANOVA), which is based on distance matrices, to determine whether there were biologically significant differences in the microbiome community structure between the different groups. In addition to assessing the taxonomic composition of the microbiome, we identified differences in metabolic functions between the different groups. Profiling phylogenetic marker genes, such as the 16S rRNA gene, cannot directly identify the metabolic or other functional capabilities of a community. Thus, we used the PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) software to predict the Kyoto Encyclopedia of Genes and Genomes (KEGG) functional profiles of the microbial communities (40). PICRUSt employs an extended ancestral state reconstruction algorithm to estimate the unknown gene content. Using the relative abundance of taxa within the community, these algorithms can predict the potential functionality of gene content based on the reference genome for each taxon. Significant results were explored using the KEGG PATHWAY online database (https://www.genome.jp/kegg/pathway.html). Baseline demographics were compared using Fisher's exact and chi-square tests for categorical

variables and independent t-tests for continuous variables. We used conventional statistical analyses, such as t-tests, Wilcoxon rank-sum tests, ANOVA, or Kruskal–Wallis tests, to identify differences in the abundance of specific known bacterial taxa or alpha diversity in different groups (41). However, when comparing low-level taxonomic differences, such as genus or species, these conventional tests are prone to false positives without proper correction for multiple comparisons (42). Thus, we employed a linear discriminant analysis (LDA) of effect size (LEfSe), a well-established method explicitly designed for microbiome data. This analysis initially computed the Kruskal–Wallis rank sum p-value to identify significantly differentially abundant features between groups, followed by performing LDA to assess the effect size of these specific attributes. Our study identified taxa with significantly different abundances and functional profiles between different groups using LEfSe and visualized them using GraphPad Prism 5 (GraphPad, San Diego, CA, USA). During this analysis, the alpha value was set to <0.05, and the threshold of the logarithmic LDA score for the discriminative feature was set to >3.0.

Results

Clinical characteristics of the study population

After applying the inclusion and exclusion criteria, 20 participants were enrolled: 10 patients with pleural empyema and 10 non-infected controls. The baseline demographics are shown in **Table 1**. The mean patient age was 66.4 ± 13.6 years, with a preponderance of men (60.0%). The groups showed no significant differences in basic information, such as age, sex, body mass index, and smoking habits. **Table 2** shows the bacterial culture results of the pleural fluid, sputum, and blood of the patients with empyema. Bacterial cultures from pleural fluid were positive in five patients. Sputum cultures were positive in six patients; among these patients, three had matching bacteria isolated from the pleural fluid. However, blood culture results were negative in all patients.

Table 1. Clinical and laboratory features of 10 patients with empyema and 10 controls

Patient characteristics	Empyema group (Infectious effusions)	Control group (Non-infectious effusions)
N	10	10
Age (years)	67.8 ± 10.9	65.0 ± 16.3
Male/Female	4/6	8/2
Body mass index	21.2 ± 4.8	24.3 ± 3.2
Smoking		
Non-smoker	6	3
Current–smoker	4	5
Previous-smoker	0	2
Comorbidities		
COPD	0	1
Alcohol.abuse	2	0
Diabetes mellitus	5	1
Hypertension	5	5
Etiology of pleural effusion		
Parapneumonics	10	0
Paraneoplastics		
Breast	0	1
Colon	0	1
Stomach	0	1
Liver	0	1
Lymphoma	0	1
Thymus	0	1
Transudate		
Heart failure	0	1
Renal failure	0	3
Hospitalization	30.4 ± 17.9	3.3 ± 3.4*
Bacterial culture positive	5	0
Laboratory results		
WBC	13.6 [11.2;17.5]	6.3 [5.1; 7.4]*
Hemoglobin	10.7 ± 2.3	11.3 ± 1.6
Platelet	410.0 ± 209.9	198.7 ± 115.1*
Albumin	2.8 ± 0.4	3.6 ± 0.7*
Creatinine	0.5 [0.4; 0.8]	1.1 [0.9; 1.2]*
Total bilirubin	0.4 [0.3; 0.5]	0.6 [0.5; 0.7]
CRP	216.4 [115.0;486.0]	2.2 [1.0; 4.7]*
Pleural fluid analysis		
Total protein (mg/dL)	3.7 ± 0.6	3.0 ± 1.2
Glucose (mg/dL)	26.0 [4.0;38.0]	119.5 [108.0;128.0]*
Lactate dehydrogenase (U/mL)	2575.5 [2002.0;4631.0]	114.5 [91.0;270.0]*
Albumin (g/dL)	1.7 ± 0.3	2.0 ± 0.9
pH	7.0 ± 0.1	7.6 ± 0.1*

*Significance level of $p < 0.05$; empyema group

Table 2. Results of bacterial culture from pleural fluid, sputum, and blood in the empyema group.

No	Antibiotics Pretreatment	Pleural Fluid Culture	Sputum Culture	Blood Culture
1	No	*Klebsiella pneumoniae*	MRSA, *Klebsiella pneumoniae*	N.D.
2	No	*Klebsiella pneumoniae*	N.D.	N.D.
3	No	N.D.	N.D.	N.D.
4	No	N.D.	CRAB (carbapenem resistance *Acinectobacter baumannii)*	N.D.
5	No	*Staphylococcus aureus*	*Staphylococcus aureus*	N.D.
6	No	N.D.	*Candida krusei*	N.D.
7	No	N.D.	N.D.	N.D.
8	No	*Staphylococcus aureus*	*Staphylococcus aureus*	N.D.
9	No	N.D.	*Candida tropicalis* *E.coli*	N.D.
10	No	*Staphylococcus aureus*	N.D.	N.D.

N.D.: not detected N.D.

Oral microbiome diversity and richness between distinct oral sites

From 100 samples obtained from 20 individuals, 4,003,812 reads passed the quality control. Alpha diversity analysis of the microbiome collected from various oral sites was conducted using the Shannon index, Chao1 index, and phylogenetic diversity. The results showed that microbiome clusters derived from various oral sites exhibited similar patterns across all participants and no significant differences were observed among the four sites (**Figure 2**).

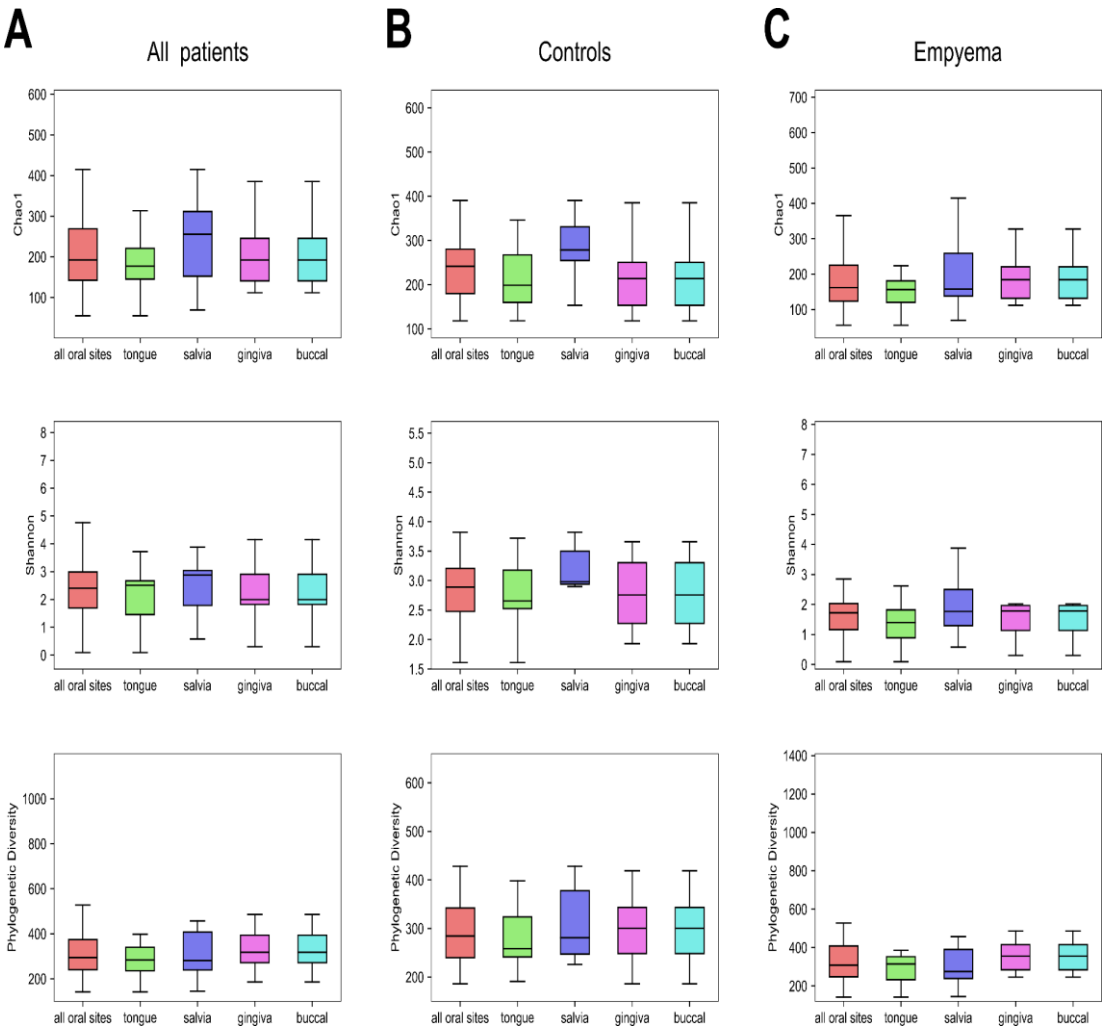

Figure 2. Comparison of alpha diversity indexes (Chao1, Shannon, and phylogenetic diversity): **(A)** samples from all patients, **(B)** samples from controls only, **(C)** oral samples from patients with empyema only.

Although none of these results were statistically significant, saliva analysis indicated that both groups had more species and a higher degree of evenness. To identify the diversity among microbial communities, we evaluated the phylogenetic distances between microbial communities at different oral sites using PCoA. PCoA plots generated from the UniFrac distance matrix showed no visual separation. The results of the beta diversity analysis showed that the microbial community members were similar among all groups **(Figure 3).** According to the PERMANOVA results, there were no significant differences between the oral sites **(Table 3).**

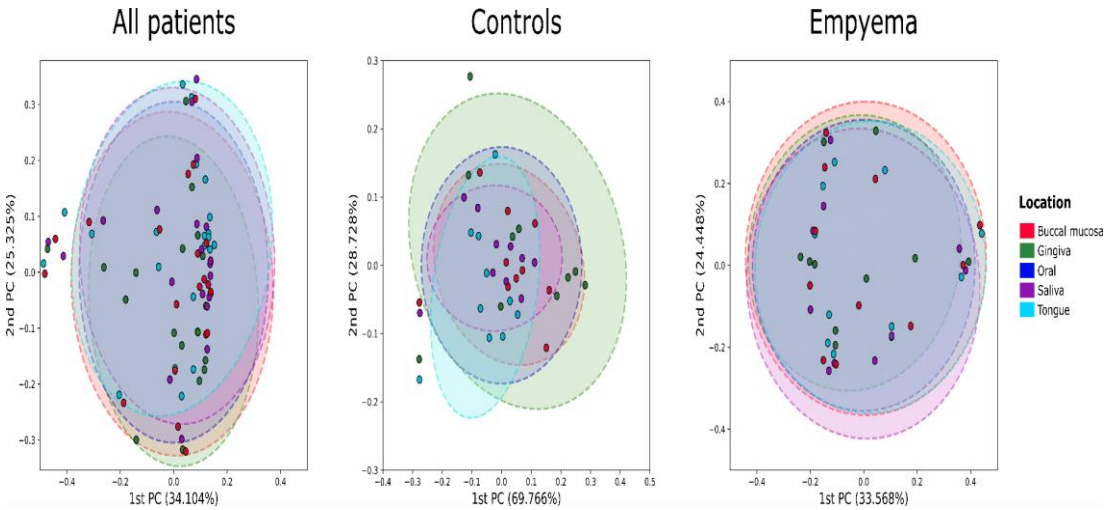

Figure 3. Principal coordinate analysis of the relative abundances of OTUs between locations: **(A)** samples from all patients, **(B)** samples from controls only, **(C)** oral samples from patients with empyema only.

Table 3. Permutation multivariate analysis of variance (PERMANOVA) to test the null hypothesis that there were no differences in the microbial community structure across locations.

Location		Empyema group		Control group	
		F	**Bray *p*-Value**	**F**	**Bray *p*-Value**
All oral sites	Tongue	0.149	0.999	1.147	0.331
	Saliva	0.079	0.981	0.194	0.951
	Gingiva	0.208	0.998	0.473	0.738
	Buccal mucosa	0.000	0.991	0.578	0.657
Tongue	Saliva	0.205	1	0.739	0.586
	Gingiva	0.302	0.988	1.511	0.182
	Buccal mucosa	0.208	0.998	2.144	0.086
Saliva	Gingiva	0.161	0.989	0.666	0.579
	Buccal mucosa	0.154	0.993	0.756	0.553
Gingiva	Buccal mucosa	0.142	1	0.277	0.907

Additionally, we conducted beta diversity analysis to identify the oral sites that were most similar to the pleural fluid. In the PCoA based on unweighted UniFrac distances, we confirmed the similarity between saliva and pleural fluid, whereas other oral sites showed a distinct separation from the pleural fluid. PERMANOVA results indicated a difference between pleural fluid and oral site samples, excluding the saliva and gingiva **(Table 4)**.

Table 4. Permutation multivariate analysis of variance (PERMANOVA) to identify oral sites most similar to pleural fluid

Location		Control group	
		F	**Bray *p*-Value**
Pleural fluid	Tongue	2.796	0.011
	Saliva	2.210	0.054
	Gingiva	1.644	0.123
	Buccal mucosa	2.179	0.049
Tongue	Saliva	0.739	0.586
	Gingiva	1.511	0.182
	Buccal mucosa	2.144	0.086
Saliva	Gingiva	0.666	0.579
	Buccal mucosa	0.756	0.553
Gingiva	Buccal mucosa	0.277	0.907

Differences in oral bacterial phyla and genera between groups (microbial taxonomy)

(1) Oral microbiome analysis at the phylum level

To evaluate the oral microbial community characteristics, we compared the relative taxonomic abundance within the oral microbiome of both controls and empyema patients. A total of 16 were identified at the phylum level, with *Bacillota, Bacteroidota, Actinomycetota*, and *Pseudomonadota* as the predominant phyla, collectively accounting for over 90% of the total phyla. When comparing the two groups, *Bacteroidota* and *Actinomycetota* were significantly abundant in the control group ($p < 0.02$, $p < 0.04$, respectively), whereas *Synergistota* was significantly abundant in the empyema group ($p < 0.03$). The taxonomic distribution of oral bacteria at the phylum level for each group is shown in **Figure 4**.

(2) Oral microbiome analysis at the genus level

A total of 24 taxa were identified at the genus level. Taxa with an average abundance value of less than 1% in the sample were labeled "ETC" (**Figure 4**). *Prevotella, Streptococcus*, and *Rothia* were predominant in all the samples. Additionally, *Prevotella, Neisseria*, and *Rothia* were detected at high levels in the control group. There were significant differences between the two groups in the comparative analysis of relative abundance. Eight genera (*Haemophilus, Rothia, Prevotella, Streptococcaceae, Neisseria,* and *Alloprevotella*) were present in the control group, indicating a significantly high relative abundance at the genus level. Four genera (*Enterobacteriaceae, Enterococcus, Staphylococcus,* and *Acinetobacter*) were identified in the empyema group, reflecting a significantly high relative abundance.

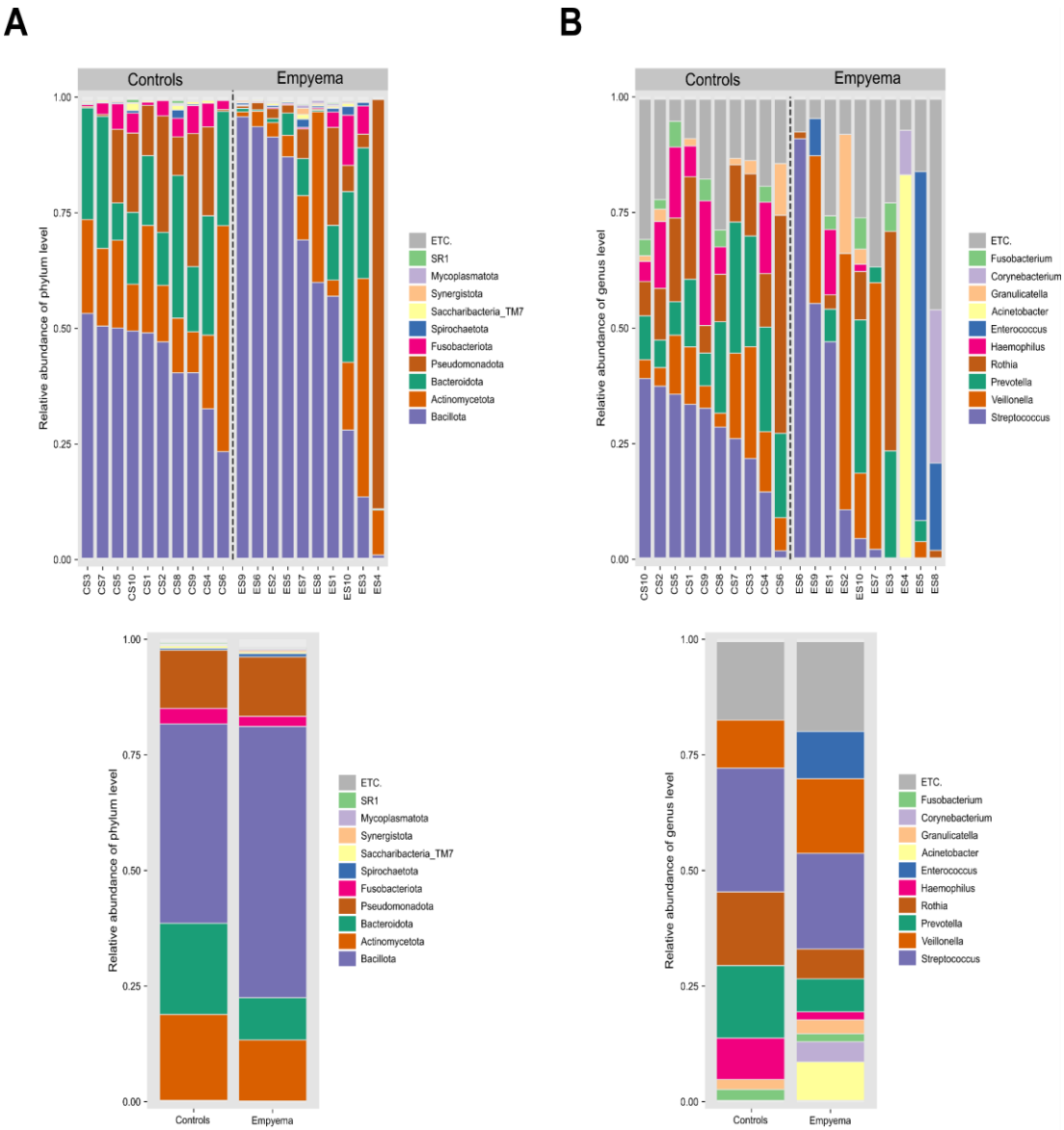

Figure 4. Composition and structure of oral microbiome community between control and empyema groups: **(A)** comparison of relative taxa abundance between empyema and control groups at phylum level. **(B)** comparison of relative taxa abundance between empyema and control groups at genus level.

(3) Diversity of oral microbiome in patients with empyema and control group

When analyzing samples from all oral sites collectively, the alpha diversity assessed through the Shannon index revealed a significant difference between the control and empyema groups (1.8 ± 1.1 vs. 2.8 ± 0.6, $p < 0.001$) **(Figure 5A).** Even when analyzing microbiomes originating from saliva separately, a distinct reduction in the diversity of oral microbiome clusters was evident (1.9 ± 1.0 vs. 3.1 ± 0.5, $p = 0.004$) **(Figure 5B).** Likewise, beta-diversity analysis revealed significant differences in the composition of microbiomes between the control and empyema groups, both when analyzing all sites collectively ($p = 0.001$) and when comparing salivary microbiomes individually ($p = 0.02$) **(Figure 5C, D).** The top 10 genera with the highest mean relative abundances were similar between cohorts, except for *Corynebacterium* and *Actinomyces*, which were highly enriched in the empyema group compared to those in the controls **(Figure 5E).**

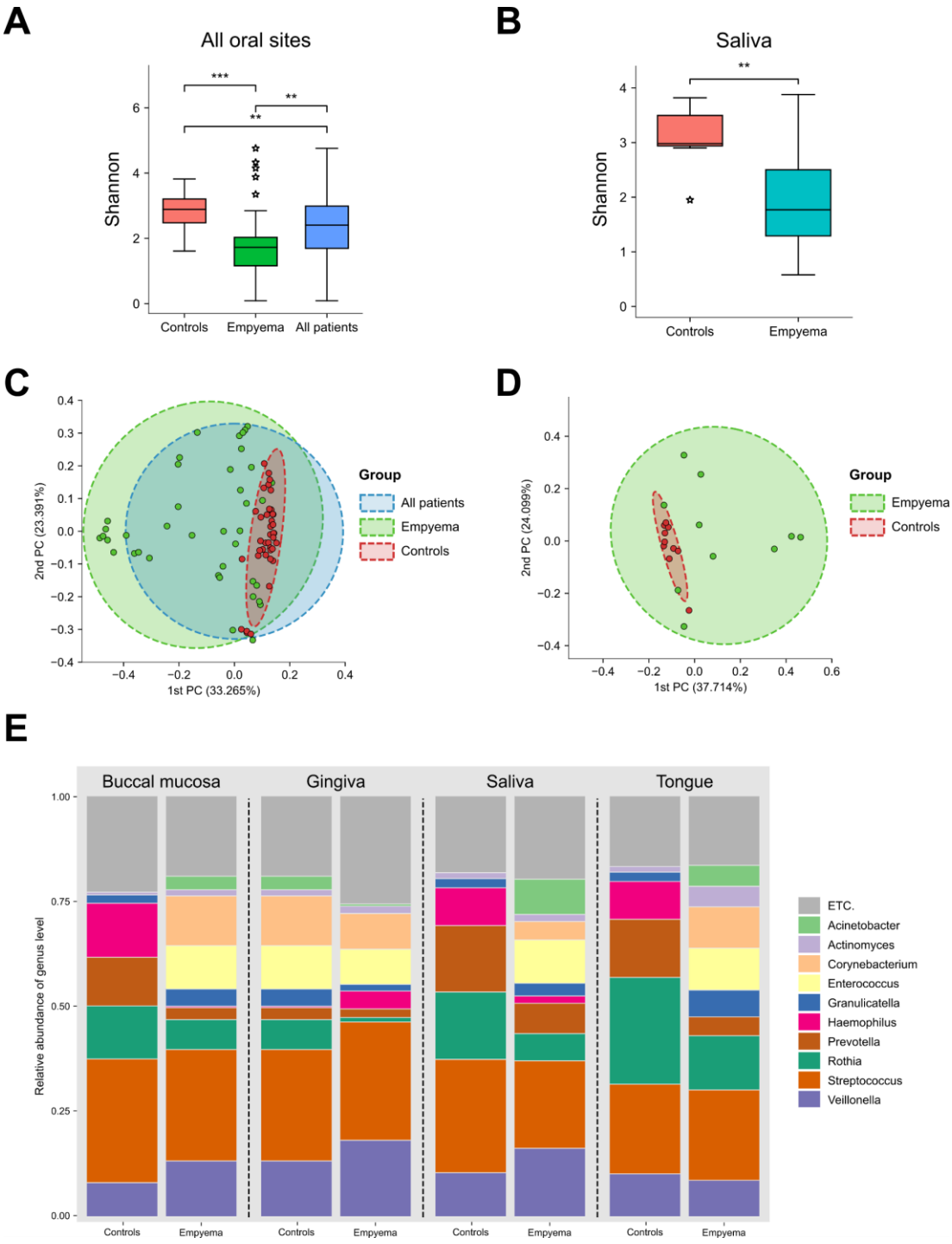

Figure 5. Comparison of oral microbiomes between control and empyema groups: (**A**) Alpha-diversity values when all sites were combined. (**B**) Alpha-diversity values when only saliva was analyzed. (**C**) Principal coordinate analysis (PCoA) of the relative abundances of OTUs between locations when all sites were combined. (**D**) PCoA of the relative abundances of

OTUs between locations when only saliva was analyzed. (**E**) Top 10 genera at each site compared between control and empyema groups. ** significance level of $p < 0.001$; *** significance level of $p < 0.0001$.

Differences in pleural bacterial phyla and genera between groups (microbial taxonomy)

(1) Pleural microbiome analysis at the phylum level

We compared the relative taxonomic abundance within the pleural microbiome of both controls and patients with empyema to evaluate pleural microbial community characteristics. A total of 12 were identified at the phylum level, with *Bacillota, Bacteroidota, Fusobacteriota*, *Actinomycetota*, and *Pseudomonadota* as the predominant phyla, collectively accounting for over 90% of the total phyla. When comparing the two groups, *Bacteroidota* and *Actinomycetota* were significantly more abundant in the control group ($p < 0.04$, $p < 0.001$, respectively), while *Fusobacteriota* was significantly more abundant in the empyema group ($p < 0.01$). The distribution of pleural bacterial taxonomy at the phylum level for each group is shown in **Figure 6**.

(2) Pleural microbiome analysis at the genus level

A total of 25 taxa were identified to the genus level **(Figure 6).** *Prevotella*, *Streptococcus*, *Veillonella*, and *Rothia* were detected at high levels in the control group. *Staphylococcus*, *Enterobacteriaceae, Aggregatibacter, Acinetobacter, and Fusobacterium* were detected in the empyema group. There was a significant difference in the comparative analysis of relative abundance between the two groups. There were seven genera–*Prevotella*, *Haemophilus*, *Veillonella, Actinomyces, Streptococcus, Neisseria, and Rothia* in the control group, reflecting a significantly high relative abundance at the genus level. In the empyema group, four genera, *Corynebacterium, Enterobacteriacea, Granulicatella, and Capnocytophaga*, were identified. Notably, the genus *Enterobacteriaceae* was exclusively present in the patients with empyema and was not detectable in the control group.

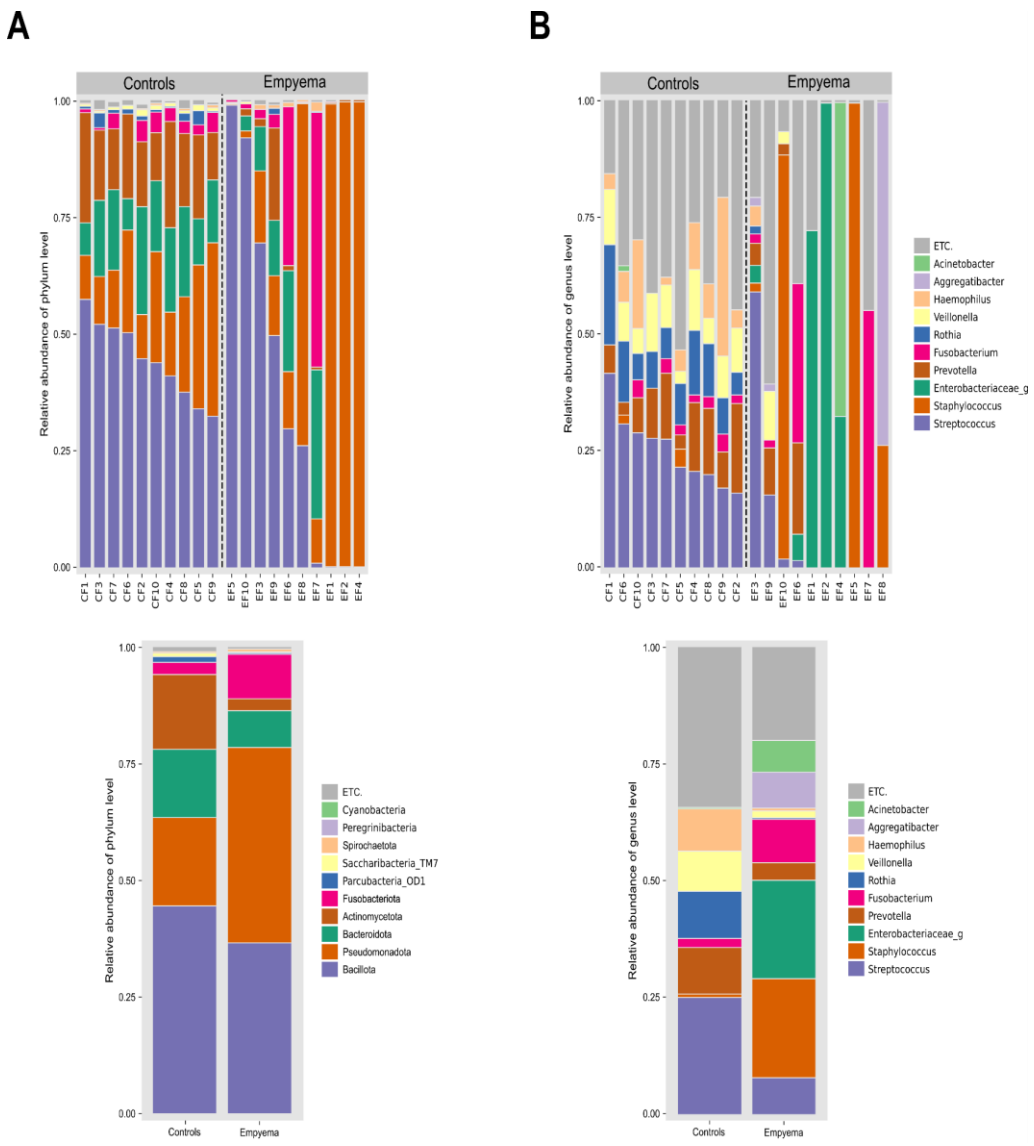

Figure 6. Composition and structure of pleural microbiome community between control and empyema groups: **(A)** comparison of relative taxa abundance between empyema and control groups at phylum level. **(B)** comparison of relative taxa abundance between empyema and control groups at genus level.

(3) Diversity of pleural microbiome in patients with empyema and control group

The alpha diversity of the pleural microbiome of patients with empyema was lower than that of controls in terms of both diversity (Shannon index, 1.2 ± 1.1 vs. 3.6 ± 0.4, $p < 0.001$, **Figure 7A**; Phylogenetic diversity, 119.7 ± 66.1 vs. 417.3 ± 152.8, $p < 0.001$, **Figure 7B**) and richness (Chao1 index, 49.2 ± 22.2 vs. 199.7 ± 98.4, $p = 0.001$, **Figure 7C**). PCoA based on UniFrac distance indicated a significant difference between the empyema and control groups. PERMANOVA showed a significant difference between the two groups ($p = 0.001$) (**Figure 7 D**).

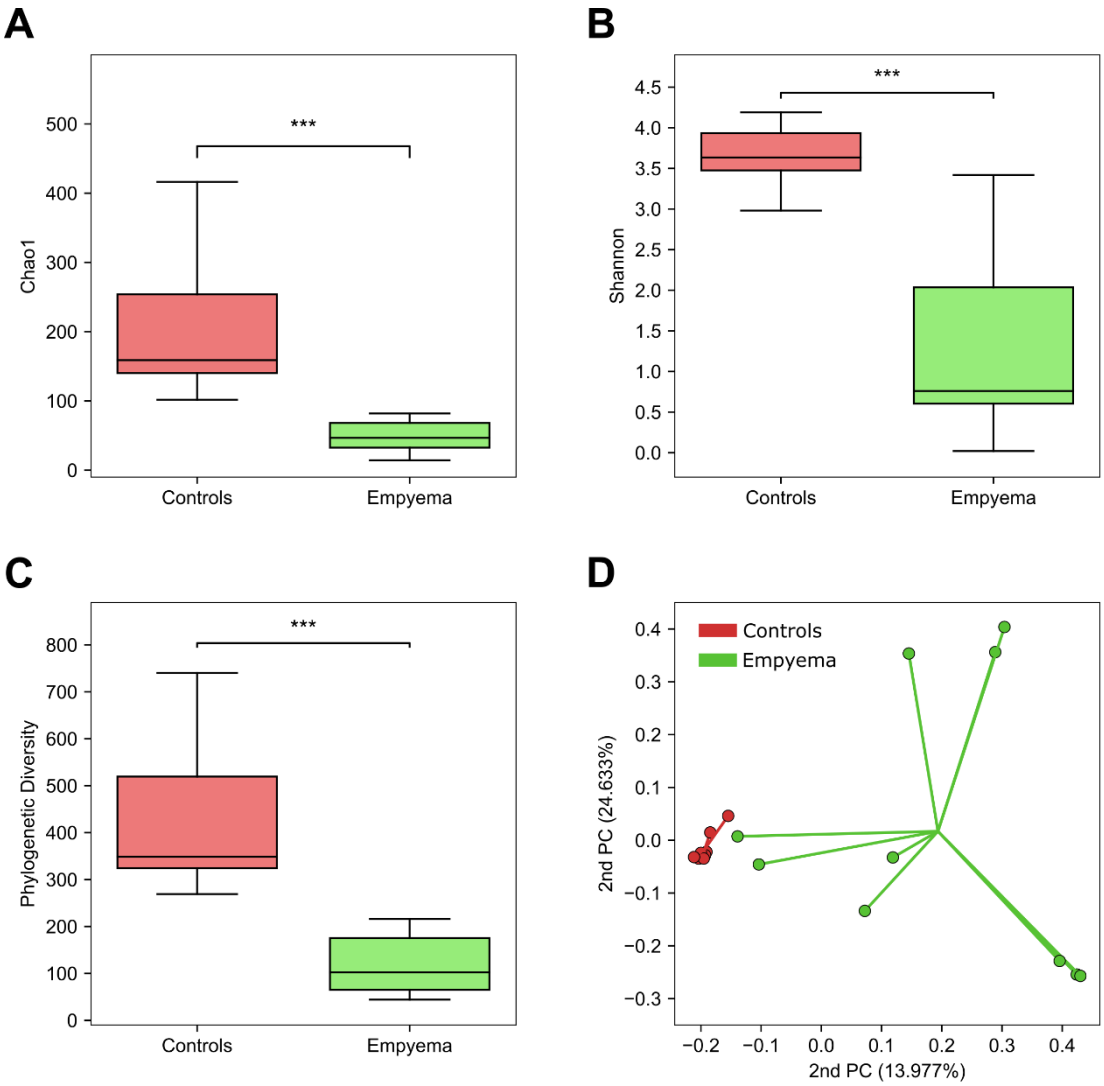

Figure 7. Comparison of pleural microbiomes between control and empyema groups: **(A~C)** comparison of alpha diversity indexes (Chao1, Shannon, and phylogenetic diversity). **(D)** principal coordinate analysis (PCoA) based on UniFrac distance.

(4) Diversity of oral and pleural microbiome in patients with empyema and control group

We compared the microbial differences between saliva and pleural fluid samples across different groups. The alpha diversity (Shannon index) was higher in the saliva and pleural fluid samples of the control group than in those of the pleural group, with no significant differences observed between the saliva and pleural fluid within the pleural group **(Figure 8A)**. Beta diversity revealed visually similar compositional differences between the groups. However, PERMANOVA indicated significant differences between groups **(Figure 8B).**

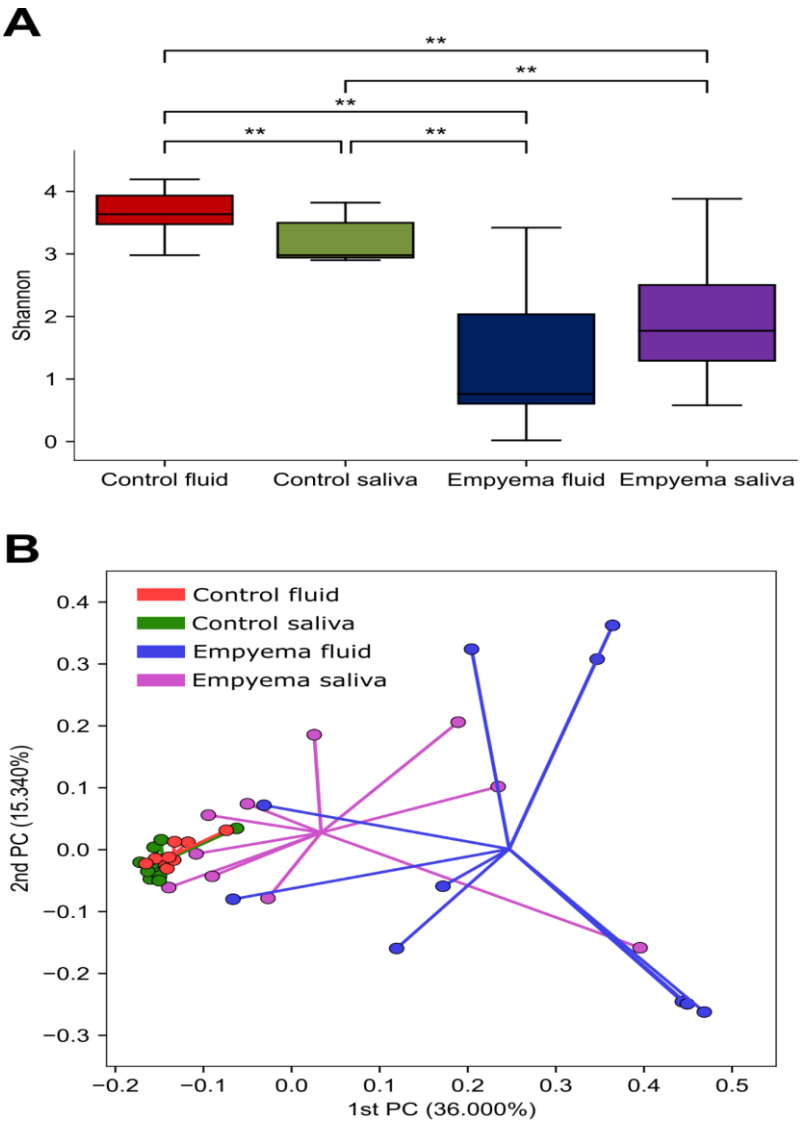

Figure 8. (A) Alpha diversity (Shannon diversity). p-values were determined using Kruskal–Wallis rank sum test. Individual comparisons using Wilcoxon rank sum tests with Benjamini–Hochberg adjustment for multiple comparisons. **(B)** Beta diversity based on UniFrac distance.

Comparison of bacterial species identified by culture and NGS

The predominant bacterial species identified via NGS corresponded with the culture results for the two groups **(Table 5,6).** In the control group, many species of periodontopathogenic bacteria or normal oral flora (*Rothia dentocariosa*; *Prevotella melaninogenica*, jejuni, denticola, and oris; *Streptococcus parasanguinis*; and *Streptococcus peroris*) were highly abundant. Blood cultures were negative for all patients. In the empyema group, five patients in the mNGS group had positive sputum cultures. Microbiomes, such as *Acinectobacter baumannii, Klebsiella pneumoniae,* and *Staphylococcus aureus,* were also detected. Five patients in the mNGS group had positive pleural effusion culture results. *Klebsiella pneumoniae* and *Staphylococcus aureus* were also detected. The mNGS pathogen detection results showed that bacteria were the most commonly identified potential pathogens, with *Streptococcus, Prevotella, Parvimonas, Porphyromonas,* and *Gemella* being the most prevalent. Among the patients who tested positive by mNGS, seven were diagnosed with mixed infection, and two or more pathogens were identified in each case. Notably, the predominant bacteria identified via NGS did not align with those detected through the culture of one effusion sample. In an effusion positive for *Staphylococcus aureus* in culture, these bacteria represented only 26.2% of the total bacterial genome, whereas NGS identified the anaerobe *Aggregatibacter segnis* (73%).

Table 5. Major bacterial species identified via 16s rRNA metagenomics in the control group.

No	NGS				Pleural fluid culture
	Bacterial species (Pleural)	**Proportion (%)**	**Bacterial species (Oral)**	**Proportion (%)**	**Bacterial species**
1	*Rothia KV831974_s group*	20.1	*Rothia KV831974_s group*	17.6	ND
	Streptococcus pneumoniae group	13.0	*Streptococcus pneumoniae group*	16.2	
	Veillonella dispar	9.7	*Streptococcus salivarius group*	11.5	
	Prevotella melaninogenica	8.4	*Streptococcus sinensis group*	10.7	
	Haemophilus parainfluenzae	6.5	*Veillonella dispar*	9.3	
	Streptococcus sinensis	6.3			
2	*Streptococcus pneumoniae*	27.5	*Streptococcus pneumoniae*	9.4	ND
	Haemophilus parainfluenzae	13.9	*Veillonella atypica*	5.3	
	Rothia KV831974_s	8.0	*Megasphaera micronuciformis*	5.3	
	Prevotella melaninogenica	5.0	*Prevotella melaninogenica*	5.3	
3	*Veillonella dispar*	18.6	*Streptococcus salivarius group*	22.0	ND
	Prevotella melaninogenica	18.0	*Veillonella dispar*	8.9	
	Rothia KV831974_s	12.6	*Prevotella melaninogenica*	8.6	
	Streptococcus salivarius	10.5	*Rothia KV831974_s*	5.8	
	Atopobium parvulum	4.6			
	Veillonella atypica	4.0			
	Haemophilus parainfluenzae group	13.3			
4	*Prevotella melaninogenica*	8.3	*Haemophilus parainfluenzae group*	8.2	ND
	Rothia KV831974_s group	8.1	*Rothia KV831974_s group*	7.8	
	Veillonella dispar	7.4	*Veillonella dispar*	7.4	
	Prevotella jejuni	6.9	*Streptococcus sinensis group*	7.3	
	Streptococcus pneumoniae group	5.6	*Streptococcus pneumoniae group*	7.0	
			Prevotella melaninogenica	5.2	
			Rothia mucilaginosa group	5.1	
			Prevotella jejuni	5.0	
5	*Streptococcus pneumoniae group*	20.8	*Streptococcus pneumoniae group*	9.3	ND
	Rothia KV831974_s group	13.3	*Enhydrobacter aerosaccus group*	7.5	
	Haemophilus parainfluenzae group	12.5	*Rothia KV831974_s group*	7.1	
	Veillonella dispar	7.4	*Streptococcus salivarius group*	6.1	
	Fusobacterium nucleatum	5.5			

	group				
	Veillonella parvula group	5.0			
6	*Rothia KV831974_s group*	46.5	*Streptococcus pneumoniae group*	18.6	ND
	Prevotella melaninogenica	18.0	*Rothia KV831974_s group*	8.7	
	Granulicatella adiacens group	11.2	*Streptococcus salivarius group*	6.4	
			Veillonella dispar	6.4	
			Haemophilus parainfluenzae group	5.4	
7	*Streptococcus sinensis group*	19.0	*Streptococcus pneumoniae group*	9.7	ND
	Veillonella dispar	12.9	*Streptococcus sinensis group*	9.3	
	Rothia KV831974_s group	11.9	*Parvimonas micra*	7.2	
	Prevotella histicola	10.8	*Rothia KV831974_s group*	6.2	
	Prevotella melaninogenica	10.6	*Veillonella dispar*	6.0	
			Prevotella HE999470_s	5.9	
8	*Streptococcus pneumoniae group*	22.7	*Streptococcus pneumoniae group*	16.1	ND
	Rothia mucilaginosa group	8.5	*Rothia mucilaginosa group*	8.3	
	Prevotella melaninogenica	4.3	*Haemophilus parainfluenzae group*	5.4	
	Porphyromonas endodontalis	4.1	*Prevotella melaninogenica*	4.5	
9	*Haemophilus parainfluenzae group*	24.3	*Haemophilus parainfluenzae group*	32.2	ND
	Streptococcus pneumoniae group	20.9	*Streptococcus pneumoniae group*	11.4	
	Prevotella melaninogenica	4.5	*Veillonella rogosae*	6.6	
	Streptococcus parasanguinis group	4.2	*Rothia KV831974_s group*	5.8	
10	*Streptococcus pneumoniae group*	21.7	*Streptococcus pneumoniae group*	18.4	ND
	Neisseria perflava	7.3	*Haemophilus parainfluenzae group*	16.8	
	Rothia KV831974_s group	4.8			

Table 6. Major bacterial species identified via 16s rRNA metagenomics in the empyema group

No	NGS				Pleural fluid culture
	Bacterial species (Pleural)	**Proportion (%)**	**Bacterial species (Oral)**	**Proportion (%)**	**Bacterial species**
1	*Enterobacteriaceae group*	72.3	*Streptococcus pneumoniae group*	44.0	*Klebsiella*
	Klebsiella FWNZ_s	25.9	*Gemella haemolysans group*	7.9	
			Haemophilus influenzae group	7.0	
2	*Enterobacteriaceae group*	99.8	*Veillonella parvula group*	37.5	*Klebsiella*
	Klebsiella singaporensis	0.1	*Granulicatella adiacens group*	25.8	
			Veillonella dispar	15.4	
			Streptococcus pneumoniae group	9.1	
3	*Streptococcus pneumoniae group*	56.6	*Rothia KV831974_s group*	47.7	ND
	Gemella haemolysans group	4.0	*Prevotella pleuritidis*	20.0	
	Enterobacteriaceae group	3.6	*Parvimonas micra*	10.7	
			Fusobacterium nucleatum group	6.0	
			Porphyromonas endodontalis	5.3	
4	*Acinectobacter baumannii*	67.4	*Acinectobacter baumannii*	83.8	ND
	Enterobacteriaceae group	32.4	*Corynebacterium minutissimum group*	6.7	
			Enterobacteriaceae group	5.6	
			Corynebacterium striatum	2.8	
5	*Staphylococcus aureus group*	99.7	*Enterococcus faecium group*	76.3	*Staphylococcus aureus group*
			Staphylococcus aureus group	4.9	
			Parascardovia denticolens	3.7	
			Veillonella dispar	2.5	
			Prevotella oralis	2.2	
6	*Fusobacterium nucleatum group*	33.6	*Streptococcus sinensis group*	91.1	ND
	Parvimonas micra	18.9	*Lactobacillus paracasei group*	2.0	
	Prevotella intermedia	16.9	*Rothia KV831974_s group*	1.1	
	Enterobacteriaceae group	5.5			
	Peptostreptococcus stomatis group	4.1			
7	*Fusobacterium nucleatum group*	53.1	*Veillonella dispar*	46.8	ND
	Porphyromonas gingivalis	31.8	*Staphylococcus aureus group*	6.9	

	Campylobacter showae group	8.7	*Veillonella parvula group*	4.6	
			Veillonella rogosae	4.1	
8	*Aggregatibacter segnis*	73.7	*Corynebacterium minutissimum group*	33.4	*Staphylococcus aureus group*
	Staphylococcus aureus group	26.2	*Leuconostoc lactis*	29.1	
			Enterococcus faecium group	18.8	
			Lactobacillus sakei group	7.8	
9	*Enterococcus faecium group*	10.7	*Streptococcus salivarius group*	55.0	ND
	Corynebacterium minutissimum group	10.4	*Veillonella dispar*	23.0	
			Enterococcus faecium group	7.9	
			Veillonella parvula group	6.3	
10	*Staphylococcus aureus group*	87.1	*Prevotella histicola*	11.0	*Staphylococcus aureus group*
	Veillonella dispar	1.9	*Veillonella dispar*	10.6	
	Prevotella melaninogenica	1.4	*Rothia KV831974_s group*	9.5	
			Fusobacterium nucleatum group	6.1	

LEfSe comparison between the empyema and control group

To investigate the bacterial taxa specifically associated with the development of empyema, an LEfSe comparison of the oral and pleural microbiomes was conducted between the control and empyema groups. An LDA score exceeding three indicated the most significant differences among taxa from the phylum to genus levels. A cladogram was used to depict the structures and dominant bacteria in the oral and pleural microbiomes of the control and empyema groups. In the oral microbiome, the genera *Acinetobacter, Staphylococcus*, and *Enterococcus* were enriched in the empyema group, while *Haemophilus, Prevotella*, and *Rothia* were enriched in the control group **(Figure 9).** In the pleural microbiome, the genus *Corynebacterium* was significantly enriched in patients with empyema, whereas, similar to the oral microbiota, *Haemophilus, Rothia,* and *Prevotella* were significantly enriched in the control group **(Figure 10).**

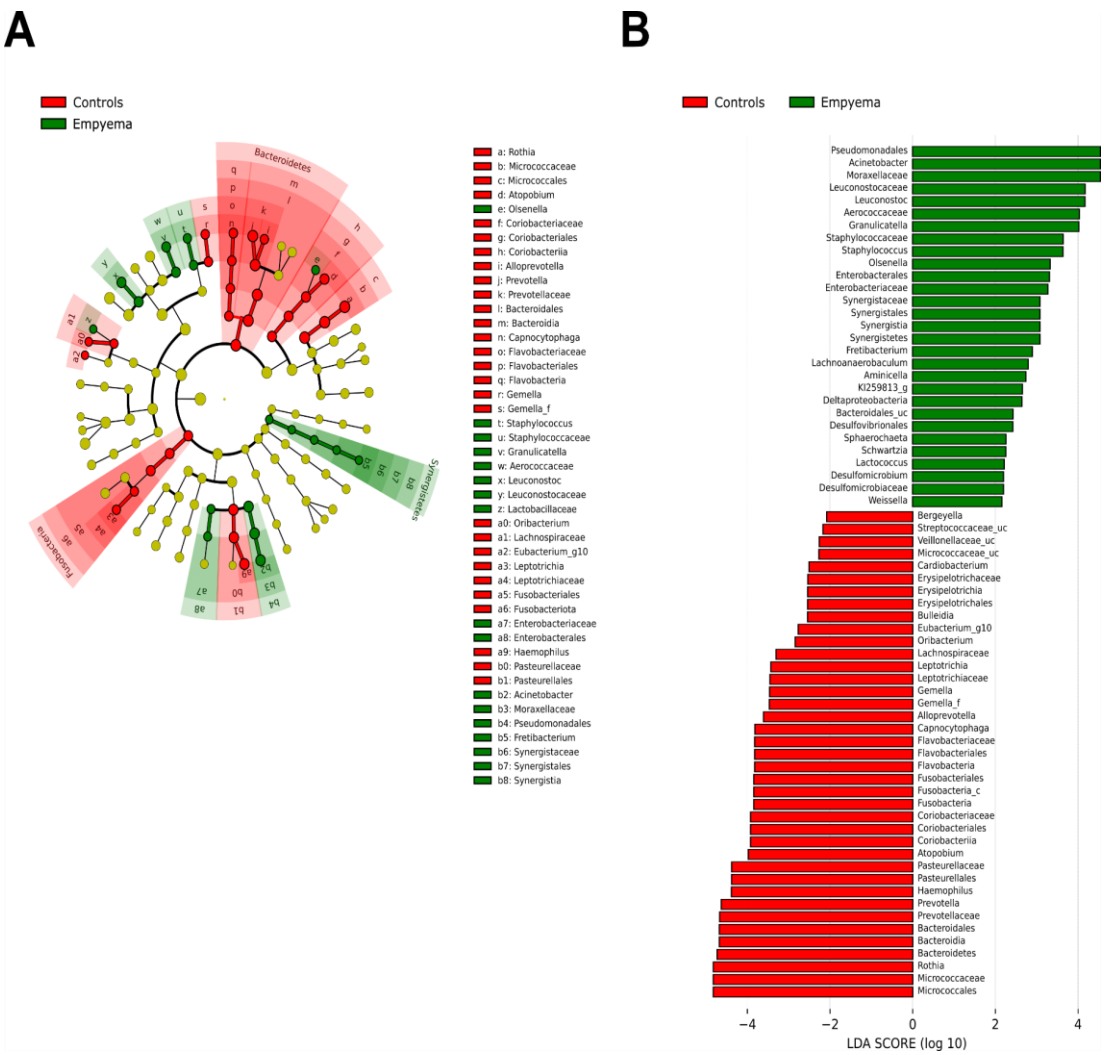

Figure 9. (A) Different structures of oral microbiome between empyema and control groups. The bacterial groups from phylum to genus level are listed from center to outside. Each circle's diameter is proportional to the bacterial taxon's abundance. Cladograms of bacterial lineages with significantly different representation in empyema and control groups. **(B)** Histogram of the linear discriminant analysis (LDA) scores for differentially abundant bacterial taxa between empyema and control groups. Only taxa meeting an LDA significant threshold > 3.0 are shown.

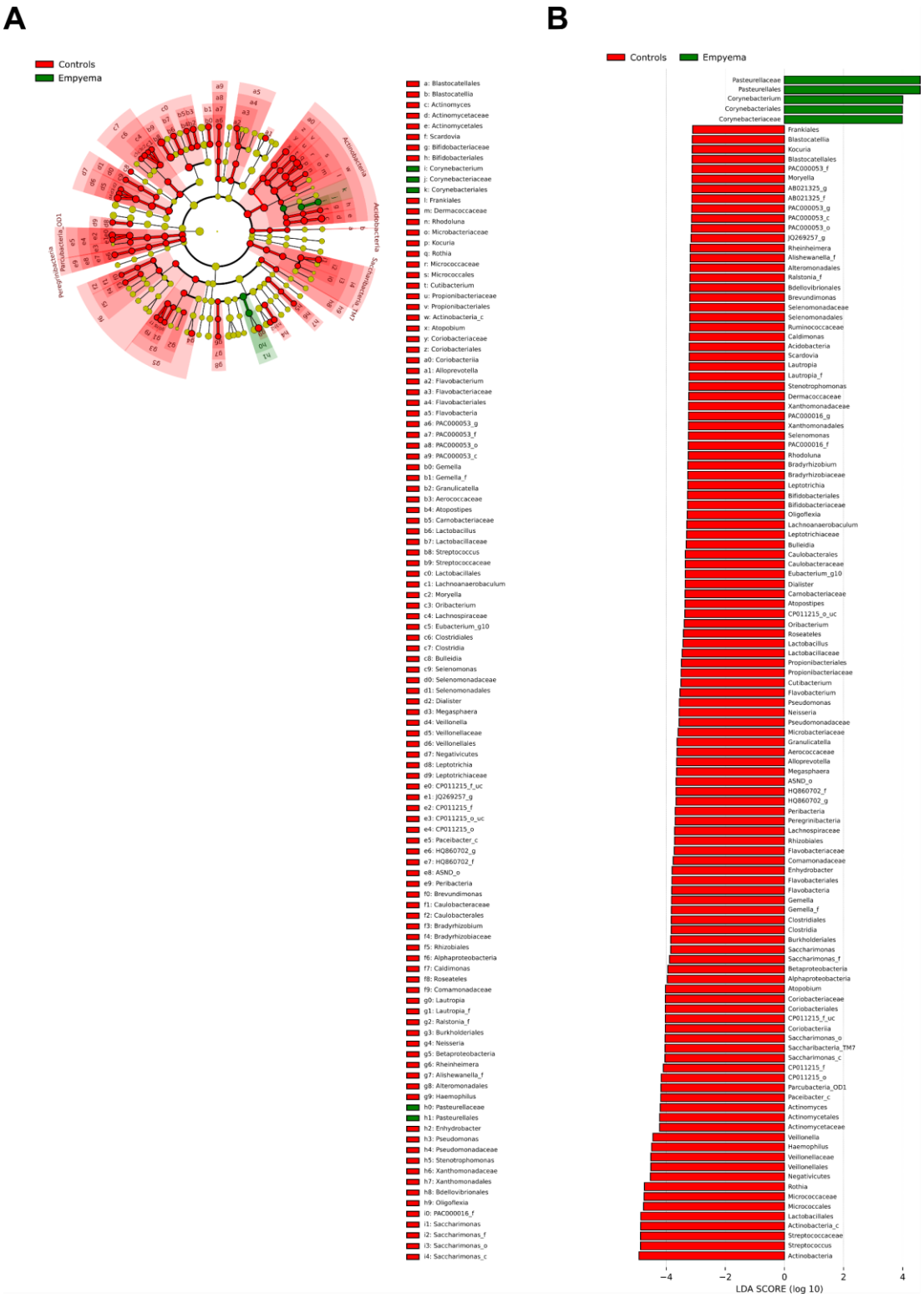

Figure 10. (A) Different structures of pleural microbiome between empyema and control groups. The bacterial groups from phylum to genus level are listed from center to outside. Each circle's diameter is proportional to the bacterial taxon's abundance. Cladograms of

bacterial lineages with significantly different representation in empyema and control groups. **(B)** Histogram of the linear discriminant analysis (LDA) scores for differentially abundant bacterial taxa in pleural microbiome between empyema and control groups. Only taxa meeting an LDA significant threshold > 3.0 are shown.

Microbiome functional profiles

Although 16S rRNA gene analysis revealed the presence of bacteria, it did not provide information on their functions. Thus, we used PICRUSt software to investigate our data, inferring functions indirectly based on known pathways of organisms categorized to a given species-level OTU. We found significant differences in the relative abundances of metabolic pathways between the oral and pleural microbiomes of the empyema and control groups **(Figure 11).** Inferences of the KEGG pathways for each group were additionally compared using LEfSe with the significance threshold set at α of 0.05 and a LDA score of 3.0. In the oral microbiome, we identified 60 metabolic pathways, of which 33 were significantly overrepresented in controls, which were related to genetic information processing, nucleotide metabolism, and metabolism of cofactors. Twenty-seven pathways were significantly overrepresented in patients with empyema, including the two-compartment system, amino acid metabolism, and xenobiotic biodegradation and metabolism. With respect to the pleural effusion, we identified 67 metabolic pathways, of which a total of 50 pathways were significantly overrepresented in controls. Only 17 pathways, including carbohydrate metabolism, were significantly over-represented in patients with empyema.

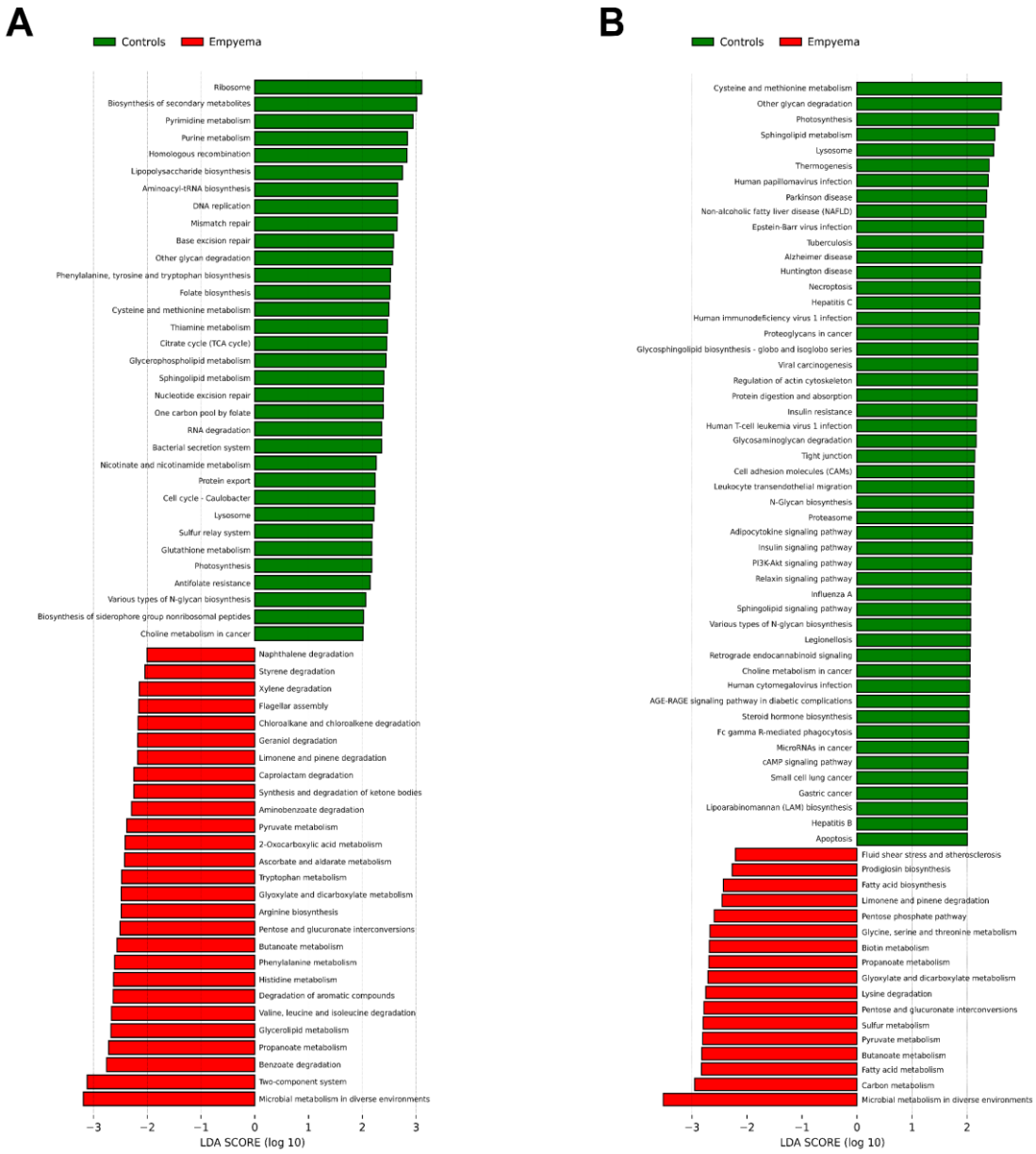

Figure 11. Different structures of predicted KEGG pathways between empyema and control groups: **(A)** LEfSe of over-represented metabolic pathways in the oral microbiome between empyema and control groups. **(B)** LEfSe of over-represented metabolic pathways in the pleural microbiome between empyema and control groups.

Discussion

Empyema imparts an enormous economic burden on individuals and society. Although previous studies have indicated that multiple factors are related to the onset and development of empyema, emerging evidence suggests that empyema is associated with the oral microbiome. One of the key findings of this study was that alpha diversity was significantly low in patients with empyema and the microbial composition differed between empyema patients and controls. We identified distinct taxonomic and functional profiles and significant differences in the microbiome structure between patients with empyema and controls.

Recently, there has been growing attention on the association between respiratory diseases and dysbiosis in oral microecosystems (43). However, the potential association between the oral microbiome and pleural infections has not yet been evaluated. To address this issue, it is necessary to determine the optimal site for oral microbiome sampling. Unlike the gut microbiome, various sites within the mouth exhibit differences in bacterial community characteristics despite their proximity, owing to factors, such as gene mutations, changes in pH, and interactions among the bacteria (44). In our study, the microbiota composition across all four sites was generally similar, but with small-scale differences. Although not statistically significant, the salivary microbiome exhibited a high diversity and a notable number of unique and shared bacterial species. Additionally, its similarity to the pleural microbiome of the control group led to the selection of saliva as a representative oral sample for this study. Saliva has proven to be the foremost compartment for exploring variations in microbial composition in various human diseases because it effectively “captures” the most accurate representation of the microbiome within the oral cavity (45, 46). However, to assess the rationale of this approach, it is necessary to further evaluate, with a larger sample size, the extent to which the variability in each oral site contributes to the heterogeneity of the pleural microbiome.

The oral cavity serves as the primary gateway for the microbiome to enter the human

body. Therefore, the microbiome in this area has the potential to spread to different body sites. The oral commensal microbiome, through harmony within itself and defense mechanisms against pathogenic microbes, regulates and maintains the host immune system (47). At the population level, oral microbiomes with higher diversity exhibit increased resistance to pathogenic microbes or antibiotics. In contrast, low beta diversity in the oral microbiome suggests a high degree of homogeneity and conservation among the microbiomes of different hosts (48). In other words, high alpha diversity and low beta diversity ensure a certain degree of resilience and resistance in maintaining the core functions of the resident microbiota in hosts with minimal variation in microbial composition despite other influencing factors (49). However, any disruption in the immune system balance can alter the symbiotic relationship, resulting in the extensive colonization and growth of opportunistic pathogens. These conditioned pathogens may initiate pathogenic processes that eventually lead to various diseases (50). Conversely, dysbiosis of the oral microbiome affects the immune system and potentially exacerbates immune disorders, suggesting that oral microbiome plays a role in the pathogenesis of various diseases. The transition from microbial balance or "symbiosis" to imbalance or "dysbiosis" due to various factors may contribute to disease pathogenesis via systemic inflammatory reactions (51). This study found a significant shift in oral microbiome communities between patients with empyema and controls. Notably, *Rothia* and *Neisseria* genera, known for their beneficial roles in maintaining NO host homeostasis, were significantly more abundant in the control group (52). Conversely, the *Veillonella* genera, which are associated with disruptions in homeostasis, exhibited a high prevalence among patients with empyema. The exclusive presence of *Enterococcus* and *Corynebacterium* in patients with empyema suggests that they may serve as microbial markers of susceptibility to pleural empyema. These microorganisms are typically removed from the mouth; however, they can persist in patients with compromised immune responses, which could explain their presence in patients with empyema. Interestingly, a detailed analysis of microbial diversity

between the two groups revealed significant differences. Patients with empyema demonstrated a notable decrease in diversity and richness compared to the control group, which is consistent with previous research findings (53, 54). Beta diversity also demonstrated marked differences in the microbial composition of patients with empyema and controls. Ultimately, a decrease in alpha diversity and proliferation of opportunistic pathogens within the oral microbiome may be involved in the progression of pleural infections. It is plausible that the infection-promoting effects of the oral microbiome on pleural effusion are attributable to overall dysbiosis, rather than to a specific pathogen. However, precise changes in the bacterial communities during the onset and progression of pleural infections could not be confirmed. Therefore, longitudinal or new cross-sectional studies are required to validate our results and elucidate the relationship among microbial colonization, dysbiosis, and infectious diseases.

Interestingly, previous studies have reported similarities between the composition of normal lungs and oral microbiome (55, 56). Our study also confirmed the similarity in the composition of pleural and oral microbiota in the control group, indicating that oral microbes mainly enter the thoracic cavity via subclinical microaspiration (57). These observations are clinically significant, as they suggest that selective pressure on bacterial reproduction rates may make a minimal contribution to the community composition of the pleural microbiome in the control group, mainly determined by immigration and elimination (58, 59). Selective pressure is the extent to which organisms adapt and reproduce in response to environmental changes. When microbiomes encounter a new environment, such as an antibiotic treatment, they experience significant selective pressure to evolve for better growth under new conditions. This results in substantial changes in microbial composition (60). However, the low beta diversity in oral and pleural microbiomes suggests homogeneity and conservation among microbiomes from different regions. In other words, the stability of oral and pleural microbiomes is likely maintained through a delicate balance between bacterial immigration and elimination. Conversely, the proximity and continuity of the oral cavity, low respiratory

tract, and pleural cavity imply that the oral microbiome could be a potential determinant of the pleural microbiome. Indeed, the oral microbiome may be the driving force underlying bacterial transformation through direct or indirect regulation of mucosal immunity, thereby affecting pathogenicity (61). Patients with a history of alcohol abuse, intravenous drug use, difficulty swallowing, and low awareness commonly exhibit poor oral hygiene. Typically, these risk factors increase the likelihood of bacterial community accumulation in the oral cavity, leading to the development of pleural effusion, particularly empyema (10, 62).

Although traditional culture-dependent pathogen detection methods are clinically meaningful, they are often associated with low specificity and sensitivity due to prior antibiotic treatment and challenges in culturing specific bacterial species. In a previous study, NGS successfully identified potential bacteria in nearly all effusion samples, but the culture positivity rate was only 42%. Our study, compared with NGS, also showed a positivity rate of 50%, similar to the earlier findings. Therefore, unlike traditional culture methods, the pathogen profiling results from NGS enable clinicians to select antibiotics with improved precision. The use of NGS analysis in the management of pleural infections has been reported to reduce antibiotic resistance, shorten hospital stays, and decrease healthcare expenditures through precise antibiotic administration. However, 16S rRNA sequencing methods have limitations that significantly limit their clinical applicability. For example, 16S rRNA methods face challenges in accurately distinguishing DNA from viable and non-viable organisms. Therefore, initial detection does not necessarily indicate a requirement for treatment and continued detection does not imply the need to prolong antimicrobial therapy in patients already undergoing treatment. In addition, 16S rRNA analysis results are susceptible to sequencing noise and DNA contamination when applied to samples with low microbial biomass, such as those from the pleural and lung environments. In this context, microbial RNA metatranscriptome sequencing may be particularly advantageous. These methods are generally effective in indicating the presence of viable microbes because detecting microbial RNA

implies viability, and RNA undergoes rapid degradation after cell lysis.

Most metabolites in the human body are thought to originate from non-human sources, implying that changes in microbial metabolic activity can directly affect human health. We verified the inferred metagenomic functions of the oral microbiome using 16S rRNA data. Despite the small sample size, we observed differences between the two groups using the LEfSe analysis. Our findings demonstrated that xenobiotic biodegradation and metabolism pathways, amino acid metabolism, and two-component systems were overrepresented in patients with empyema compared to those in controls. Increased metabolic activity associated with xenobiotic biodegradation and metabolic pathways in the oral cavity of patients with empyema is not surprising. Such metabolites typically arise from the release of industrial compounds, and multiple studies have consistently reported that this phenomenon is directly associated with high rates of respiratory diseases following exposure to poor air quality (63). Recent studies have reported an association between air pollution and pneumonia-related pleural effusion (64). Pathways associated with amino acid metabolism were significantly over-represented in the empyema group. Previous studies have demonstrated the crucial role of commensal microbiota in the synthesis and extraction of available amino acids as well as their association with intestinal dysbiosis and protein-energy malnutrition (65). Thus, the oral microbiome influences the nutritional status of the host by participating in amino acid extraction and metabolism. Consequently, this interaction suggests a significant interdependence between commensal microbiota and the host. In our study, two-component systems (TCS) were predominant in the oral microbiome of patients with empyema. Several studies have reported that TCS is closely related to biofilm synthesis, growth, and bacterial virulence and viability (66). In addition, the expression of antibiotic resistance determinants in pathogenic bacteria may be regulated by TCS (67). This suggests that the oral microbiome of patients with empyema is likely to actively adapt to various environments, thereby affecting pleural infections. These findings suggest that the functional potential of the oral microbiome

may be imbalanced because of microbial differences in composition between patients with empyema and controls. However, because PICRUSt2 only predicts microbial function based on 16S rRNA reads, additional powerful work, such as dual-transcriptomic approaches, is required in a longitudinal study to confirm these results. There are some limitations to consider when interpreting our findings. First, our study was limited by its single-center design and the available sample size, which allowed us to identify informative variability in the microbiota profiles. Based on the microbiome-based power calculation, the current sample size of 20 resulted in a post-hoc power of 95.4%, considering an α-error of 0.05. We identified significant microbial differences among the different groups, even with this small sample size. Second, this study used a cross-sectional design that displayed the microbiome community at a single time point. Thus, there are limitations in definitively explaining the causal relationship between oral dysbiosis and infection progression. Additional longitudinal studies are required to determine whether oral dysbiosis plays a causative role in pleural empyema or is a consequence of its onset of pleural empyema. Exploration of these differences may help reveal the underlying molecular mechanisms that drive the initiation and progression of pleural empyema and unmask the infectious processes that lead to pleural empyema. Third, the study was confined to a single racial group (Asians), potentially limiting generalizability, as microbiota composition can vary according to racial characteristics. Fourth, data on inflammation or immune markers related to pleural empyema are missing. Future studies should incorporate flow cytometry for immune markers to provide further evidence of changes in bacterial community composition and diversity. Despite strict enrolment criteria, the overall oral health parameters of the participants were unidentified. This study did not cover specific confounding factors, such as oral health parameters, diet, lifestyle, and family history.

Despite these limitations, our findings have important implications for clinical practice. To our knowledge, this is the first study to assess compositional changes (dysbiosis) and predict KEGG pathways in the oral and pleural microbiomes of patients with empyema and controls.

Based on our preliminary findings, further investigation into the potential role of the oral microbiome in empyema progression is required. Furthermore, our study extends this observation to patients with empyema and may facilitate the development of clinical therapeutic strategies for monitoring and altering oral dysbiosis in patients with pleural effusion. Our results suggest that patients with oral microbiome dysbiosis are at high risk for pleural empyema. Hence, these patients may require intensive monitoring and care to improve their outcomes. We expect that our findings will contribute to our understanding of the oral microbiome in patients with pleural empyema.

Conclusions

We conducted a comprehensive characterization of the oral and pleural microbiomes in individuals diagnosed with pleural empyema and juxtaposed them with a control group. Our investigations revealed distinct bacterial communities and pronounced variations in composition, structure, and metabolic attributes between the cohorts. Moreover, our analysis indicated that dysbiosis, marked by a reduction in autochthonous bacterial abundance and shifts in bacterial functionality, may contribute to the pathogenesis of pleural empyema. These findings underscore the necessity for further investigation to deepen our understanding of the intricate interplay between the oral microbiome and empyema pathology.

References

1. Roy B, Shak HJ, Lee YCGJJoL, Medicine P. Pleural fluid investigations for pleural infections. 2021. 2021;6.

2. Grijalva CG, Zhu Y, Nuorti JP, Griffin MR. Emergence of parapneumonic empyema in the USA. Thorax. 2011;66(8):663-8.

3. Corcoran JP, Wrightson JM, Belcher E, DeCamp MM, Feller-Kopman D, Rahman NM. Pleural infection: past, present, and future directions. The Lancet Respiratory Medicine. 2015;3(7):563-77.

4. Strange C, Sahn SA. The definitions and epidemiology of pleural space infection. Seminars in respiratory infections. 1999;14(1):3-8.

5. Chalmers JD, Singanayagam A, Murray MP, Scally C, Fawzi A, Hill AT. Risk factors for complicated parapneumonic effusion and empyema on presentation to hospital with community-acquired pneumonia. 2009;64(7):592-7.

6. ALEMÁN C, ALEGRE J, MONASTERIO J, SEGURA RM, ARMADANS L, ANGLÉS A, et al. Association between inflammatory mediators and the fibrinolysis system in infectious pleural effusions. Clinical Science. 2003;105(5):601-7.

7. Alemán C, Alegre J, Monasterio J, Segura RM, Armadans L, Anglés A, et al. Association between inflammatory mediators and the fibrinolysis system in infectious pleural effusions. Clinical science (London, England : 1979). 2003;105(5):601-7.

8. Idell S, Girard W, Koenig KB, McLarty J, Fair DS. Abnormalities of pathways of fibrin turnover in the human pleural space. The American review of respiratory disease. 1991;144(1):187-94.

9. Kroegel C, Antony VB. Immunobiology of pleural inflammation: potential implications for pathogenesis, diagnosis and therapy. The European respiratory journal. 1997;10(10):2411-8.

10. Lisboa T, Waterer GW, Lee YC. Pleural infection: changing bacteriology and its implications. Respirology (Carlton, Vic). 2011;16(4):598-603.

11. Tobin CL, Lee YC. Pleural infection: what we need to know but don't. Current opinion in pulmonary medicine. 2012;18(4):321-5.

12. Maskell NA, Batt S, Hedley EL, Davies CW, Gillespie SH, Davies RJ. The bacteriology of pleural infection by genetic and standard methods and its mortality significance. American journal of respiratory and critical care medicine. 2006;174(7):817-23.

13. Birkenkamp K, O'Horo JC, Kashyap R, Kloesel B, Lahr BD, Daniels CE, et al. Empyema management: A cohort study evaluating antimicrobial therapy. The Journal of infection. 2016;72(5):537-43.

14. Corcoran JP, Wrightson JM, Belcher E, DeCamp MM, Feller-Kopman D, Rahman NM. Pleural infection: past, present, and future directions. The Lancet Respiratory medicine. 2015;3(7):563-77.

15. Dyrhovden R, Nygaard RM, Patel R, Ulvestad E, Kommedal Ø. The bacterial aetiology of pleural empyema. A descriptive and comparative metagenomic study. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2019;25(8):981-6.

16. CUTLER EC. THE EXPERIMENTAL PRODUCTION OF ABSCESS OF THE LUNG. American Journal of Diseases of Children. 1929;38(4):683-98.

17. Kobashi Y, Mouri K, Yagi S, Obase Y, Oka M. Clinical analysis of cases of empyema due to Streptococcus milleri group. Japanese journal of infectious diseases. 2008;61(6):484-6.

18. Sudhakara P, Gupta A, Bhardwaj A, Wilson A. Oral Dysbiotic Communities and Their Implications in Systemic Diseases. Dentistry journal. 2018;6(2).

19. Tlaskalová-Hogenová H, Stěpánková R, Kozáková H, Hudcovic T, Vannucci L, Tučková L, et al. The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-

free and gnotobiotic animal models of human diseases. Cellular & molecular immunology. 2011;8(2):110-20.

20. Tanner A, Maiden M, Macuch P, Murray L, Kent Jr RJJocp. Microbiota of health, gingivitis, and initial periodontitis. 1998;25(2):85-98.

21. Yang S-F, Huang H-D, Fan W-L, Jong Y-J, Chen M-K, Huang C-N, et al. Compositional and functional variations of oral microbiota associated with the mutational changes in oral cancer. Oral Oncology. 2018;77:1-8.

22. Segata N, Haake SK, Mannon P, Lemon KP, Waldron L, Gevers D, et al. Composition of the adult digestive tract bacterial microbiome based on seven mouth surfaces, tonsils, throat and stool samples. Genome biology. 2012;13(6):R42.

23. Bartlett JG. How important are anaerobic bacteria in aspiration pneumonia: when should they be treated and what is optimal therapy. Infectious disease clinics of North America. 2013;27(1):149-55.

24. El-Solh AA, Pietrantoni C, Bhat A, Aquilina AT, Okada M, Grover V, et al. Microbiology of severe aspiration pneumonia in institutionalized elderly. American journal of respiratory and critical care medicine. 2003;167(12):1650-4.

25. Azarpazhooh A, Leake JL. Systematic review of the association between respiratory diseases and oral health. Journal of periodontology. 2006;77(9):1465-82.

26. Ren Y, Liang J, Li X, Deng Y, Cheng S, Wu Q, et al. Association between oral microbial dysbiosis and poor functional outcomes in stroke-associated pneumonia patients. BMC Microbiology. 2023;23(1):305.

27. Dyrhovden R, Eagan TM, Fløtten Ø, Siljan W, Leegaard TM, Bø B, et al. Pleural empyema caused by Streptococcus intermedius and Fusobacterium nucleatum - a distinct entity of pleural infections. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2023.

28. Dalil Roofchayee N, Marjani M, Dezfuli NK, Tabarsi P, Moniri A, Varahram M, et

al. Potential diagnostic value of pleural fluid cytokines levels for tuberculous pleural effusion. Scientific reports. 2021;11(1):660.

29. Bäumler AJ, Sperandio V. Interactions between the microbiota and pathogenic bacteria in the gut. Nature. 2016;535(7610):85-93.

30. Zhao T, Wei Y, Zhu Y, Xie Z, Hai Q, Li Z, et al. Gut microbiota and rheumatoid arthritis: From pathogenesis to novel therapeutic opportunities. Frontiers in immunology. 2022;13:1007165.

31. Pathak JL, Yan Y, Zhang Q, Wang L, Ge L. The role of oral microbiome in respiratory health and diseases. Respiratory medicine. 2021;185:106475.

32. Mayhew D, Devos N, Lambert C, Brown JR, Clarke SC, Kim VL, et al. Longitudinal profiling of the lung microbiome in the AERIS study demonstrates repeatability of bacterial and eosinophilic COPD exacerbations. Thorax. 2018;73(5):422-30.

33. Dickson RP, Erb-Downward JR, Prescott HC, Martinez FJ, Curtis JL, Lama VN, et al. Cell-associated bacteria in the human lung microbiome. Microbiome. 2014;2(1):28.

34. Gilbreath JJ, Semino-Mora C, Friedline CJ, Liu H, Bodi KL, McAvoy TJ, et al. A core microbiome associated with the peritoneal tumors of pseudomyxoma peritonei. Orphanet journal of rare diseases. 2013;8(1):105.

35. Mason KL, Erb Downward JR, Mason KD, Falkowski NR, Eaton KA, Kao JY, et al. Candida albicans and bacterial microbiota interactions in the cecum during recolonization following broad-spectrum antibiotic therapy. Infection and immunity. 2012;80(10):3371-80.

36. Kim JY, Yi MH, Kim M, Lee S, Moon HS, Yong D, et al. Measuring the absolute abundance of the microbiome by adding yeast containing 16S rRNA gene from a hyperthermophile. MicrobiologyOpen. 2021;10(4):e1220.

37. Pielou EC. The measurement of diversity in different types of biological collections. Journal of Theoretical Biology. 1966;13:131-44.

38. Goodrich JK, Di Rienzi SC, Poole AC, Koren O, Walters WA, Caporaso JG, et al.

Conducting a microbiome study. Cell. 2014;158(2):250-62.

39. Navas-Molina JA, Peralta-Sánchez JM, González A, McMurdie PJ, Vázquez-Baeza Y, Xu Z, et al. Advancing our understanding of the human microbiome using QIIME. Methods in enzymology. 2013;531:371-444.

40. Langille MG, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nature biotechnology. 2013;31(9):814-21.

41. Xia Y, Sun J. Hypothesis Testing and Statistical Analysis of Microbiome. Genes & diseases. 2017;4(3):138-48.

42. Staley C, Sadowsky MJ. Practical considerations for sampling and data analysis in contemporary metagenomics-based environmental studies. Journal of microbiological methods. 2018;154:14-8.

43. Mark Welch JL, Dewhirst FE, Borisy GG. Biogeography of the Oral Microbiome: The Site-Specialist Hypothesis. Annual review of microbiology. 2019;73:335-58.

44. Sultan AS, Kong EF, Rizk AM, Jabra-Rizk MA. The oral microbiome: A Lesson in coexistence. PLoS pathogens. 2018;14(1):e1006719.

45. Farrell JJ, Zhang L, Zhou H, Chia D, Elashoff D, Akin D, et al. Variations of oral microbiota are associated with pancreatic diseases including pancreatic cancer. 2012;61(4):582-8.

46. Yang F, Zeng X, Ning K, Liu K-L, Lo C-C, Wang W, et al. Saliva microbiomes distinguish caries-active from healthy human populations. 2012;6(1):1-10.

47. Hans M, Madaan Hans V. Epithelial antimicrobial peptides: guardian of the oral cavity. International journal of peptides. 2014;2014:370297.

48. D'Agostino S, Ferrara E, Valentini G, Stoica SA, Dolci M. Exploring Oral Microbiome in Healthy Infants and Children: A Systematic Review. International journal of environmental research and public health. 2022;19(18).

49. Rosier BT, Marsh PD, Mira A. Resilience of the Oral Microbiota in Health: Mechanisms That Prevent Dysbiosis. Journal of dental research. 2018;97(4):371-80.

50. Xun Z, Zhang Q, Xu T, Chen N, Chen FJFim. Dysbiosis and ecotypes of the salivary microbiome associated with inflammatory bowel diseases and the assistance in diagnosis of diseases using oral bacterial profiles. 2018;9:1136.

51. Brenner DR, McLaughlin JR, Hung RJ. Previous lung diseases and lung cancer risk: a systematic review and meta-analysis. PloS one. 2011;6(3):e17479.

52. Marsh PD. In Sickness and in Health-What Does the Oral Microbiome Mean to Us? An Ecological Perspective. Advances in dental research. 2018;29(1):60-5.

53. Libertucci J, Young VB. The role of the microbiota in infectious diseases. Nature Microbiology. 2019;4(1):35-45.

54. Soffritti I, D'Accolti M, Fabbri C, Passaro A, Manfredini R, Zuliani G, et al. Oral Microbiome Dysbiosis Is Associated With Symptoms Severity and Local Immune/Inflammatory Response in COVID-19 Patients: A Cross-Sectional Study. 2021;12.

55. Bassis CM, Erb-Downward JR, Dickson RP, Freeman CM, Schmidt TM, Young VB, et al. Analysis of the upper respiratory tract microbiotas as the source of the lung and gastric microbiotas in healthy individuals. mBio. 2015;6(2):e00037.

56. Segal LN, Alekseyenko AV, Clemente JC, Kulkarni R, Wu B, Gao Z, et al. Enrichment of lung microbiome with supraglottic taxa is associated with increased pulmonary inflammation. Microbiome. 2013;1(1):19.

57. Dickson RP, Erb-Downward JR, Freeman CM, McCloskey L, Falkowski NR, Huffnagle GB, et al. Bacterial Topography of the Healthy Human Lower Respiratory Tract. mBio. 2017;8(1).

58. Charlson ES, Bittinger K, Haas AR, Fitzgerald AS, Frank I, Yadav A, et al. Topographical continuity of bacterial populations in the healthy human respiratory tract. American journal of respiratory and critical care medicine. 2011;184(8):957-63.

59. Bahrani-Mougeot FK, Paster BJ, Coleman S, Barbuto S, Brennan MT, Noll J, et al. Molecular analysis of oral and respiratory bacterial species associated with ventilator-associated pneumonia. Journal of clinical microbiology. 2007;45(5):1588-93.

60. Baishya J, Wakeman CA. Selective pressures during chronic infection drive microbial competition and cooperation. npj Biofilms and Microbiomes. 2019;5(1):16.

61. Li Y, Wang K, Zhang B, Tu Q, Yao Y, Cui B, et al. Salivary mycobiome dysbiosis and its potential impact on bacteriome shifts and host immunity in oral lichen planus. International journal of oral science. 2019;11(2):13.

62. Shinzato T, Uema H, Inadome J, Shimoji K, Kusano N, Fukuhara H, et al. [Bacteriological and clinical studies in 23 cases of thoracic empyema--the role of oral streptococci and anaerobes]. Nihon Kyobu Shikkan Gakkai zasshi. 1993;31(4):486-91.

63. Manisalidis I, Stavropoulou E, Stavropoulos A, Bezirtzoglou E. Environmental and Health Impacts of Air Pollution: A Review. 2020;8.

64. Bai KJ, Chuang KJ, Chen JK, Tsai CY, Yang YL, Chang CC, et al. Alterations by Air Pollution in Inflammation and Metals in Pleural Effusion of Pneumonia Patients. International journal of environmental research and public health. 2019;16(5).

65. Guda P, Guda C, Subramaniam S. Reconstruction of pathways associated with amino acid metabolism in human mitochondria. Genomics, proteomics & bioinformatics. 2007;5(3-4):166-76.

66. Sultan M, Arya R, Kim KK. Roles of Two-Component Systems in Pseudomonas aeruginosa Virulence. International journal of molecular sciences. 2021;22(22).

67. Tiwari S, Jamal SB, Hassan SS, Carvalho P, Almeida S, Barh D, et al. Two-Component Signal Transduction Systems of Pathogenic Bacteria As Targets for Antimicrobial Therapy: An Overview. Frontiers in microbiology. 2017;8:1878.

국문 요약

배경: 다양한 질병의 진행과 관련된 구강 미생물 군집에 대한 연구는 점점 더 늘어나고 있다. 그러나 흉막 감염 환자의 구강 미생물 군집에 관한 체계적인 연구에 대해서는 아직까지 알려져 있지 않다. 따라서 본 연구에서는 16S 리보솜 RNA 유전자 시퀀싱을 기반으로, 흉막 감염환자의 흉수와 비 감염 환자의 흉강 및 구강 내 미생물 구성 및 특성을 평가하고자 한다.

방법: 총 20 명 (흉막 감염군 10 명, 대조군 10 명)이 연구에 참여하였으며, 이들 모두에서, 구강 내 샘플 (타액, 잇몸, 혀, 볼 점막)과 흉수를 채취하였다. 각 샘플에서 유전자 DNA 추출 후, 16S 리보솜 rRNA 유전자 상의 V3-V4 초가변영역을 대상으로 polymerase chain reaction 증폭 및 정제하고 차세대 염기서열 분석장비를 이용해 메타지놈 시퀀싱 및 생물정보학적 분석이 수행되었다. 환자의 의료 기록은 후향적으로 검토하였다.

결과: 구강 샘플 부위 간의 구강 내 미생물의 알파 다양성 비교에서 모두 풍요도(richness)와 균등도(evenness)에서 유의한 차이를 보이지 않았으나, 상대적으로 타액 샘플에서 차이가 나는 경향을 보였다. 베타 다양성 분석 역시 각 샘플 부위 간의 클러스터링 양상이 유사함을 보였다. 흉막 감염군과 대조군 사이의 구강 및 흉강 내 미생물 군집 비교에서는 상당한 차이를 보였다. 흉막 감염 군은 대조군에 비해 알파 및 베타 다양성이 유의하게 낮았다. 미생물의 구성 역시 차이가 있었으며, 흉막 감염군은 대조군에 비해 구강 미생물 군집에서 *Acinetobacter, Staphylococcus* 및 *Enterococcus* 속이 증가되어 있었다. 또한 구강 미생물의 기능적인 프로파일을 분석하였을 때, 흉막 감염 군에서는 주로 two-compartment system, 아미노산 대사, 그리고 xenobiotic 생분해 및 대사 경로가 우세하였다.

결론: 본 연구에서는 흉막 감염 환자의 구강 미생물 군집이 비 감염 환자의 구강 미생물 군집과 차이가 있음을 확인하였고, 흉막 감염 환자의 구강 및 흉강 내 미생물 군집의 대사 기능을 예측하였다. 이러한 발견은 흉수를 가진

환자에서 미생물 구성의 변화가 흉막 감염의 발병 및 진행과 연관이 있을 수 있음을 시사한다.

중심 단어: 세균, 흉막 감염, 미생물 군집, 구강 건강