TITLE

MICROBIOLOGICAL, IMMUNOLOGICAL, AND BIOCHEMICAL FEATURES OF MECHANICAL **V**ENTILATION-**A**SSOCIATED **P**NEUMONIA (**MICROVAP**) DEVELOPMENT

A

DISSERTATION

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By

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DEDICATION

To my parents, brothers, nephew, and niece, who have taught me the importance of love, perseverance, and strength. During this time, they have shown me that the path to success is built within the family and is a challenge shared by all. I also want to dedicate this work to my friends and team, who have allowed me to learn not only in the professional realm but also on a personal level. My greatest lesson is that one never reaches anywhere alone, as you will always have the support of your family, colleagues, friends, and mentors who will guide and support you in difficult times.

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ABSTRACT (ENGLISH)

This study focuses on the complex relationship between positive pressure mechanical ventilation, the pulmonary microbiome, cytokine dynamics, and metabolomic profiles in patients with ventilator-associated pneumonia (VAP). Our research emphasizes how alterations in microbial dynamics are associated with the onset of VAP, underscoring the pivotal role of microbiomes in maintaining respiratory health, particularly in the context of viral respiratory diseases such as COVID-19. We observed significant variations in microbial abundances, which have implications for the development and progression of VAP. Furthermore, our analysis of cytokine dynamics revealed a distinctive immunological modulation in VAP patients, characterized by an enhanced response of neutralizing antibodies coupled with reduced levels of proinflammatory cytokines. Additionally, our metabolomic analysis provides deeper insights into the metabolic adaptations during the progression of VAP, offering perspectives on tissue repair processes and stress responses. Collectively, our investigation contributes valuable knowledge on the pathogenesis of VAP and emphasizes the importance of maintaining a balanced respiratory microbiome and an appropriate immune response to mitigate adverse outcomes associated with mechanical ventilation and the development of VAP. Further studies are required to fully elucidate the underlying mechanisms and potential therapeutic targets to improve patient outcomes in critical care settings.

ABSTRACT (SPANISH)

El presente estudio se enfoca en la compleja relación entre la ventilación mecánica con presión positiva, el microbioma pulmonar, la dinámica de citoquinas y los perfiles metabolómicos en pacientes con neumonía asociada a ventilación (VAP). Nuestra investigación destaca cómo las alteraciones en la dinámica microbiana están asociadas con el inicio de VAP, subrayando el papel crucial de los microbiomas en el mantenimiento de la salud respiratoria, especialmente en el contexto de enfermedades respiratorias virales como COVID-19. Observamos variaciones significativas en las abundancias microbianas, lo que tiene implicaciones en el desarrollo y progresión de VAP. Además, el análisis de la dinámica de citoquinas reveló una modulación inmunológica distintiva en pacientes con VAP, caracterizada por una respuesta aumentada de anticuerpos neutralizantes junto con niveles reducidos de citoquinas proinflamatorias. Adicionalmente, nuestro análisis metabolómico proporciona una comprensión más profunda de las adaptaciones metabólicas durante la progresión de VAP, ofreciendo perspectivas sobre los procesos de reparación tisular y las respuestas al estrés. En conjunto, nuestra investigación aporta valiosos conocimientos sobre la patogénesis de VAP y enfatiza la importancia de mantener un microbioma respiratorio equilibrado y una respuesta inmune adecuada para mitigar los resultados adversos asociados con la ventilación mecánica y el desarrollo de VAP. Se necesitan más estudios para esclarecer completamente los mecanismos subyacentes y los posibles objetivos terapéuticos para mejorar los resultados de los pacientes en entornos de cuidados críticos.

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Understanding the presence and function of a diverse lung microbiome in acute lung infections, particularly ventilator-associated pneumonia (VAP), is still limited, evidencing significant gaps in our knowledge.

1. Areas Covered**:** In this comprehensive narrative review, we aim to elucidate the contribution of the respiratory microbiome in the development of VAP by examining the current knowledge on the interactions among microorganisms. By exploring these intricate connections, we endeavor to enhance our understanding of the disease's pathophysiology and pave the way for novel ideas and interventions in studying the respiratory tract microbiome.
2. Expert Opinion: The conventional perception of lungs as sterile is deprecated since it is currently recognized the existence of a diverse microbial community within them. However, despite extensive research on the role of the respiratory microbiome in healthy lungs, respiratory chronic diseases, and acute lung infections such as pneumonia are not fully understood. It is crucial to investigate further the relationship between the pathophysiology of VAP and the pulmonary microbiome, elucidating the mechanisms underlying the interactions between the microbiome, host immune response, and mechanical ventilation for the development of VAP.

Keywords: Lung microbiome, Lung microbiota, Dysbiosis, immune response, ventilator-associated pneumonia, mechanical ventilation.

3. Articles highlights

- The lung microbiome is a diverse and dynamic ecosystem unique to everyone. Current knowledge is based on the description of bacterial communities. Still, it is crucial to characterize the virome and mycobiome, connecting the clinical outcomes associated with each patient with VAP.

- Ventilator-associated pneumonia (VAP) is a serious complication in intensive care units, but identifying its causative agent is challenging due to limitations in traditional tests. Exploring the dynamics of microbial communities during VAP development through techniques like DNA sequencing offers new opportunities for understanding its relevance.

- The oral microbiome plays a pivotal role in developing VAP, primarily through the microaspiration of microorganisms during mechanical ventilation. Additionally, the role of respiratory colonization by *Candida spp*. may have clinical implications in VAP, although its precise role remains intricate and multifaceted.

- The development of VAP involves complex interactions between the lung microbiome and host immune responses. Multifaceted interactions suggest that several factors influence the development of VAP beyond the presence of a single pathogen.

- To gain a deeper understanding of the microbial communities linked to ventilator-associated pneumonia (VAP), adopting a comprehensive multibiome approach is crucial by integrating several omics techniques. These robust methodologies will enable us to unravel the essential characteristics of the VAP-associated microbiome.

- Probiotics have emerged as a promising strategy for preventing and treating VAP. Their administration can aid in restoring the balance of the lung microbiome, thereby

reducing the colonization of pathogenic bacteria in the lungs and modulating the immune response.

A. Introduction

The human microbiome, found in various parts of the body such as the skin, mouth, gut, and reproductive tract, interacts with the immune system and protects against harmful pathogens, playing a crucial role in maintaining overall health. The microbiome presents challenges for researchers due to the vast microbial diversity and dynamic nature of these communities, which change in response to age, genetics, immune response, diet, and medication. Since approximately 2006, extensive research has been conducted on the gut, skin, and upper respiratory tract microbiomes. However, the recent focus on studying the lung microbiome has made it a crucial and actively researched field, aiming to enhance our understanding of respiratory diseases and develop more accurate methods for their diagnosis and treatment (1-4).

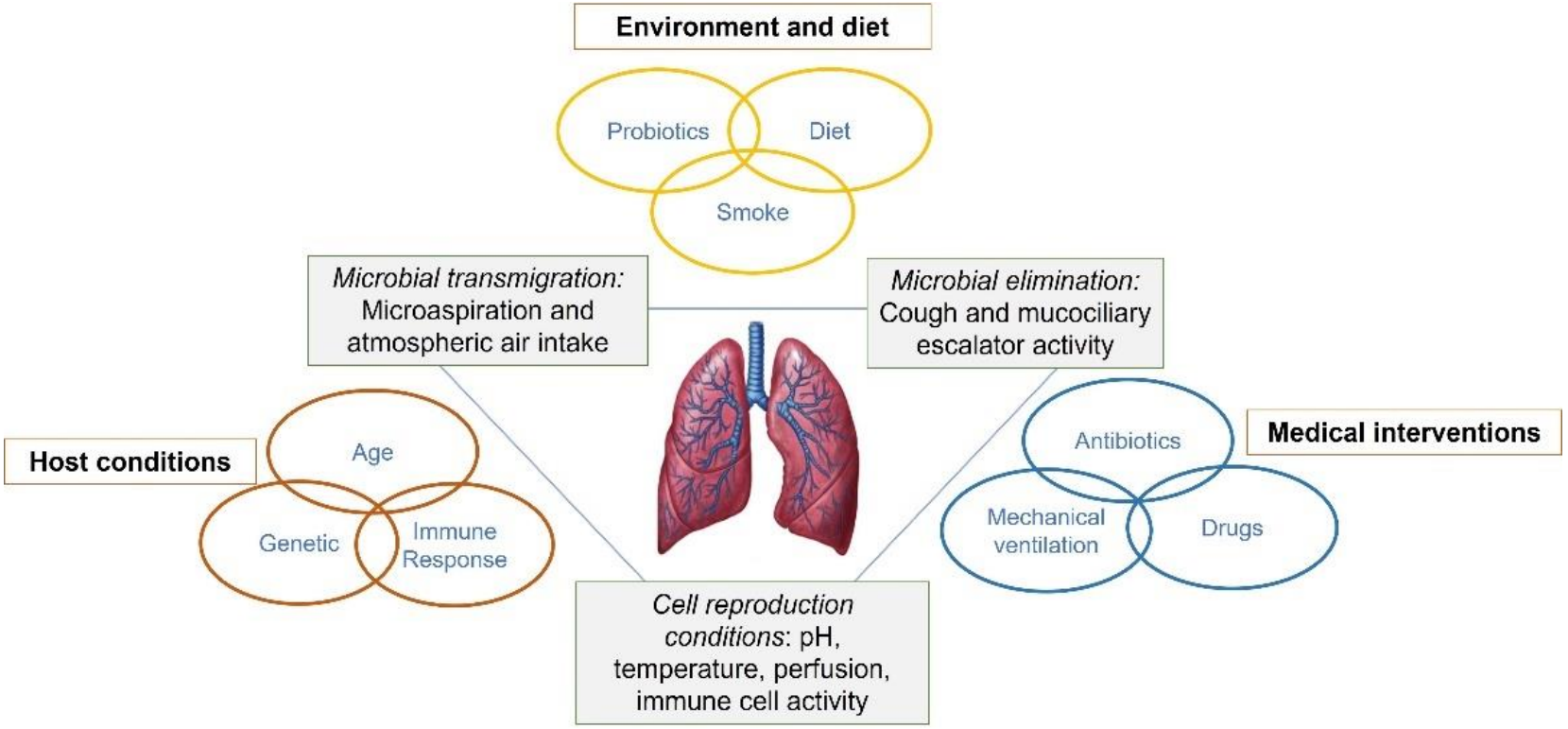
The lung microbiome in healthy individuals constitutes a dynamic and diverse ecosystem, encompassing bacteria, viruses, and fungi, albeit with a lower cellular density than in other microbiomes (5, 6). It primarily originates from the upper respiratory tract and supraglottic regions. Nevertheless, its composition displays individual variation and is intricately linked to lung physiology, immune responses, and clinical outcomes in chronic lung disease patients (7-10). The establishment of the lung microbiome is governed by a balance of at least three ecological mechanisms (11, 12). Firstly, microorganisms are transported from the upper to the lower respiratory tract through the microaspiration of oral

secretions (13, 14). Secondly, microorganisms are cleared through mucociliary clearance, coughing, and the host's immune defenses. Thirdly, these microorganisms carve out their ecological niches, influenced by temperature, mucous secretion, pH levels, nutrient availability, and oxygen levels (15-17).

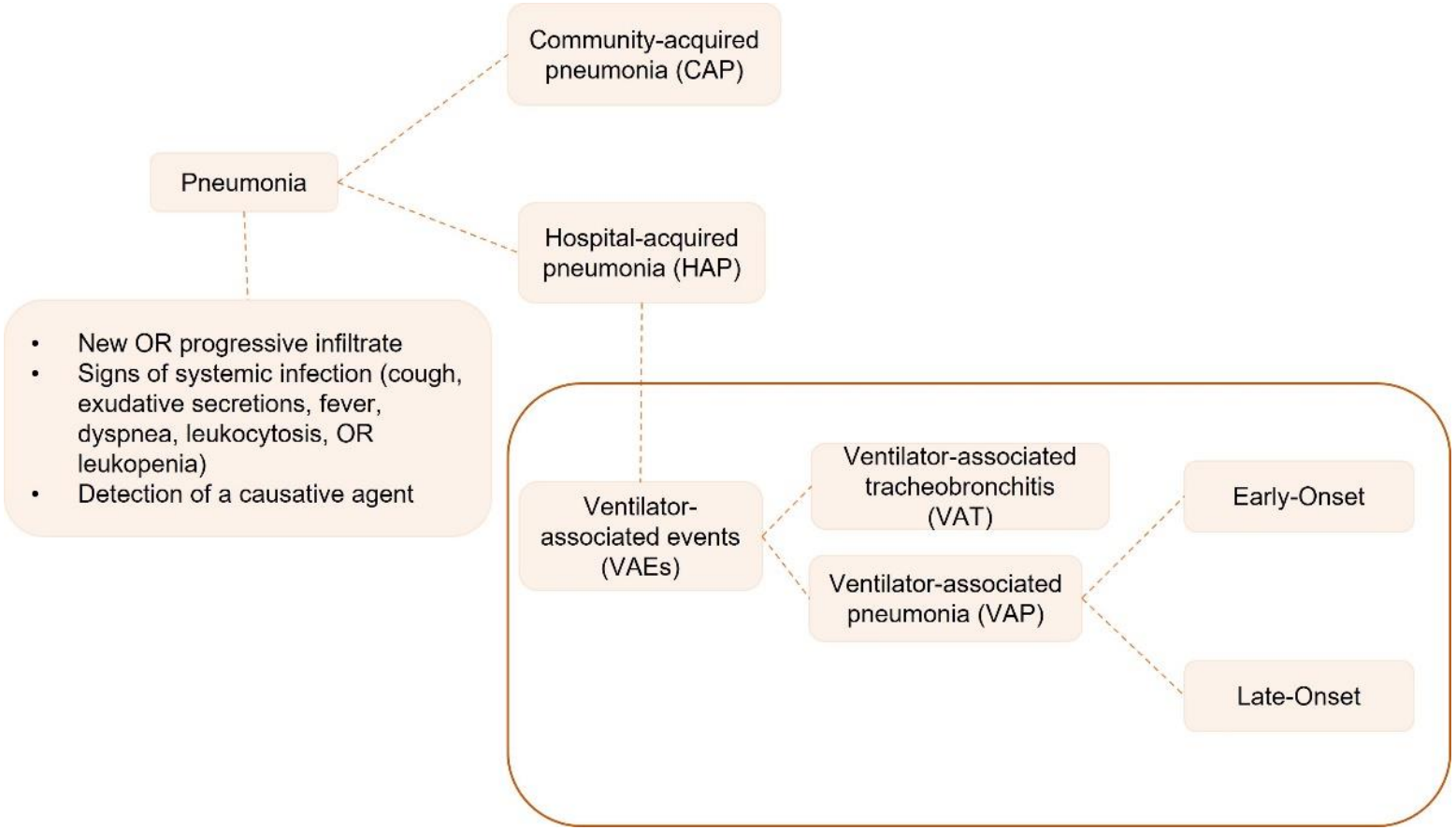
Disruption of the lung microbiota's balance, known as dysbiosis, can trigger acute and chronic lung diseases by provoking the release of proinflammatory cytokines, accelerating tissue aging, and inducing exaggerated immune responses (1-4). In healthy lungs, dysbiosis may result from various factors, including immunosuppression, antibiotic use, toxin exposure, air pollution, smoking, and pre-existing chronic lung diseases (**Fig. 1.1**) (5-7). Nevertheless, the precise cause-and-effect relationship and the mechanisms connecting dysbiosis to the development of pneumonia, particularly in the context of ventilator-associated pneumonia (VAP), are still subjects of ongoing investigation.

Pneumonia, characterized by inflammation of the alveoli, is a leading cause of infectious mortality worldwide and poses significant public health challenges (8-10). This respiratory condition comprises several categories, such as community-acquired pneumonia (CAP) and hospital-acquired pneumonia (HAP), each linked to specific causative agents, therapeutic approaches, and clinical outcomes (**Fig. 1.2**) (11, 12). However, it is essential to recognize the complexity inherent in the accurate classification and diagnosis of pneumonia, particularly ventilator-associated pneumonia (VAP). This complexity stems from variations in clinical presentations, patient comorbidities, and the wide range of microorganisms involved. Therefore, improving our understanding of the categories of

pneumonia, including VAP, is essential to advance patient care and the development of effective preventive strategies.



1. **Figure 1.1:Healthy lungs**. Several factors influence lung microbial community compositions.



2. **Figure 1.2: Classification of pneumonia**. Similarities and differences

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This review aims to present the current evidence regarding the association between the lung microbiome and the development of ventilator-associated pneumonia (VAP), focusing on the pathophysiology and immunology of the disease. Additionally, potential research areas will be discussed, which may guide us toward new approaches for preventing, diagnosing, and treating this significant disease.

1. Lung Microbiome: a new concept in pneumonia

In the not-so-distant past, there was a belief that pneumonia could be prevented, stemming from the mistaken notion that the lungs were devoid of microbial life (11, 14). However, current knowledge has unveiled the intricate and diverse nature of pneumonia. Today, we understand that the lungs harbor various species from the *Proteobacteria, Firmicutes*, and *Bacteroidetes* phyla (15, 16). Dominant genera include *Streptococcus spp., Prevotella spp., Fusobacteria spp.,* and *Veillonella spp*., while *Haemophilus spp*. and *Neisseria spp*. are less common (17-20). Despite the lungs typically presenting a hostile environment for microorganisms, pneumonia disrupts this balance, allowing for rapid infiltration and proliferation (21). This triggers a swift transition into a state of dysbiosis, influencing microbiota abundance, diversity, and composition, closely linked to the host's immune status (22-24).

These changes have been associated with the emergence of lung lesions, such as infiltrates induced by the influx of neutrophils, consolidations, nodules, or cavities. These manifestations can indicate inflammation, infection, or lung tissue damage, depending on the immune response's atypical reaction to invading microorganisms or metabolites

produced by microorganisms (25, 26). However, numerous aspects of these processes remain unknown (27).

2. Ventilator Associated Pneumonia (VAP)

Pneumonia associated with mechanical ventilation (VAP) is a severe complication that can affect a substantial percentage of patients on mechanical ventilation, with rates ranging from 5% to 40% (28). VAP impacts patient health and has significant implications in terms of hospitalization duration, which can extend from 6 to 25 days, and the lengthening of the intubation period, which can reach up to 11 days (29). This, in turn, translates to a substantial increase in hospital costs. Its prevalence varies from 9 to 18 cases per 1,000 ventilator days, mainly depending on hospital-specific prevention strategies, with worrying mortality rates, including a crude mortality rate that can reach up to 76% (30, 31). Moreover, it accounts for a significant proportion of in-hospital deaths, with an attributable impact of up to 13% (32, 33).

Various approaches exist for the prevention of VAP, often incorporated into ventilation bundles that encompass non-pharmacologic measures (such as hand hygiene, oral care, patient positioning, and drainage of subglottic secretions) and pharmacologic measures (such as the use of antibiotics for selective digestive decontamination and probiotics) (34, 35). It's important to note that there is no conclusive evidence to support the efficacy of these strategies, leading to conflicting recommendations in current guidelines. These data underscore the importance of a thorough understanding of this disease and

highlight our significant challenges. This in-depth knowledge is essential for successfully implementing effective measures for preventing and controlling VAP in healthcare settings.

Diagnosing VAP poses significant challenges due to its complexities. It typically occurs in patients undergoing invasive mechanical ventilation for over 48 hours, characterized by new pulmonary infiltrates on radiological images and symptoms such as fever, purulent discharge, leukocytosis, impaired oxygenation, or worsening hypoxia (12, 33, 36, 37). Precise identification of the microbiological cause is crucial for follow-up interventions and appropriate antimicrobial treatment (38). However, these diagnostic criteria present substantial challenges, particularly as mechanically ventilated patients with similar symptoms attributable to other causes complicate the exclusive attribution of symptoms to VAP. Furthermore, we encounter significant obstacles in identifying the etiological cause of Ventilator-Associated Pneumonia (VAP). Factors such as antibiotic effects, challenging-to-culture microorganisms, delayed results, and PCR limitations contribute to the diagnostic process's complexity (39). Innovative culture-independent techniques, like molecular panels, sequencing, and PCR, offer promising alternatives, enabling the detection of various microorganisms, and providing insights into the significance of the pulmonary microbiome (40-43).

3. The lung microbiome and ventilator-associated pneumoniae VAP

3.1 Interaction of the Oral Microbiome and the Development of VAP

The oral microbiome and its influence on the pulmonary microbiome have become crucial areas of study for understanding respiratory infections such as Ventilator Associated

Pneumonia (VAP)(40). The oral microbiome contributes to the development of VAP, mainly through the microaspiration of microorganisms during mechanical ventilation. It starts with colonizing dental plaque and oral mucosa using bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa,* and *Enterobacteriaceae* (44). Supported by risk factors such as those related to the host, healthcare personnel, and devices such as ventilator circuits and endotracheal tubes (45).In healthy individuals, bacterial communities in the upper respiratory tract (URT) and lower respiratory tract (LRT) share similar compositions, predominantly featuring bacterial families like *Prevotella*, *Streptococcaceae*, and *Veillonellaceae* (46, 47). However, intubated patients exhibit greater variability in bacterial communities, indicating a dynamic interaction between the oral and lung microbiomes (46). These oral changes may facilitate the colonization of pathogenic bacteria in the lower respiratory tract (40, 48, 49). Associations have been identified between VAP and oral bacteria such as *Enterococcus faecalis*, *Fusobacterium periodonticum, Gemella morbillorum, Neisseria mucosa, Propionibacterium acnes, Prevotella melaninogenica, Streptococcus oralis, Streptococcus sanguinis,* and *Treponema denticola, Treponema socranscki*i y *Veillonella parvula* (49).

Continuous microaspiration of oropharyngeal colonization into the lower respiratory tract, emerges as a significant pathway for oral microorganisms to colonize the lungs, increasing the risk of pulmonary infections like VAP (50, 51). Dental plaques provide a suitable environment for pathogen growth and accumulation. Following intubation, bacterial density, and biofilm formation in the oral cavity increase (40, 49, 52). Findings from recent studies indicate similarities between oropharyngeal and pulmonary pathogens

in VAP patients (53). Furthermore, research supports the idea that pathogenic oral microorganisms are genetically related to lung isolates in VAP patients, suggesting that the oral microbiome can serve as a reservoir for pathogens that colonize the lower respiratory tract (40, 52). Inadequate oral hygiene has been associated with an increased risk of VAP, highlighting the need for good oral health to prevent respiratory infections (49).

In addition to the oral bacteriome, fungi such as *Candida spp.* play a major role in the respiratory tract of healthy adults. *Candida spp*. is often considered a colonizer in the airways, given the rarity of *Candida pneumonia* (54, 55). However, isolation rates of *Candida spp*. in tracheal aspirates from patients with suspected mechanical ventilation-associated pneumonia (VAP) vary (10%-56%), and its role in VAP remains a matter of debate (53, 56). Some studies suggest non-benign effects, with *Candida* colonization correlating with prolonged mechanical ventilation, increased ICU stay, and increased mortality (53, 57). Other studies support these findings, linking Candida colonization with worse outcomes and an increased risk of *multidrug*-resistant bacterial infections (58, 59). However, conflicting research suggests that it may be a marker of disease severity and immunosuppression (56). Respiratory colonization by *Candida spp*. is common and may have clinical implications in VAP, although its exact role remains complex and multifaceted (58).

3.2 Interaction of the Lung Microbiome and the Development of VAP

Recent research has prominently utilized 16S ribosomal RNA (rRNA) gene sequencing to delve into microbial metataxonomy and profile bacterial communities in both healthy and afflicted individuals, even encompassing atypical microorganisms (41, 60-63). Within 16S

ribosomal RNA (rRNA) gene sequencing, two pivotal metrics come to the forefront: alpha diversity and beta diversity, offering critical insights into the lung microbiota's composition and structure (23, 32). A noteworthy discovery is that reduced alpha diversity, detectable within just 48 hours after tracheal intubation, correlates with extended ventilation time but not necessarily with the onset of ventilator-associated pneumonia (VAP) or the administration of antibiotics (18, 32). Specifically, taxa like *Streptococcus*, *Lactobacillares*, and *Prevotella* at the time of intubation exhibit reduced abundance in subjects who subsequently develop VAP, in contrast to taxa such as *Haemophilus, Moraxella, Streptococcus mitis/pneumoniae, Staphylococcus aureus,* and *Prevotella melaninogenica*, which are more prevalent at the time of intubation (25, 60, 64-66).

Additionally, research reveals that samples from patients with positive microbiological cultures in respiratory specimens or those categorized as microbiota manifest lower alpha diversity, characterized by dominance of 2 or 3 bacterial genera like *Prevotella*, *Staphylococcus,* and *Pseudomonas*. At the same time, some display an overabundance of the genus *Corynebacterium* (64, 67, 68). This underscores the role of microbial communities in modulating growth, virulence, biofilm formation, quorum sensing, and antibiotic resistance, emphasizing that disease assessment should not rely solely on taxon dominance. Beta diversity, influenced by disease presence and severity, may also impact susceptibility to pulmonary infection. However, inter-patient taxonomic variability complicates comparisons (25, 64, 66).

Airway dysbiosis appears to be more pronounced in VAP-developing patients than in those who don't, potentially linked to the relative abundance of genera such as *Burkholderia*, *Bacillales* (with *Staphylococcus aureus* as the prominent species), and to a lesser extent, *Pseudomonadales* (64, 65, 69). Changes in the relative abundance of taxa like *Streptococcus, Lactobacilli,* and *Prevotella* are also implicated in VAP development (21, 23). The presence of anaerobes and facultative anaerobes appears to be affected, potentially linking the absence of these bacteria to increased susceptibility to respiratory infections, especially in older adults. However, consensus on VAP patients' most prevalent bacterial taxa remains elusive (30, 35, 36, 70).

Patients with VAP show elevated levels of both human and bacterial DNA at diagnosis compared to controls with similar ventilation durations, reflecting the presence of host inflammatory cells in VAP or active infections (18, 45, 46). Establishing a specific threshold to distinguish VAP-developing patients from non-developers is an ongoing endeavor. It's essential to note that the mere detection of bacterial DNA doesn't confirm microbial activity, as DNA presence could result from colonization, excretion, or non-infectious deceased organisms (18, 47). Although dysbiosis is notably more pronounced in VAP-developing patients than non-developers, alpha diversity alone may not be a reliable marker for diagnosing lower respiratory tract infections, as certain studies suggest (29) **(Table 1.1)**.

Author	Year	Type of study	Sample origin	Sample size	Patients characteristics	Analysis	Main findings
Fenn D. et al	2022	Post HOC study	ETA, BAL	90 samples	90 (37 with positive culture) IMV adult patients	16S rRNA gene sequencing (V4-V5 hypervariable regions)	Patients with positive cultures exhibited microbiome dysbiosis, reduced alpha diversity, and higher pathogenic bacteria levels (0.45 vs. 0.02). The compositional variance's association with culture positivity had modest accuracy (AUROCC 0.66-0.71). Elevated IL-1β correlated with reduced species diversity ($rs = -0.33$, $p < 0.01$) and more pathogenic bacteria ($rs = 0.28$, $p = 0.013$). Pathogen abundance thresholds improved diagnostic accuracy (AUROCC 0.89-0.998) over untargeted 16s rRNA detection.
Tarquinio KM. et al	2022	Multicenter prospective cohort	TA	221 serial samples	61 IMV pediatric patients	16S rRNA gene sequencing (V1-V3 hypervariable regions)	Out of 221 samples from 58 patients, 197 (89%) met the >1000 reads criteria with an average of 43,000 reads per sample. Subjects had a median of 3 samples each (IQR: 2–5) and a median VAIN score of 2 (IQR: 1–3). *Proteobacteria* dominated, followed by *Firmicutes* and *Actinobacteria*. Alpha diversity inversely correlated with intubation days ($p = .032$) and VAIN score ($p = .016$). High VAIN scores linked to decreased Mycobacterium *obuense* and increased *Streptococcus peroris, Porphyromonadaceae, Veillonella atypica*, and other taxa.

Harrigan JJ. Et al	2022	Prospective cohort	ETA	1066 serial samples	83 IMV adult patients	16S rRNA gene sequencing	Cross-sectional MDIs, characterized by low Shannon diversity and high total bacterial abundance, were linked to VA-LRTI risk, although with broad posterior credible intervals. However, persistent disruption in the lower respiratory microbiome demonstrated a stronger association with VA-LRTI risk, where each day of Shannon diversity <2.0 was associated with an odds ratio for VA-LRTI.
Sole ML. et al	2021	Prospective descriptive subanalysis	OS, TA	160 samples	16 (7 cases, 9 controls) IMV adult patients	16S rRNA gene sequencing	The study included mostly male participants (69%) of White ethnicity (63%) with an average age of 58 years. They underwent mechanical ventilation for an average of 9.36 days. Predominant bacterial taxa were *Prevotella, Staphylococcus, Streptococcus, Stenotrophomonas,* and *Veillonella*. Tracheal α-diversity decreased over time in both the total group (P = .002) and the control group (P = .02). β-Diversity was lower (P = .04) in the control group (1.905) compared to the intervention group (2.607).
Mourani PM. et al	2021	Multicenter prospective cohort	ETA, TA	2202 samples	366 pediatric IMV patients	16S rRNA gene sequencing (V4-V5 hypervariable regions)	Initial bacterial load (TBL) didn't differ, but VAP subjects had lower Shannon diversity and lower abundance of *Streptococcus, Lactobacillales,* and *Prevotella*. Higher TBL on subsequent days lowered VAP risk (HR: 0.39; CI: 0.23, 0.64), with consistent findings in matched analysis and clustering. Dominant VAP pathogens: *Prevotella species* (19%), *Pseudomonas aeruginosa* (14%), and *Streptococcus mitis/pneumoniae* (10%), along with *Mycoplasma* and *Ureaplasma* in some subjects.

Baek MG. et al	2020	Prospective cohort	ETA	180 samples	60 IMV adult patients (41 with pneumonia diagnosis)	16S rRNA gene sequencing (V3-V4 hypervariale regions)	In the pneumonia group, Corynebacterium was more abundant, and the NHAI group had lower α-diversity. The β-diversity analysis also revealed significant microbiome differences (weighted UniFrac distance, Adonis, $p<0.001$). Notably, the NHAI group exhibited higher *Corynebacterium* levels and lower *Granulicatella, Staphylococcus, Streptococcus*, and *Veillonella* abundances than the non-NHAI group.
Otsuji K. et al	2019	Prospective Cohort	TA	116 samples	22 IMV adult patients	16S rRNA gene sequencing	Genus-based principal component analysis revealed unclear microbiota changes in saliva but lower anaerobe levels in tracheal aspirate. A Poisson regression model confirmed anaerobe reduction ($p < 0.001$), mainly occurring during mechanical ventilation before antibiotics. Post-antibiotics, the tracheal aspirate featured *Enterobacter spp., Corynebacterium spp., Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus*, and *Granulicatera adiacens.*
Sommerstein R. et al	2019	Prospective cohort	OS	116 samples	10 (5 VAP and 5 NO VAP) IMV adult patients	16S rRNA gene sequencing	Five patients developed VAP, with three having *Enterobacteriaceae* and two *Haemophilus influenzae* as causative pathogens. Locally weighted polynomial regression revealed lower alpha-diversity in *Enterobacteriaceae* VAP patients from days two to five of mechanical ventilation compared to controls. Enterobacteriaceae detection in the oropharynx began on day two, with a single operational taxonomic unit in 2/3 enterobacterial VAP patients.

Emonet S. et al	2019	Case control study in a cohort.	OS, ETA	240 samples	54 (16 cases, 38 controls) IVM adult patients	16S rRNA gene sequencing (first V1-V6 region, followed bya nested PCR of V3-V4 region)	Metataxonomic analysis revealed a strong association between low Bacilli relative abundance in oropharyngeal secretions at intubation and subsequent VAP development. On the day of VAP, both tracheal and oropharyngeal secretions in VAP patients had significantly higher human and bacterial DNA quantities compared to matched controls with similar ventilation times. Molecular techniques identified VAP pathogens found by culture, as well as numerous challenging-to-culture bacteria like *Mycoplasma spp*. and anaerobes.
Kitsios GD. et al	2018	Prospective cohort	ETA, OS	110 samples	56 IMV adult patients	16S rRNA gene sequencing and Luminex multianalyte panel by (RAGE [receptor of advanced glycation end products], soluble TNFR1 [tumor necrosis factor receptor 1], IL-10, fractalkine, and angiopoietin-2)	Sequencing revealed low-diversity lung communities dominated by clinically isolated pathogens (>50% relative abundance), showing significant concordance with culture results ($p = 0.009$). Notably, sequencing detected dominant pathogens in 20% of culture-negative patients receiving broad-spectrum antibiotics. Regardless of culture outcomes, pathogen dominance correlated with increased plasma markers of host injury (RAGE) and inflammation (IL-6, TNFR1) ($p < 0.05$), compared to those without dominant lung pathogens. Machine-learning algorithms identified pathogen abundance by sequencing as the most informative predictor of culture positivity.

Huebinger RM. *et al.*	2018	Prospective cohort	BAL	22 Samples	51 IMV adult patients	16S rRNA gene sequencing (V4 hypervariale regions)	Microbiome diversity analysis found that culture-positive samples had the lowest diversity (Shannon-Wiener index: 0.77 ± 0.36), while culture-negative samples had the highest (3.97 ± 0.65). Culture-negative samples lacked a dominant bacterial genus. Respiratory tract flora lavages were more similar to culture-positive samples. Cytokine analysis revealed elevated levels (IFN-g, IL-17F, IL-1B, IL-31, TNF-a) in culture-positive and respiratory tract flora groups. Culture-positive samples exhibited a stronger immune response and reduced bacterial genera diversity.
Shimizu K. et al	2018	Randomized controlled trial	BAL	72 Samples	72 IMV adult patients	16S and 23S rRNA-targeted reverse-transcription quantitative polymerase chain reaction (RT-qPCR)	The Synbiotics group exhibited significantly lower incidence rates of enteritis (6.3% vs. 27.0%) and VAP (14.3% vs. 48.6%) compared to the No-Synbiotics group ($p < 0.05$). However, there were no significant differences in bacteremia or mortality between the two groups. Fecal analysis showed that the Synbiotics group had significantly higher levels of Bifidobacterium/Lactobacillus. In terms of fecal organic acids, the Synbiotics group had notably higher total organic acid concentration, during the first week ($p < 0.05$).

Lamarche. et al	2018	Prospective cohort	ETA, GA, SS, BS	64 samples	34 IMV adult patients	16S rRNA gene sequencing (V3 hypervariale region)	Sampling occurred at a median of 3 days (lower respiratory and gastric aspirates; IQR 2–4) and 6 days (stool; IQR 4.25–6.75) post-ICU admission. Biogeographical distinction between the lower respiratory and gastrointestinal microbiota was lost during critical illness. Respiratory tract microbial diversity inversely correlated with APACHE II score ($r = -0.46$, $p = 0.013$) and associated with hospital mortality (Median Shannon index: Discharged alive; 1.964 vs. Deceased; 1.348, $p = 0.045$).
Zakharkina T. *et al.*	2017	Prospective cohort	ETA, BAL	111 samples	35 patients	16S rRNA gene sequencing	The duration of mechanical ventilation was associated with a decrease in α diversity (Shannon index; fixed-effect regression coefficient (β): −0.03 (95% CI −0.05 to −0.005)), while antibiotic therapy administration was not significant (fixed-effect β: 0.06; 95% CI −0.17 to 0.30). There was a significant difference in β diversity change between patients who developed VAP and control patients, as observed with Bray-Curtis distances (p=0.03) and Manhattan distances (p=0.04). Positive correlations with the change in β diversity were found for Burkholderia, Bacillales.
Smith et al	2016	Prospective cohort	BAL	15 samples	15 IMV adult patients	16S rRNA gene sequencing (V4 hypervariable regions)	Culture negative BAL samples were dominated by three phyla: Proteobacteria (46.98%), Firmicutes (19.14%), and Bacteroidetes (18.51%). Differences between individual samples were more apparent at genus level classification where Streptococcus, Hydrogenophaga, and Haemophilus were among the most common genera present in samples

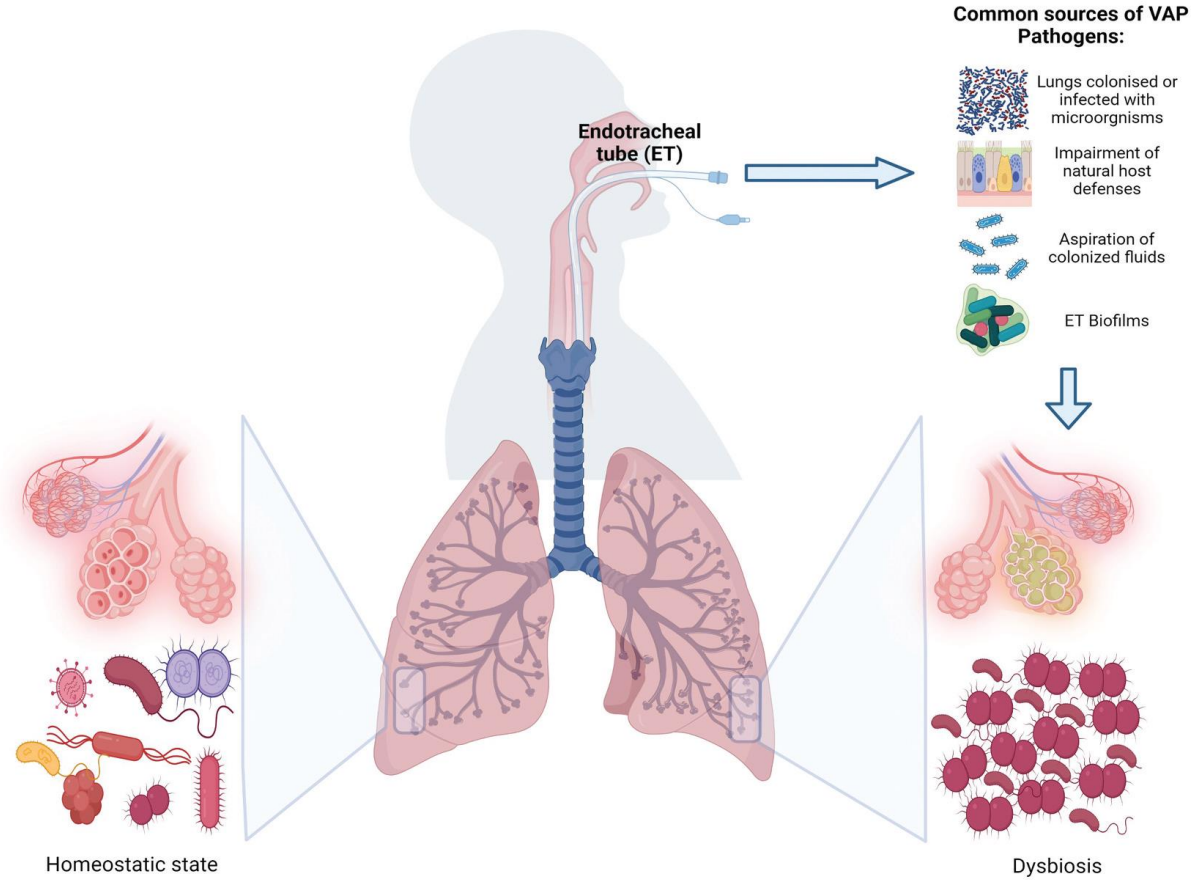
May AK. et al	2015	Prospective cohort	BAL	51 samples	48 IMV adult patients	16S rRNA gene sequencing	49 samples showed strong concordance (>95% by k statistic) in identified pathogens and closely correlated with clinical cultures. Regression analysis of bacterial DNA quantity in paired samples revealed a significant positive correlation (r = 0.85). In a convenience sample, polymerase chain reaction analysis of serial HCH/HME samples for bacterial DNA indicated an increase in load preceding pneumonia suspicion.
Toma I. et al	2014	Case series	BA	27 samples	61 IMV adult patients	NGS of essentially full-length PCR-amplified 16S ribosomal DNA	sufficient DNA was obtained from 72% of samples, with 44% (27 samples) suitable for NGS amplification. Among these sequenced samples, 20 had bacterial culture growth, and NGS coincided with microbiological identification in 17 (85%) cases. Even in 7 samples without bacterial growth but with sequenced amplimers, NGS identified bacterial species. Notably, diverse bacterial species were identified from the same genus as the predominant cultured pathogens, and NGS consistently identified a greater number of bacterial genera compared to standard microbiological methods.
abbreviations: BAL (bronchoalveolar lavage), ETA (endotracheal aspirate), BA (bronchial aspirate), GA (gastric tube aspirate), SS (stool samples), BS (Buccal samples), OS (oropharyngeal swab), PS, TA (tracheal aspirate), IMV (Invasive mechanical ventilation)							

1. **Table 1.1: Lung microbiome or Microbiota Associated with Ventilator Associated Pneumonia**.

These investigations have reshaped our comprehension of ventilator-associated pneumonia (VAP), unveiling a complex interplay involving the pulmonary microbiome, host lung cells, and metabolites (25, 47, 71). This intricate relationship blurs the boundary between commensal microorganisms and pathogens, suggesting that VAP development is influenced by various factors beyond a single pathogen's presence (**Figure 1.3**) (50). While 16S rRNA gene sequencing has significantly advanced our understanding of the pulmonary microbiome's role in VAP, it's crucial to acknowledge its limitations, including limited taxonomic resolution, inherent amplification biases, and a lack of functional insights, among other constraints (48, 49). These limitations have practical implications, such as the challenge of detecting microorganisms at low concentrations and gaining a comprehensive understanding of microbial interactions in the pulmonary environment (13).

3.3 Interaction of the Lung Microbiome and Mucosal Immunity, and Its Association with VAP

The pulmonary immune system encompasses various innate and adaptive immunity components, including pulmonary epithelial cells, alveolar macrophages, neutrophils, innate lymphocytes, and cytokines/chemokines, which initiate immune responses and maintain pulmonary homeostasis (4, 50-52). Complex lung microbial communities play a crucial role in regulating the immune system, establishing a symbiotic relationship (72-74). Although the link between the lung microbiome and innate or adaptive responses remains incompletely understood, it influences host immune responses in health and disease (44, 51, 55, 56).



3. Figure 1.3: Pathophysiology of Ventilator-Associated Pneumonia.

The lung microbiome plays different roles in innate immune cells (75). Dendritic cells, antigen-presenting, and processing cells, activate various T-cell responses (76). Dysbiosis of the lung microbiome can modify dendritic cell processes, increasing cytokines that stimulate pro-inflammatory cytokine production (5, 75-77). Alterations in the lung microbiota also result in deficiencies in Th17 and regulatory T cells, which regulate homeostasis and maintain pulmonary immune tolerance (5, 72). Additionally, pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and NOD-type receptors (NLRs), play critical roles in host-microbiome interactions (4, 78, 79). Bacterial metabolites and products of bacterial metabolism may also contribute to changes in inflammatory cytokine levels in the airways (80, 81).

The relationship between the lung microbiome and inflammation is crucial for protecting against pathogenic microorganisms (24). However, dysbiosis of the lung microbiome can lead to inflammation and reduced microbiome complexity generates a bidirectional inflammation-dysbiosis cycle (73, 82-84). The lung microbiome and host immunity interactions are complex and fundamental to developing or progressing various respiratory diseases (24, 82, 85). Understanding the underlying mechanisms and consequences of these interactions is crucial for a better understanding of the host-microbiota interaction during diseases like ventilator-associated pneumonia (VAP).

Certain microorganisms, such as those belonging to the Proteobacteria phylum, can trigger excessive or inappropriate lung inflammatory responses, contributing to tissue damage and disease progression (76). Dysbiosis caused by different phyla, such as Firmicutes, Proteobacteria, and Bacteroidetes, is associated with variations in immune cell

percentages and cytokine production (86, 87). The relative abundance of specific bacteria can influence the production of pro-inflammatory and anti-inflammatory cytokines, affecting immune responses (86, 87). These findings challenge the traditional understanding of pneumonia and highlight the importance of longitudinal studies to comprehend the complex interactions between the lung and microbial communities during disease development.

3.4 Other Factors Contributing to Pulmonary Dysbiosis and Facilitating the Development of VAP

Changes in microbial community structure have been suggested as potential biomarkers to identify VAP or early pneumonia (67). However, multiple factors can affect pulmonary dysbiosis and contribute to the development of VAP. One example is the formation of biofilms on endotracheal tubes (ETT), a mechanism used frequently by *Pseudomonas aeruginosa* to generate VAP (64, 88, 89). Hotterbeekx *et al.* discovered that 89% of the phyla in ETTs are Proteobacteriaceae, followed by Phylobacteriaceae (86%) and Enterobacteriaceae (77%). However, analyzing biofilm formation can only be done after extubation and does not establish a causal link to VAP (90).

Antibiotic use is another crucial contributor to this problem, as it can potentially modify the composition of the airway microbiota, resulting in a reduction in alpha diversity. However, it is crucial to recognize that increased antibiotic use alone does not explain the decreased diversity in the pulmonary microbiome (13, 23). Antibiotic use has also been associated with the emergence of antimicrobial resistance among commensal lung microbiota (46). In addition, antibiotics may also contribute to fungal colonization and

an increased incidence of allergic reactions. However, despite recognizing antibiotic exposure as a risk factor for the development of mechanical ventilation-associated pneumonia (VAP), the existing body of research remains insufficient to definitively establish the efficacy and impact of antibiotics in preventing VAP (46, 65). This inadequacy is mainly due to significant variations and inconclusive findings in various studies (65, 91).

4. Modulating Lung Microbiota to Prevent the Development of VAP: Exploring Strategies

Several efforts have been made to modulate the lung microbiota for therapeutic benefits in respiratory diseases using probiotics. However, the few clinical trials conducted in VAP have targeted the lower gut microbiota rather than the respiratory microbiota, so the effects on the respiratory microbiota remain to be determined. Recent data suggest that enteric probiotics may reduce the incidence of VAP through local and systemic effects. They have the potential to control the growth of pathogenic bacteria, modulate bacterial translocation in the gut-lung axis, decrease the microbial load in the respiratory tract, and improve gastrointestinal barrier function (92, 93). In addition, probiotics have demonstrated immune-enhancing effects in the lower respiratory tract, potentially regulating microbial composition and metabolism and improving host immune response. However, a study by Johnstone *et al.* contradicts these findings by demonstrating that using probiotics does not reduce the risk of VAP. Furthermore, the study underlines the importance of assessing potential adverse effects and considering different patient populations, as the results cannot

be generalized to all critically ill patients. It is important to note that this study focused on a specific probiotic strain (94).

No human studies have been conducted to evaluate "respiratory probiotics" (i.e., viable microbiota instilled or aerosolized into the lower respiratory tract). Clearly, larger, well-designed, and statistically powered clinical trials are still needed to further evaluate this promising intervention's efficacy (71, 93). Similarly, bacteriophage therapy targeting potentially pathogenic bacteria in the respiratory tract may have a role, according to emerging evidence in the gut, although experience in the respiratory tract remains anecdotal (95, 96).

5 New Perspectives: The Future of Lung Microbiome in VAP

The study of the lung microbiome is a relatively new field of research that initially focused on microbial ecology and changes in the diversity or relative abundance of microorganisms, mainly bacteria, in different lung diseases. However, there are still many gaps in this field. Therefore, it is vital to continue generating reports on the lung microbiome to understand the microbiota profile better and clarify its role in the pathophysiology of VAP. Longitudinal studies within rigorous protocols with clear design guidelines, such as screening methods, experimental execution, and data analysis, are also needed. It is essential that studies are replicated to establish the presence or absence of a causal relationship between microbiota, disease, and lung injury (97-99). A thorough characterization of the host-specific response to microbial composition, both locally and systemically, is required to clarify whether the altered lung microbiome contributes to

pathogenesis or is a marker of injury and inflammation, pivotal in establishing multidrug-resistant microorganisms.

Finally, the new challenge of studying the lung microbiome leads us towards metagenomics, lung metatranscriptomics, and multiple omics approaches, seeking a comprehensive understanding of bacterial, viral, and fungal communities simultaneously **(Figure 1.4)** (100, 101). We must focus on developing new diagnostic features and more effective therapeutic strategies with clinical impact, looking for biomarkers that allow us to assess disease progression, therapeutic targets that safely and effectively restore pulmonary dysbiosis, and therapeutic manipulation of the microbiome, eliminating pro-inflammatory taxa, and establishing algorithms or biological patterns, ultimately generating precision medicine (16, 102).

TECHNIQUE	FUNDAMENTAL	LIMITACIONS
Metataxonomy	Sequencing of specific genes, 16S rRNA or 18S rRNA (taxonomic composition)	• Limited Taxonomic Resolution • Lack of Functional Information • Sequence Contamination • Dominant Sequence Interference
Metagenomics	Sequencing and analyzing all DNA (genetic and functional diversity)	• Sample Complexity • Identification of Novel Microorganisms • Metabolite Analysis • Does Not Distinguish Viability
Transcriptomics	Systematic study of metabolites (metabolic pathways)	• Difficulty in Metabolite Identification • Interpretation Challenges
Metabolomic	Study of mRNA and other types of RNA (actively transcribed genes)	• Does Not Report on Metabolites • State-Dependent

4. **Figure 1.4: Techniques of the study of lung microbiome.**

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B. Expert opinion

There are still significant gaps in our knowledge regarding mechanical ventilation-associated pneumonia (VAP) and its relationship with the pulmonary microbiome. These gaps include the crucial need for viable predictive biomarkers that can detect alterations in the microbial community, enabling early identification of patients at risk of developing VAP or the specific microorganisms causing VAP. Early identification is essential for accurate diagnosis and implementing targeted and effective antibiotic treatment, even for other pharmacological therapies.

To advance our understanding of the intricate interplay between the pulmonary microbiome and the development of VAP, it is imperative to move beyond metataxonomy. We must delve deeper into the complexities surrounding the pulmonary microbiome, including its interactions with the host immune response, mechanical ventilation, and the factors contributing to the occurrence of VAP. Only through this comprehensive research can we gain the necessary insights to embrace precision in personalized medicine and effectively combat this disease.

Within the field of pulmonary microbiome research, there is a growing anticipation for the potential use of probiotics as a preventive measure against VAP. Probiotics have exhibited promising benefits in modulating the microbiota and reducing VAP incidence. Nevertheless, it is essential to approach this prospect with caution, as further empirical evidence and robust clinical trials are imperative to establish its efficacy and safety in this context.

In summary, it is imperative to intensify our efforts to address the unmet needs in VAP research. This involves identifying and developing predictive biomarkers and a deeper understanding of the intricate interplay between the pulmonary microbiome and various factors. Also, a meticulous evaluation of preventive interventions, such as probiotics, and their effectiveness. These endeavors can potentially revolutionize the early diagnosis, treatment, and prevention of VAP, paving the way for improved patient outcomes and significantly reducing the burden of this condition.

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II. GOALS AND HYPOTHESIS OF DISSERTATION RESEARCH PROJECT

The human body hosts at least 100 trillion (10^14) microbial cells, surpassing the number of human cells. As a result, individuals exhibit significant variations in the taxonomic content of their microbiota, which undergoes drastic changes even within the same person during development due to various environmental factors (Dominguez-Bello, Godoy-Vitorino, Knight, & Blaser, 2019; Pascale *et al.*, 2018). Microbial colonization, primarily in the intestine, begins at birth through the maternal microbiota from the genital tract, colon, and the general environment (Chen, Sun, & Zhang, 2017). Factors such as diet, antibiotic use, delivery mode (cesarean or vaginal), breastfeeding, and gestational age influence the microbiome composition (Penders *et al.*, 2006). The microbiota contributes to the synthesis of certain vitamins and functions in the immune system, influencing the maturation of immune cells and the normal development of immune functions, impacting both innate and adaptive immunity (Chen *et al.*, 2017; Clemente, Ursell, Parfrey, & Knight, 2012).

For a long time, lower respiratory pathways were believed to be sterile, and only a few studies dared to challenge this paradigm. However, recent data demonstrate the isolation of oral microorganisms in lower respiratory samples. While contamination from exposure to upper respiratory pathways during sample collection has been blamed on some occasions (Segal, Rom, & Weiden, 2014), recent studies have successfully characterized the pulmonary microbiome, a community of microorganisms residing in the lung without causing infection (Budden *et al.*, 2019).

Importantly, current evidence indicates a positive correlation between increased microbiome diversity and lung health (Budden *et al.*, 2019). However, various diseases have been associated with altered immune responses and dysbiosis (altered microbiota composition). These associations extend to diseases related to energy metabolism, blood pressure control, coagulation risks or even behavioral disorders (Budden *et al.*, 2019; Dominguez-Bello *et al.*, 2019; Gholizadeh *et al.*, 2019; Littman & Pamer, 2011). In lung diseases, associations have been established with asthma, chronic obstructive pulmonary diseases, cystic fibrosis, and exacerbations of bronchiectasis (Budden *et al.*, 2019). However, the relationship between the lung microbiome and its association with lung mucosal inflammatory response remains unclear.

Understanding the pulmonary microbiome and its role in diseases such as Ventilator-Associated Pneumonia (VAP) is of paramount importance, given the current ambiguity regarding whether alterations in microbial diversity are the cause or a consequence of immune dysregulation and mucosal inflammation. Despite acknowledgment that dysbiosis may influence the progression of respiratory illnesses, the focus of research to date has been predominantly on characterizing microbial composition, rather than delving into the interactions between host, pathogens, and the functional implications of these imbalances in the lower respiratory tract. Furthermore, the specific impact of mechanical ventilation on the pulmonary microbiome's biodiversity and its contribution to VAP remains an underexplored field. Therefore, investigating these aspects is not merely imperative to bridge the existing knowledge gaps but is also crucial for the development of more accurate diagnostic methods, the optimization of treatment protocols,

and most fundamentally, the formulation of innovative preventative strategies that could enhance outcomes for critically ill patients.

Therefore, we aim to characterize the immunological, cellular, biochemical, and microbiome changes linked to mechanical ventilation, which influence the onset of Ventilator-Associated Pneumonia (VAP) in adult ICU patients. Our hypothesis is that mechanical ventilation leads to distinct alterations in the pulmonary microbiome, immune, biochemical, and cellular responses, which together facilitate the development of VAP in intubated, critically ill adults. By understanding these changes, our research seeks to uncover the underlying mechanisms of VAP, with the goal of improving prevention and treatment strategies for this serious condition.

Aim 1: Investigate the relationship between positive pressure mechanical ventilation and changes in pulmonary microbiome biodiversity. *Hypothesis:* Patients who develop VAP will exhibit a greater change in both alpha and beta diversity, alongside reduced diversity in follow-up samples compared to baseline samples. These factors are anticipated to be determinants in the development of ventilator-associated pneumonia in adult hospitalized patients in the intensive care unit who undergo intubation. To test this hypothesis, we will analyze the lung microbiome in bronchoalveolar lavage fluid using rRNA sequencing from intubated patients without preexisting pulmonary conditions. Changes in microbiota will be assessed through alpha and beta diversity analysis. Over time, we will categorize patients who develop VAP and those who do not, then examine samples collected at the onset of intubation and, in the case of VAP development, on the

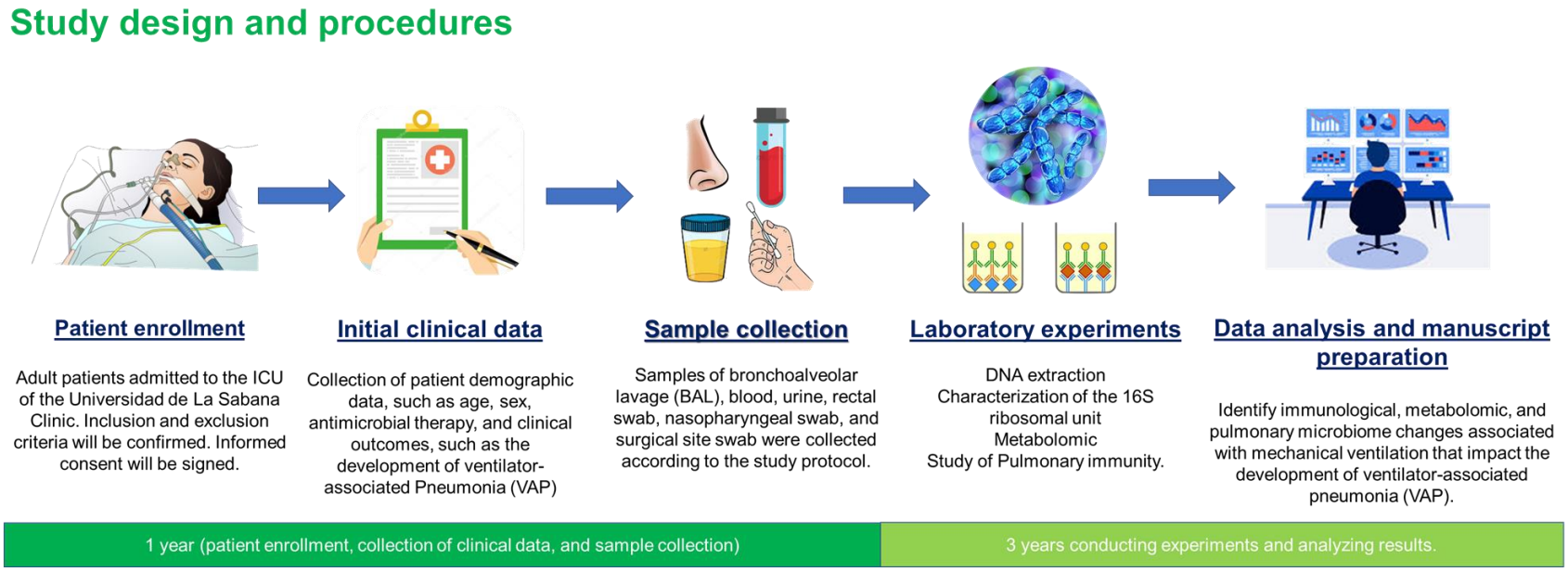
day of diagnosis. For patients without VAP, we will analyze samples obtained after a specific duration of intubation.

Aim 2: Examine the link between pulmonary microbiome composition and innate pulmonary immunity. *Hypothesis:* We posit that in intubated patients, there is a significant correlation between the composition of the pulmonary microbiome and the production of key cytokines such as IL-1β, IL-6, and TNF-alpha, which serve as markers of the innate pulmonary immune response. It is anticipated that specific microbial profiles are associated with distinct levels of these cytokines, potentially influencing susceptibility to respiratory diseases, including the possible development of Ventilator-Associated Pneumonia (VAP), and the host's capacity to effectively combat such conditions. To test this hypothesis, we will analyze three key proinflammatory cytokines in the innate pulmonary immune response using immunofluorescence assays with magnetic beads employing the Milliplex methodology. Cytokine production will be quantified based on the fluorescence emitted by the beads, and comparative analysis will be conducted among the various study groups.

Aim 3: Characterize patterns within the pulmonary microbiome, immunological factors, or biochemical biomarkers that can predict the early diagnosis of VAP during the onset of mechanical ventilation. *Hypothesis:* It is proposed that by characterizing patterns within the pulmonary microbiome, immunological factors, and metabolomic biomarkers, distinctive signatures correlating with the early diagnosis of Ventilator-Associated Pneumonia (VAP) during intubation can be identified. These signatures may offer valuable predictive indicators, enabling more timely and effective intervention and management of VAP in intubated patients. To test this hypothesis, in addition to the

analyses conducted on the pulmonary microbiome and the host's innate immune response, a metabolomic analysis will be carried out on bronchoalveolar lavage fluid using GC-TOF-MS (Gas Chromatography-Time of Flight Mass Spectrometry). The metabolites obtained will be subject to a comparative analysis among the different study groups.

This **innovative project** will advance our understanding of the complex interactions between the pulmonary microbiome, the host immune response, and the metabolomic profile in developing ventilator-associated pneumonia. By exploring these interrelationships in detail, we hope to gain profound insights into the impact of mechanical ventilation and the pathophysiology of the disease. The data collected through this analysis can potentially drive significant advances in the diagnosis, therapeutics, and prevention of VAP, thus contributing to the advancement of critical care medicine **(Fig.2.1)**.



5. Figure 2.1: Timeline of Clinical Research Phases from Enrollment to Publication.

III. MAJOR ALTERATION OF LUNG MICROBIOME AND THE HOST REACTION IN CRITICALLY ILL COVID-19 PATIENTS WITH HIGH VIRAL LOAD.

A. Abstract

1. Background

Patients with COVID-19 under invasive mechanical ventilation are at higher risk of developing ventilator-associated pneumonia (VAP), associated with increased healthcare costs, and unfavorable prognosis. The underlying mechanisms of this phenomenon have not been thoroughly dissected. Therefore, this study attempted to bridge this gap by performing a lung microbiota analysis and evaluating the host immune responses that could drive the development of VAP.

2. Materials and methods

In this prospective cohort study, mechanically ventilated patients with confirmed SARS-CoV-2 infection were enrolled. Nasal swabs (NS), endotracheal aspirates (ETA), and blood samples were collected initially within 12 hours of intubation and again at 72 hours post-intubation. Plasma samples underwent cytokine and metabolomic analyses, while NS and ETA samples were sequenced for lung microbiome examination. The cohort was categorized based on the development of VAP. Data analysis was conducted using RStudio version 4.3.1.

3. Results

In a study of 36 COVID-19 patients on mechanical ventilation, significant differences were found in the nasal and pulmonary microbiome, notably in Staphylococcus and

Enterobacteriaceae, linked to VAP. Patients with VAP showed a higher SARS-CoV-2 viral load, elevated neutralizing antibodies, and reduced inflammatory cytokines, including IFN-δ, IL-1β, IL-12p70, IL-18, IL-6, TNF-α, and CCL4. Metabolomic analysis revealed changes in 22 metabolites in non-VAP patients and 27 in VAP patients, highlighting D-Maltose-Lactose, Histidinyl-Glycine, and various phosphatidylcholines, indicating a metabolic predisposition to VAP.

4. Conclusions

This study reveals a critical link between respiratory microbiome alterations and ventilator-associated pneumonia in COVID-19 patients, with elevated SARS-CoV-2 levels and metabolic changes, providing novel insights into the underlying mechanisms of VAP with potential management and prevention implications.

4 Background

Since the emergence of the highly contagious Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) in 2019, the COVID-19 pandemic has rapidly spread worldwide, leading to profound global health and economic consequences (103). The World Health Organization (WHO) has reported a staggering 770 million cases and nearly 7 million deaths globally by July 2023 (104, 105). Notably, 5% to 12% of patients progress to severe or critical stages, necessitating invasive mechanical ventilation (IMV) and significantly increasing mortality rates (106-108). However, IMV often triggers complications, including secondary infections, which can worsen clinical outcomes and extend stays in intensive care

units (ICUs) and hospitals (106, 109). Critically ill COVID-19 patients often experience bacterial superinfections, further complicating their condition.

In the intensive care setting, individuals with severe COVID-19 pneumonia show a marked propensity for respiratory superinfections, with mechanical ventilation-associated pneumonia (VAP) being especially prevalent. This tendency is thought to be associated with SARS-CoV-2 virus-induced alterations of the pulmonary microbiota (110, 111). The occurrence of VAP and other superinfections may be attributed to the invasion of new pathogens or bacterial strains, which diversify from primary SARS-CoV-2 infection (112, 113). Data suggests that at least 32% of these patients will develop bacterial superinfections, increasing morbidity and mortality rates (114, 115). However, the exact prevalence and impact of initial bacterial superinfections on progression to VAP in patients with severe COVID-19 pneumonia are not yet fully understood (113). The dynamics of the pulmonary microbiome are thought to play an integral role in initiating and shaping the course of superinfections and influencing patient response to treatment. Understanding these interactions is essential to improve therapeutic strategies and patient outcomes in severe cases of COVID-19 (116).

The lungs harbor a diverse microbiome comprising approximately 100 different bacteria, viruses, and fungi (16, 87). This complex microbiome is crucial in maintaining immune balance and significantly influences the severity and duration of respiratory infections, such as SARS-CoV-2 (103, 117). The intricate interplay between the commensal microbiota and the immune system is vital for regulating immune responses, with microbiota-derived metabolites mediating these interactions. Additionally, metabolic

changes have been observed, but their connection to bacterial superinfections in severe COVID-19 patients remains unclear (103, 118, 119). Changes in the microbiome-immune system interplay due to host-microbiome dysbiosis may lead to dysregulated immune responses and conditions like systemic inflammation (120, 121). It is crucial to comprehend these interactions. This study explores how SARS-CoV-2 affects the lung microbiome in critically ill COVID-19 patients on mechanical ventilation. We analyze the microbiome, metabolites, and host immune response to understand better the underlying mechanisms responsible for VAP in 36 mechanically ventilated COVID-19 patients.

B. Material and methods

This prospective cohort study was conducted at Clinica Universidad de La Sabana in Chia, Colombia, between January 2021 and July 2021, including all critically ill COVID-19 patients requiring invasive mechanical ventilation admitted to the ICU. The attending physicians prospectively gathered data by reviewing medical records and laboratory results in the platform for data storage REDCap every time the patient was screened and selected. Nasal swabs (NS), Endotracheal aspirates (ETA), and blood samples were collected in the initial 12 hours following intubation, and a follow-up was conducted 72 hours post-intubation. Then, we performed microbiological analysis, cytokines, and metabolomic characterization. The Institutional Review Board (IRB) of Clinica Universidad de La Sabana approved the study, and all patients provided informed consent to participate (CUS-20190903).

1. Study population.

Patients diagnosed with COVID-19 and required ICU admission and invasive mechanical ventilation within 12 hours of hospital admission for more than 72 hours were included in this study (**Table 3.1**). The severity of COVID-19 was classified based on WHO guidelines, and critical illness was identified in patients who needed invasive mechanical ventilation, extracorporeal membrane oxygenation (ECMO), or suffered from end-organ dysfunction (122). We excluded pregnant patients who had been invasively ventilated in another hospital. Patients who had been administered more than two doses of antibiotics before intubation, those who had IMV for over 24 hours before the sample collection, and patients who had a documented coinfection within 48 hours of admission were also excluded. Demographic data, comorbidities, symptoms, physiological variables, systemic complications, and laboratory reports from the first 24 hours of admission were recorded and monitored every 48 hours until the patient was extubated. We retrospectively reviewed the data from medical records at the time of hospital discharge to ensure the accuracy of the recorded information uploaded to the REDCap platform hosted at the Universidad de La Sabana (Plataforma REDCap - Universidad de La Sabana [Internet]. Universidad de La Sabana; [2023]. Disponible en: https://redcap.unisabana.edu.co/).

Characteristic	All n = 36	VAP baseline n = 24	No-VAP baseline n = 12	p-value	VAP follow-up n = 25	No VAP follow-up n =11	p-value
Demographic							
Male. N (%)	22 (61)	14 (58)	8 (66)	0.90	14 (56)	7 (63)	1
Age. Median (IQR)	56.0 (49.7-64.2)	57.5 (50.0-64.2)	54.5 (47.7-61.5)	0.51	57.5 (50.0-64.2)	54.0(46.5-64.0)	0.54
Comorbid conditions. N (%)							
Anemia	1 (2.8)	0 (0)	1 (8.3)	0.71	0 (0)	1 (9.1)	0.68
Cancer	1 (2.8)	1 (4.2)	0 (0)	1	1 (4.0)	0 (0)	1
Diabetes mellitus	2 (5.6)	1 (4.2)	1 (8.3)	1	1 (4.0)	1 (9.1)	1
Coronary disease	1 (2.8)	1 (4.2)	0 (0)	1	1 (4.0)	0 (0)	1
COPD	1 (2.8)	1(4.2)	0 (0)	1	1 (4.0)	0 (0)	1
Arterial hypertension	12 (33.3)	10 (41.7)	2 (16.7)	0.26	10 (40.0)	2 (18.2)	0.32
Obesity	9 (25.0)	6 (25.0)	3 (25.0)	1	6 (24.0)	3 (27.3)	1
No background	18 (50.0)	12 (50.0)	6 (50.0)	1	12 (48.0)	5 (45.5)	1
Physiological variables during the first 24 hours of admission. Median (IQR)							
Heart rate. BPM	93.5 (77.2-106.0)	85.5 (73.5-103.5)	98.0 (90.7-125.5)	0.07	83.5 (63.7-99.0)	82.0 (67.0-87.0)	1

Respiratory rate. RPM	24.0 (20.0-30.0)	24.0 (20.0-25.7)	24.5 (20.0-40.0)	0.31	24.0 (20.0-24.2)	24.0 (20.0-24.0)	0.91
Temperature. °C	36.6 (36.5-36.9)	36.5 (36.5-36.9)	36.9 (36.4-37.0)	0.28	36.9 (36.6-37.5)	37.0 (37.0-37.4)	0.22
SBP. mmHg	118.0 (105.0-134.2)	119.5 (107.5-133.2)	115.0 (101.5-137.8)	0.76	128.0 (117.8-144.5)	126.0 (105.5-146.5)	0.63
DBP. mmHg	65.5 (58.7-73.2)	66.0 (59.5-73.2)	65.5 (57.0-69.5)	0.67	66.0 (60.2-72.5)	71.0 (65.0-78.5)	0.27
PAM. mmHg	84.3 (75.2-89.5)	85.5 (75.2-89.5)	83.1 (73.7-88.5)	0.62	86.8 (81.2-98.1)	90.6 (81.5-94.8)	0.80
SPO2. (%)	85.5 (80.7-90.0)	84.0 (80.0-90.0)	90.0 (81.0-93.7)	0.12	90.0 (87.5-92.0)	90.0 (84.5-92.0)	0.97
Glasgow	8.5 (6.0-15.0)	8.0 (6.0-15.0)	14.0 (6.0-15.0)	0.68	6.0 (6.0-6.2)	6.0 (6.0-7.0)	0.96
Laboratory variables at admission. Median (IQR)							
WBC, cell x 103	10.7 (8.10-14.0)	9.9 (7.1-13.0)	13.0 (11.0-16.5)	0.06	9.4 (7.9-13.5)	12.9 (9.4-16.9)	0.39
Neutrophiles, (%)	85.5 (80.7-90.2)	86.5 (81.0-90.5)	82.5 (79.7-89.5)	0.34	84.0 (81.7-90.2)	89.0 (80.0-92.0)	0.48
Hemoglobin, g/dL	14.8 (13.8-16.0)	14.8 (13.9-16.0)	14.8 (13.5-16.0)	0.91	12.5 (11.2-14.0)	11.6 (11.1-12.0)	0.13
Platelet, cell x 103	230.0 (180.0-280)	226.5 (150.0-252.5)	275.0 (205.8-356.8)	**0.02**	200.0 (167.5-252.5)	243.0 (189.30-315.0)	0.31

Creatinine, mg/dL	0.9 (0.8-1.1)	1.0 (0.9-1.4)	0.8 (0.7-0.9)	**0.04**	1.3 (0.9-2.2)	0.8 (0.7-1.4)	0.10
BUN, mg/dL	20.0 (15.0-26.0)	22.5 (16.2-29.7)	15.0 (13.0-18.0)	**0.01**	32.5 (22.7-45.0)	24.0 (18.0-41.5)	0.27
Blood glucose, mg/dL	142.0 (124.5-185.0)	145.0 (129.2-180.0)	142.0 (121.0-210.0)	0.98	150.0 (130.0-180.0)	150.0 (150.0-170.0)	0.71
Sodium, mEq/L	139.0 (136.8-140.2)	139.0 (137.5-140.5)	139.0 (136.0-139.0)	0.78	144.0 (139.8-145.2)	145.0 (143.0-147.5)	0.18
Potassium, mEq/L	4.3 (4.0-4.5)	4.3 (4.1-4.6)	4.2 (4.0-4.5)	0.50	4.8 (4.2-5.2)	4.2 (4.1-4.8)	0.22
pH	7.31 (7.20-7.41)	7.33 (7.20-7.41)	7.25 (7.17-7.38)	0.62	7.34 (7.20-7.42)	7.42 (7.30-7.45)	0.24
PCO2, mmHg	46.0 (34.0-58.2)	45.0 (34.0-53.2)	57.5 (34.0-65.2)	0.38	46.5 (43.0-54.5)	46.0 (44.5-53.0)	0.78
PaO2, mmHg	68.5 (59.0-75.2)	64.5 (59.0-73.0)	79.0 (65.5-89.7)	**0.04**	64.0 (60.2-66.0)	59.0 (57.0-67.5)	0.83
FiO2	70.0 (45.0-90.0)	80.0 (45.0-91.2)	52.5 (43.7-82.5)	0.27	40.0 (39.2-48.2)	40.0 (35.0-52.5)	0.66
HCO3, mmol/L	24.0 (20.1-26.0)	23.5 (19.7-26.0)	24.0 (21.7-27.0)	0.55	26.0 (21.0-29.2)	30.0 (27.5-31.0)	0.13
Acid lactic, mmol/L	1.4 (1.1-2.1)	1.5 (1.1-2.1)	1.3 (1.1-1.7)	0.46	1.3 (1.0-1.8)	1.1 (0.9-1.2)	0.31
Outcomes. Median (IQR)							
Length of stay in ICU, days (IQR)	8.0 (4.0-14.0)	15.0 (9.0-24.0)	6.0 (3.0-11.0)	**<0.01**	15.0 (9.0-24.0)	10.0 (6.0-13.5)	**<0.01**

Length of stay in the hospital, days (IQR)	13.0 (7.0-29.0)	29.0 (12.0-48.5)	11.0 (4.0-18.0)	<**0.01**	29 (12.0-48.5)	15.0 (10.5-22.5)	**0.02**
Intubation time, days (IQR)	5.0 (3.0-9.0)	9.0 (7.0-14.0)	3.0 (2.0-5.0)	<**0.01**	9.0 (7.0-14.0)	6.0 (5.0-8.5)	<**0.01**
Hospital Mortality (%)	30 (83.3)	19 (79.2)	11 (91.7)	0.63	19 (76.0)	10 (90.9)	0.70
Mortality 28d (%)	30 (83.3)	19 (79.2)	11 (91.7)	0.63	19 (76.0)	10 (90.9)	0.70
Mortality 90d (%)	31 (86.1)	20 (83.3)	11 (91.7)	0.86	20 (80.0)	10 (90.9)	0.94
Scores. Median (IQR)							
SOFA	8.0 (7.0-9.0)	8.0 (7.2-9.0)	8.0 (6.0-9.0)	**0.01**	9.0 (7.0-10.0)	9.0 (7.0-10.0)	0.85
APACHE	15.0 (10.0-20.0)	17.0 (14.0-23.2)	14.0 (9.0-19.2)	**0.01**	17.0 (14.0-19.0)	14.0 (11.0-20.0)	0.10
CPIS	2.0 (10.0-4.0)	2.0 (1.0-3.0)	2.0 (1.0-5.0)	0.30	3.0 (1.0-4.0)	2.0 (1.0-2.30)	<**0.01**

2. Table 3.1: Demographic information, clinical characteristics, and laboratory test. patients were stratified into two groups: those with VAP and those without VAP

.

2. Recollection and sample processing

ETA and NS samples were meticulously collected following established protocols employing sterile saline (0.9%). Immediately post-collection, these samples were frozen at -80°C segregated into distinct aliquots for future sequencing and metabolomics analyses. Prior to these analyses, the samples underwent thawing and thorough mixing to eradicate any particulate matter. Concurrently, blood samples were obtained through an intravenous catheter, utilizing 5- or 10-mL Becton Dickinson Vacutainers (red top tubes), and then centrifuged at 1,970 x g for 10 minutes. Subsequently, the supernatant was methodically apportioned into aliquots and preserved at -80°C for ensuing processing. To maintain consistency in handling and storage, thereby minimizing potential contamination or degradation risks, the research team collected all blood samples, ensuring rigorous standardization and enhancing the accuracy of the analyses. Samples were obtained from eligible patients on invasive mechanical ventilation within the initial 24 hours (day 0) and subsequently on days 3, 5, and 7 or the day of diagnosis of mechanical VAP.

3. Diagnosis Criteria for VAP

The diagnosis of VAP was based on current clinical guidelines published by the Infectious Diseases Society of America and the American Thoracic Society (IDSA/ATS) for the management and diagnosis of VAP (12). Diagnostic criteria included patients on mechanical ventilation for at least 72 h, a new or progressive radiographic infiltrate, and at least two of the following symptoms: fever (body temperature > 38 °C), purulent tracheal secretions, or leukocytosis or leukopenia (leukocyte count > 10,000/μL or < 4,000/μL, respectively). Patients were included in the VAP category only if, after being intubated to the ICU for 48

hours or more, they had at least one respiratory pathogen isolated from their ETA (>106 CFU) or bronchoalveolar lavage (>104 CFU) that is known to cause pneumonia.

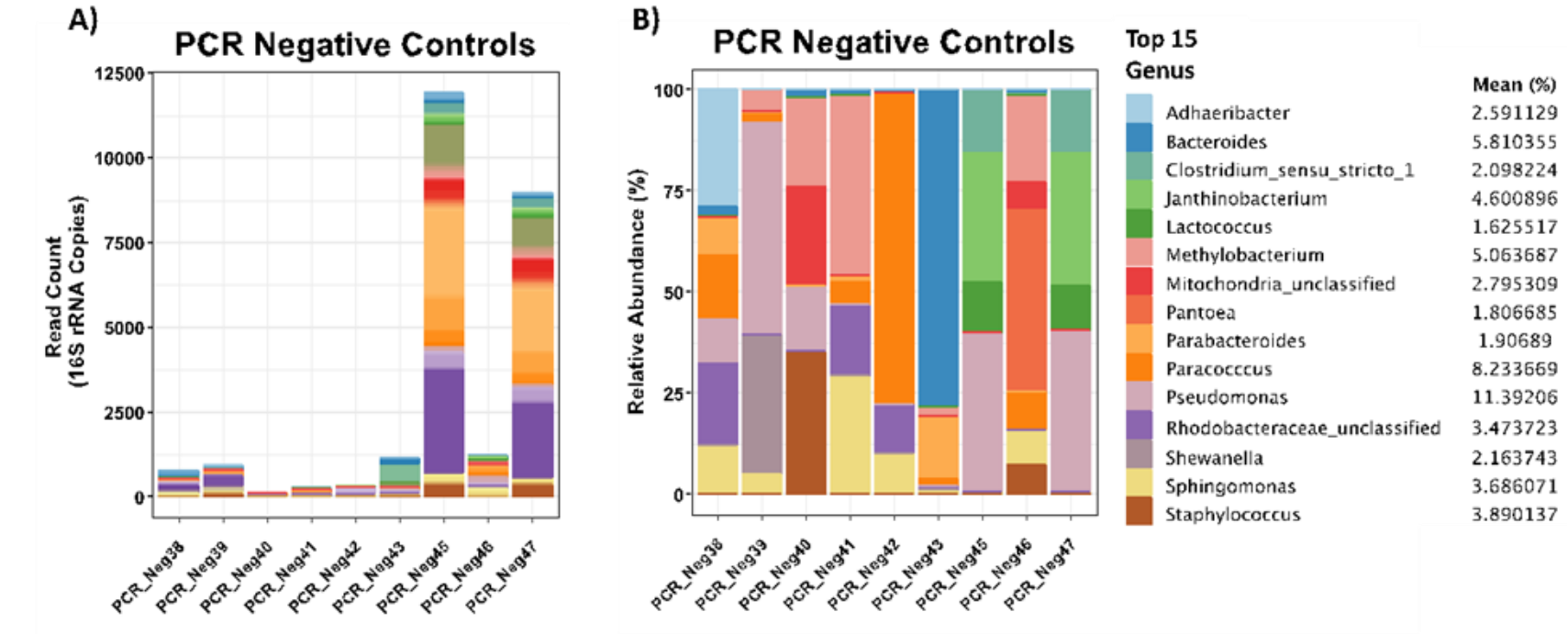
4. DNA extraction

DNA isolation was performed using the DNeasy® Blood & Tissue Kit from QIAGEN, a commercially available kit. Initially, a 500 µL sample obtained from either an ETA or NS was centrifuged at 6,750 x g for 10 minutes at room temperature. Subsequently, the supernatant was removed, and the pellet was resuspended in 200 µL of PBS. The isolation process followed the manufacturer's instructions. The quality and concentration of DNA samples were assessed using the NanoDrop™ One instrument.

5. 16S rRNA amplification and sequencing

Amplification and sequencing of the V4 region of the 16S rRNA gene were performed using primers 515-533F forward (GTGCCAGCMGCCGCGGTAA) and 806-787R reverse (GGACTACHVGGGTWTCTAAT) with 8-bp barcode and Illumina adaptor (123). The polymerase chain reaction (PCR) was carried out using approximately 100 ng of gDNA per sample and Thermo Fisher Platinum Taq DNA Polymerase (Cat# 10966–026, Life Technologies, Carlsbad, CA). The amplification conditions were as follows: 94°C for 5 min, 94°C for 30 s, 55°C for 30 s, 72°C for 30 s for 35 cycles, 72°C for 7 min. The libraries were purified using QIAquick PCR purification kit to remove primer-dimers and short reads (<100bp) and quantified using Qubit 1X dsDNA HS Assay (Cat# 28106, QIAGEN, Hilden, Germany). The libraries were normalized, and fragment size was examined using a sensitivity DNA Kit (Cat# 5067–4,626, Agilent, Santa Clara, CA). The library pool was sequenced using the Illumina MiSeq system as instructed by the manufacturer (Cat# MS-

102- 3,003, Illumina Inc., La Jolla, USA). A low amount of environmental and reagent contamination was detected in most of the PCR-negative controls (**Supplemental Fig. 3.1**).



Samples	PCR_Neg 38	PCR_Neg 39	PCR_Neg 40	PCR_Neg 41	PCR_Neg 42	PCR_Neg 43	PCR_Neg 45	PCR_Neg 46	PCR_Neg 47
Read Count	759	950	118	274	328	1,154	11,934	1,230	8,978

6. **Figure 3.1 Supplemental: Low environmental and reagent contamination detected in most PCR negative controls (molecular grade water).A**. Read count ranges from 274-11,934. The high amount of sample contamination was detected in PCR-negative controls 45 and 47. **B.** Abundance plot of the top 15 Genus showed mostly environmental- and reagent-associated taxa with some host-associated taxa. Moving forward, the environmental- and reagent-associated taxa were removed from the Nasal and SOT sample.

The bioinformatic analysis involved demultiplexing and generating fastq files using CASAVA v1.8.2 (Illumina Inc., La Jolla, CA). The fastq files were filtered using KneadData to remove low-quality reads (<Q30), end trimming, and contamination from host mitochondrial sequences (124). An in-house bioinformatic pipeline supported by Mothur (125) and Uparse (126) with SILVA 16S rRNA database (version 123) was used to assign Operational taxonomical units (OTUs) at 97% sequence similarity (127). The relative abundance and diversity plots were generated using R packages phyloseq and ggplot 2 (128).

6. Cytokines/Chemokines/growth factor measures

The analysis of various protein targets was conducted utilizing the Invitrogen™ multiplexed immunoassay panel, specifically, the Cytokine/Chemokine/Growth Factor 45-Plex Human ProcartaPlex™ Panel 1 (Cat #EPX450-12171-901, ThermoFisher Scientific, Vienna, Austria), in accordance with the manufacturer's instructions. Serum samples were processed using a compatible Luminex 200 instrument (Luminex Corporation, Austin, Texas, USA), utilizing lot# 313189-002 for bead mixes, detection antibody mixes, and standard mixes, all prepared as per manufacturer's instructions. To ensure accuracy, the combined standards were diluted fourfold and run in duplicate alongside two blanks containing assay buffer only. Prior to analysis, samples were thawed on ice, subjected to centrifugation at 1,000 x g for 10 minutes, and the supernatant was analyzed without further dilution.

Following data collection, quality control measures were implemented according to a specified protocol (129). All samples had a bead count exceeding 100, with a minimum requirement of 30 beads. After analysis with the Luminex, Mean Fluorescence Intensity (MFI) was provided and was transformed to Net MFI after subtracting the background from

the blank wells. Using the ProcartaPlex Analysis App (ThermoFisher Scientific, Vienna, Austria), concentration values were generated via transformation of Net MFI based on the standard curves for each analyte, as we previously reported for saliva (130) and serum(131) . Target concentrations were adjusted to standardized values. Values labeled OOR< or OOR> were adjusted to match the lowest (Standard 7) or highest (Standard 1) limit of detection, respectively. After this transformation, all values were log10-transformed. The samples from the VAP-COVID and NO VAP-COVID groups were analyzed separately for each target using Mann-Whitney tests. Results were visually represented through box graphs displaying mean values and standard deviations.

The 45-plex panel comprised five distinct target groups, which encompassed a comprehensive range of analytes. These target groups encompassed:

1. Th1/Th2 (GM-CSF, IFN gamma, IL-1 beta, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12p70, IL-13, IL-18, TNF alpha).
2. Th9/Th17/Th22/Treg (IL-9, IL-10, IL-17A (CTLA-8), IL-21, IL-22, IL-23, IL-27).
3. Inflammatory Cytokines (IFN alpha, IL-1 alpha, IL-1RA, IL-7, IL-15, IL-31, TNF beta).
4. Chemokines (Eotaxin (CCL11), GRO alpha (CXCL1), IP-10 (CXCL10), MCP-1 (CCL2), MIP-1 alpha (CCL3), MIP-1 beta (CCL4), RANTES (CCL5), SDF-1 alpha).
5. Growth Factors (BDNF, EGF, FGF-2, HGF, NGF, PDGF-BB, PIGF-1, SCF, VEGF-A, VEGF-D).

This comprehensive panel allowed for the simultaneous measurement of a wide array of biomarkers, enabling a thorough assessment of the immune and inflammatory responses under investigation **(Supplemental table 3.1)**.

Cytokine	Upper Limit	Lower Limit
BDNF	7949.16	2.02
EGF	15894.37	2.65
Eotaxin (CCL11)	2852.60	0.69
FGF-2	13120.67	3.39
GM-CSF	158342.87	20.04
GRO alpha (CXCL1)	3121.73	2.29
HGF	25360.16	5.90
IFN alpha	2572.49	0.61
IFN gamma	33816.64	9.16
IL-1 alpha	2008.79	0.80
IL-1 beta	10651.66	2.56
IL-1RA	153583.11	43.45
IL-2	18373.37	9.72
IL-4	49186.16	15.14
IL-5	37812.50	9.81
IL-6	30036.61	12.90
IL-7	3022.18	0.71
IL-8 (CXCL8)	10614.14	2.46
IL-9	29574.15	7.99
IL-10	6302.29	1.54
IL-12p70	30133.89	7.84
IL-13	32286.79	4.16
IL-15	17623.40	3.45
IL-17A (CTLA-8)	10793.27	3.08
IL-18	17235.49	14.81
IL-21	64061.97	15.83
IL-22	159893.49	24.25
IL-23	63804.35	28.25
IL-27	79444.34	18.42
IL-31	46053.98	11.86
IP-10 (CXCL10)	7418.33	2.09
LIF	7038.35	4.21
MCP-1 (CCL2)	16034.22	4.24
MIP-1 alpha (CCL3)	763.22	1.84
MIP-1 beta (CCL4)	51933.97	8.07
NGF beta	25686.50	6.36
PDGF-BB	18481.39	3.84

PlGF-1	6221.12	1.45
RANTES (CCL5)	267.88	1.45
SCF	4035.00	0.88
SDF-1 alpha	29185.67	16.64
TNF alpha	20976.44	5.45
TNF beta	24683.66	6.24
VEGF-A	19967.79	5.29
VEGF-D	6896.20	2.04

3. Table 3.1 Supplemental: The ranges of concentrations (pg/ml) for each target Lot # 313189-002.

7. Untargeted Metabolomic Analysis

The untargeted metabolomic investigation employed two methods: RP-LC-QTOF-MS and HILIC-LC-QTOF-MS. Sample preparation involved adding cold methanol (3:1 ratio) to plasma, vortexing for 5 minutes, and centrifugation at 7,310 x g for 10 minutes at 4°C. The analysis integrated an Agilent 1260 Infinity LC System with a 6545 Q-TOF LC/MS system from Agilent Technologies in Waldbronn, Germany. A 2 µL sample was injected into a ZORBAX Eclipse Plus C18 column (2.1 x 50 mm, 1.8 µm particle size) at 60°C. Mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) with a flow rate of 0.6 mL/min.

The HILIC-LC-QTOF-MS analysis involved injecting 5 µL of the sample into an Infinity Lab Poroshell HILIC-Z column (2.1 x 100 mm, 1.9 µm particle size) maintained at a constant temperature of 30°C. The mobile phases comprised 10% (200 mM ammonium format in Milli-Q water, pH 3) with 90% water (phase A) and 10% (200 mM ammonium format in water, pH 3) mixed with 90% acetonitrile (phase B). The flow rate remained constant at 0.6 mL/min, employing a gradient elution program. Data acquisition was conducted in negative electrospray ionization mode (ESI-), covering a mass-to-charge ratio spectrum from 50 to 1100 m/z.

8. Statistical analysis

Statistical analysis was performed using GraphPad Prism 9 software and R statistical framework (version 4.3.1). Initially, we used the Shapiro–Wilk test to assess the data distribution rigorously. Descriptive statistics were systematically applied to summarize the data set, encompassing the mean with standard error and the median coupled with the

interquartile range (IQR). Chi-square tests were judiciously applied for categorical variables to compare patient characteristics between distinct groups, while independent t-tests were utilized for continuous variables.

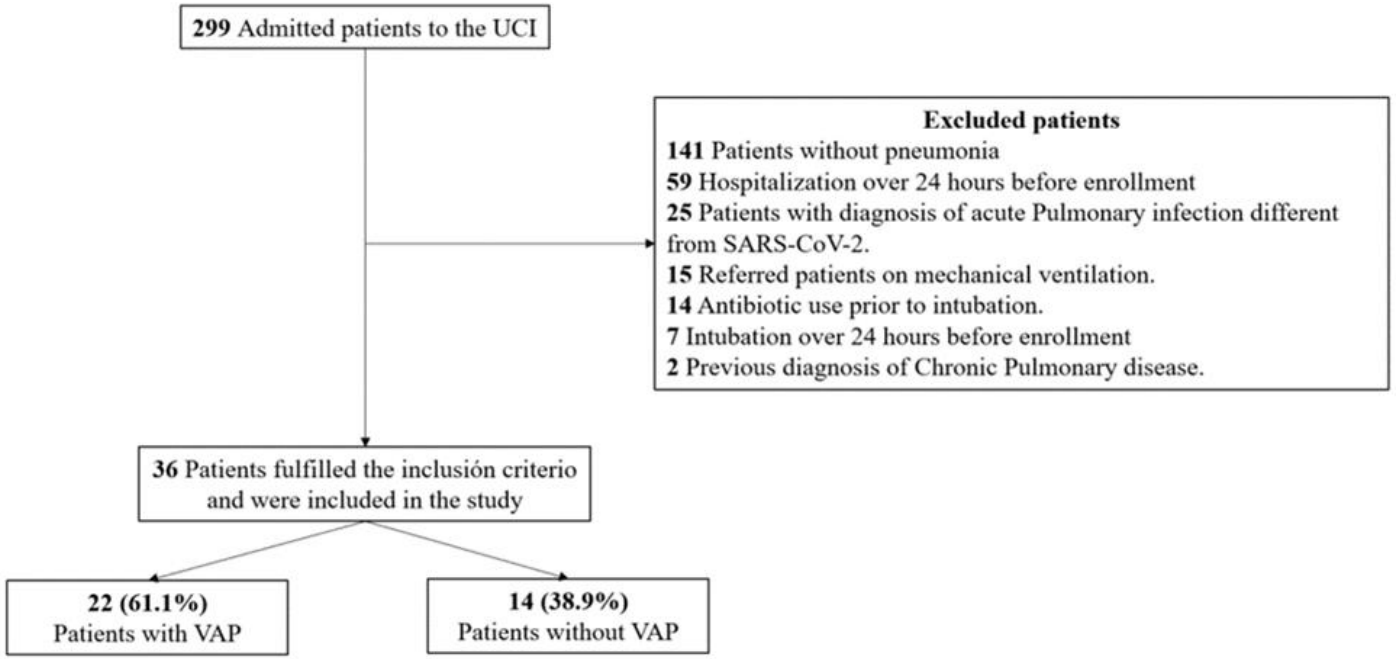
We estimated microbial diversity using the sophisticated vegan package implemented within the R environment. Alpha diversity was meticulously evaluated employing both Shannon and Chao1 indices. The significance of differences in alpha diversity between groups was determined by applying Wilcoxon's rank sum test or the Mann–Whitney U-test. The selection of these tests was contingent on whether the data were paired or unpaired. Beta diversity was quantified using the Bray-Curtis dissimilarity index and the weighted UniFrac distance. Principal Coordinate Analysis (PCoA) was conducted to assess beta diversity across varying groups. This involved using permutational multivariate analysis of variance (PERMANOVA), incorporating 9,999 permutations facilitated by the adonis2 function in the Vegan R package (v2.6-4).

To analyze the differences between groups, ratios were evaluated employing Fisher's exact probability test. Furthermore, correlations between clinical indicators and the lung microbiota were analyzed using Spearman Correlation Analysis. Throughout, a p-value threshold of less than 0.05 was adhered to, denoting statistical significance in all analytical determinations. For metabolomics comparative analysis, two-sample t-tests were applied, and the mean of groups was used to calculate the fold-change values.

C. Results

106 samples were collected from 36 COVID-19 patients undergoing mechanical ventilation in the ICU. This collection comprised 36 NS and 70 ETA samples (**Fig. 3.1**). Utilizing 16S RNA gene sequencing, the study delved into investigating the microbial composition within the respiratory tracts of these patients. The cohort was characterized by its diversity, encompassing individuals who either developed or did not develop VAP, thereby permitting a thorough evaluation of microbial diversity in severe COVID-19 cases. Demographic data, clinical characteristics, and laboratory test results are systematically presented in **Table 3.1**.

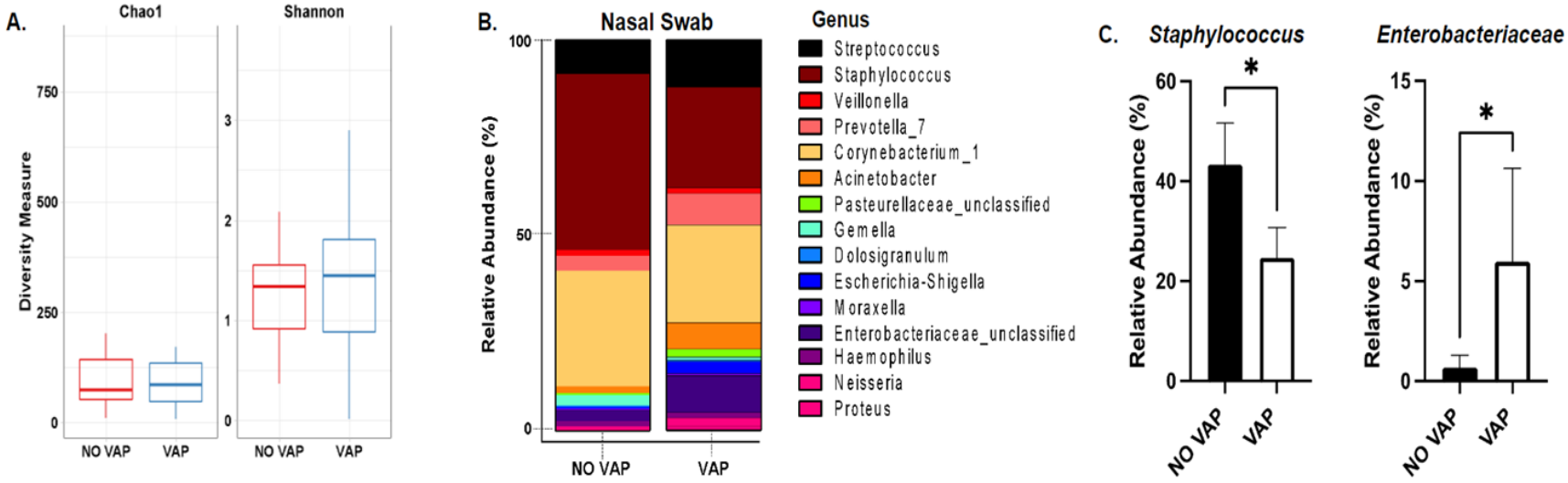
The median age of the cohort was 56 years, with an IQR of 49.7 to 64.2 years. A noteworthy finding was the prevalent administration of antimicrobials among these patients; 52.7% (19/36) received antimicrobials at ICU admission. When comparing patients with and without VAP, those admitted exhibited a markedly prolonged median duration of ICU length of stay (VAP: 15.0 days [IQR: 19.0-24.0] vs. No VAP: 6.0 days [IQR: 3.0-11.0], $p < 0.01$), extended length of hospital stays (VAP: 29.0 days [IQR: 12.0-48.5] vs. No VAP: 11.0 days [IQR: 4.0-18.0], $p < 0.01$), and an increased length of invasive mechanical ventilation (VAP: 9.0 days [IQR: 7.0-14.0] vs. No VAP: 3.0 days [IQR: 2.0-5.0], $p < 0.01$). These findings underscore the significant disparities between patients who developed VAP and those who did not.



7. Figure 3.1: Study Flow Chart. Flow diagram for the study showing the number of patients included in the analysis.

1. COVID-19 Patients with VAP and without VAP show differential nasal microbiome abundance changes upon ICU admission.

We first used Chao and Shannon diversity measures to test for differences in microbial abundance changes between the groups. Although no significant alterations were discerned among the groups in the overall microbial composition (**Fig. 3.2A**), further investigations were conducted to probe for specific abundance shifts among the predominantly present organisms within the samples. This in-depth analysis was designed to unearth subtle discrepancies potentially obscured in the broader comparative framework, yielding a more intricate and nuanced understanding of microbial dynamics. These showed significant differences between the VAP and NO VAP groups (**Fig 3.2B**), specifically in bacteria from the genus *Staphylococcus* and *Enterobacteriaceae* (**Fig 3.2C**). *Staphylococci* are Gram-positive bacteria that are common skin, pulmonary, and oral commensals, and members of this genus can also be pathobionts (132, 133). In contrast, members of the genus *Enterobacteriaceae* are part of a family of Gram-negative bacteria that includes pathogens such as *Klebsiella*, *Enterobacter*, *Citrobacter*, *Salmonella*, *Escherichia*, *Shigella*, *Proteus*, *Serratia* among others (134). These data suggest a possible shift in nasal colonizers that may predispose the patient to VAP from the members of the *Enterobacteriaceae* bacterial genus.

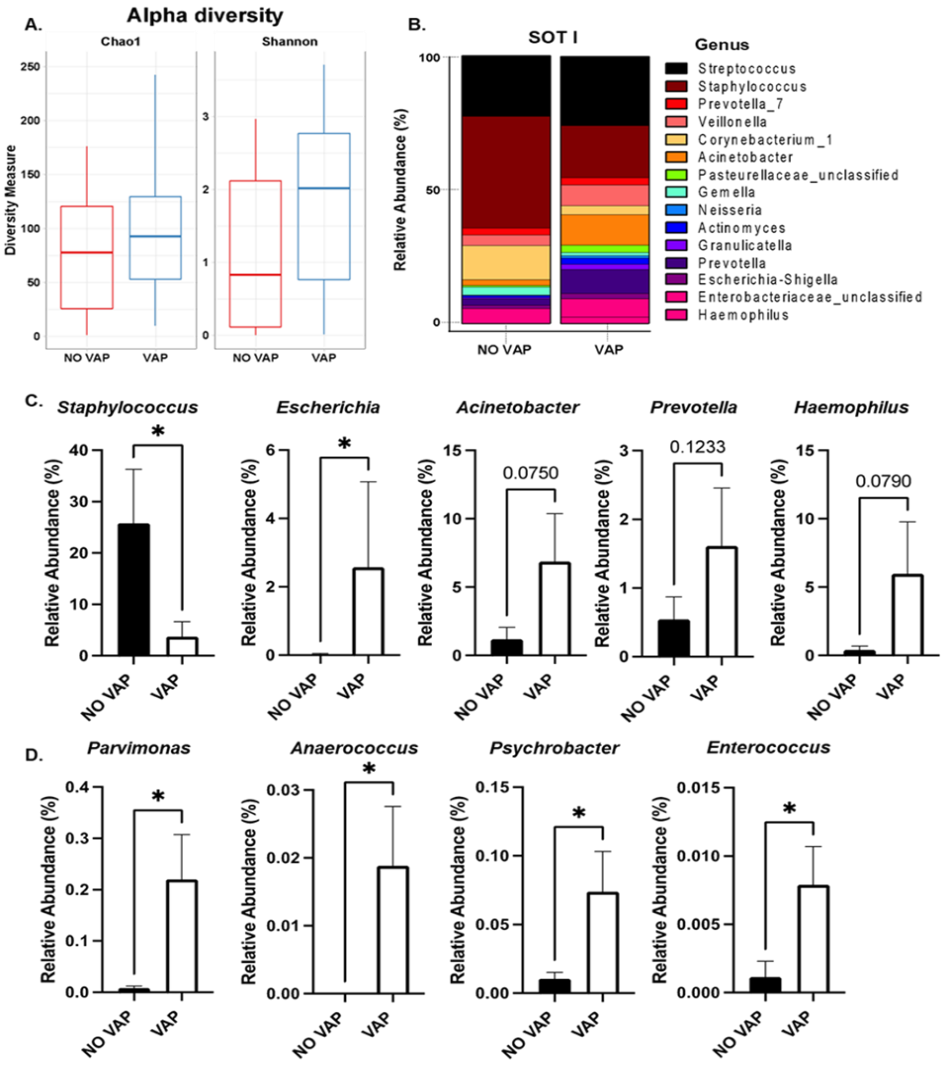


8. Figure 3.2: Nasal swabs of patients with COVID-19 that develop ventilator-associated pneumonia showed differential abundance of Staphylococcus and Enterobacteriaceae. A. Alpha diversity of nasal microbiome from COVID-19 patients that developed VAP or did not (NO VAP). **B**. Percent relative abundance of the top 15 most abundant microbes in the nasal cavity. **C.** Relative abundance bar graphs of Staphylococcus and Enterobacteriaceae genus. Student's t-test was used to calculate the p-value. Asterisks denote the level of significance observed: * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.

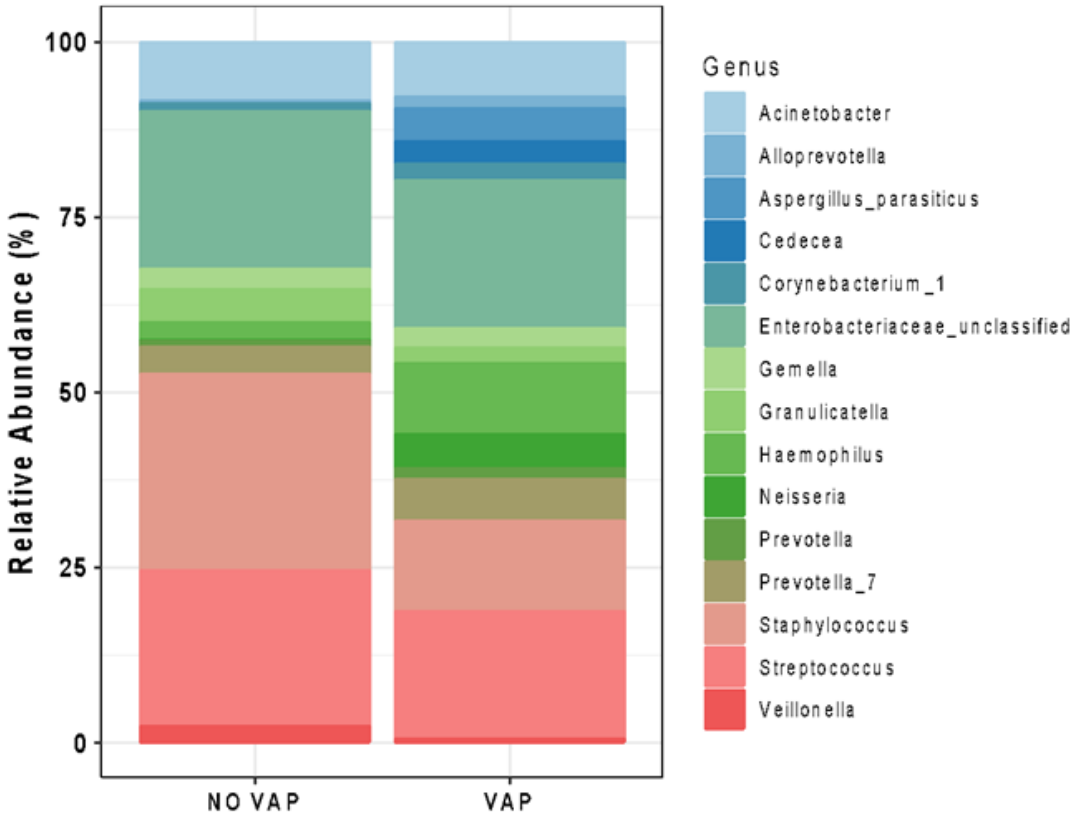
2. Endotracheal aspirates from COVID-19 patients who develop VAP have a reduction of Staphylococcus and increased Gram-negative bacterial pathogens.

To further assess pulmonary microbiome changes in the cohort, ETA samples were collected from patients upon intubation and at a follow-up time point (72 hours). At baseline, the Chao test did not show changes in total microbial richness. However, an increase in the Shannon index showed that richness and evenness were higher in the VAP group (**Fig. 3.3A**). Changes in abundance of the top 15 microbial genus showed drastic differences between the group who developed VAP and those who did not (**Fig. 3.3B**). Statistical analysis of the most abundant genus revealed a reduction in *Staphylococcus* and an increase in members of the *Enterobacteriaceae* group (**Figure 3.3C**). More precisely, a significant alteration in the abundance of *Escherichia* was observed, alongside a notable trend approaching significance in *Acinetobacter*.

Furthermore, increases in *Prevotella* and *Haemophilus* were also detected. However, these changes did not reach statistical significance (**Fig. 3.3C**). We also tested for significant changes in less abundant bacteria as they may influence the growth of pathogens by alteration of the local microenvironment. Of note, we observed a significant increase in the abundance of *Parvimonas, Anaerococcus, Psychrobacter*, and *Enterococcus* (**Fig. 3.3D**). Upon testing microbial changes in a follow-up time point, similar trends in microbial abundance were observed (**Supplemental Fig. 3.2**). Taken together, these results suggest that patients who develop VAP have an altered nasal and pulmonary microbiome that may predispose them to this severe form of disease.



9. Figure 3.3: Endotracheal aspiration of patients with COVID-19 shows differential abundance of pulmonary microbiome upon mechanical ventilation. A. Alpha diversity of pulmonary microbiome from COVID-19 patients that developed VAP or did not (NO VAP). **B.** Percent of relative abundance of the top 15 most abundant microbes in the lungs. **C.** Relative abundance bar graphs of Staphylococcus, Escherichia, Acinetobacter, Prevotella, and Haemophilus genus. **D.** Relative abundance bar graphs of *Parvimonas, Anaerococcus, Psychrobacter,* and *Enterococcus* genus. Student's t-test was used to calculate the p-value. Asterisks denote the level of significance observed: * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.



10. Figure 3.2 supplemental: Endotracheal aspiration of follow-up. Second endotracheal aspiration of patients with COVID-19 who developed ventilator-associated pneumonia shows the differential abundance of pulmonary microbiome.

3. A higher abundance of SARS-CoV-2 in serum correlates with dynamic changes in nasal and pulmonary microbiome in VAP patients.

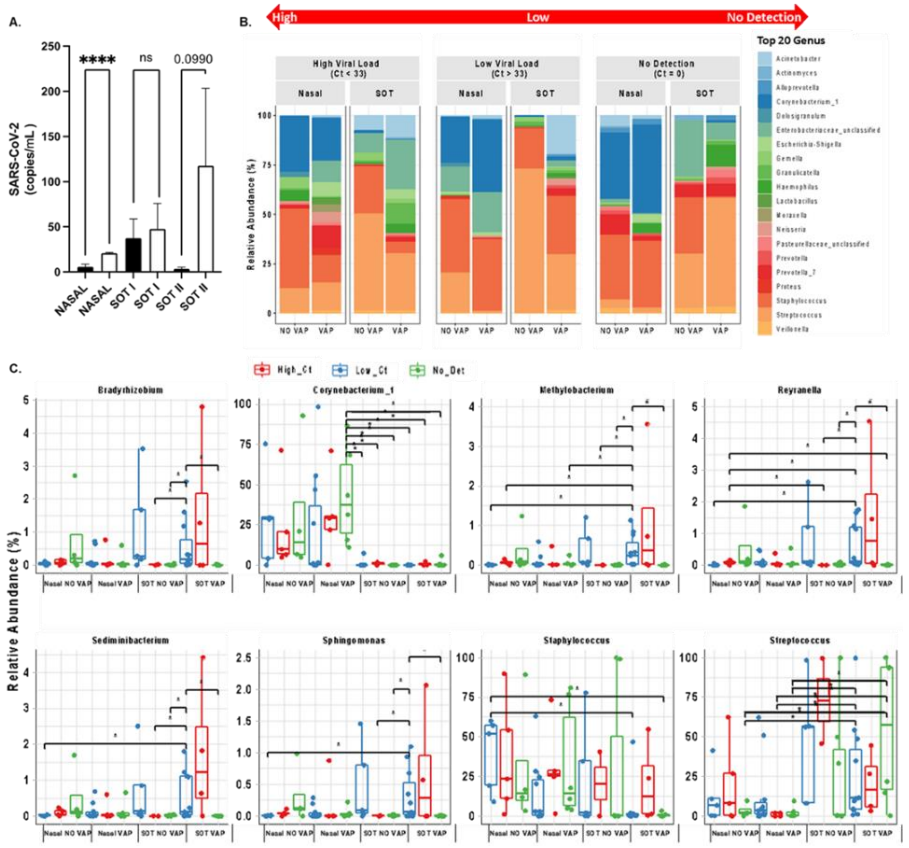
To determine the potential association between serum viral load and shifts in nasal and pulmonary microbiota, the research quantified levels of SARS-CoV-2 in the nasopharynx and lungs of the cohort at key intervals: upon hospital admission, during mechanical ventilation and at a subsequent follow-up. The findings indicated that patients who developed VAP exhibited higher Log copies/mL of SARS-CoV-2 at admission (the initial assessment point), as determined via quantitative real time polymerase chain reaction (RT-PCR) (**Fig. 3.4A**). Notably, significant variations in bacterial abundance were observed among patients with differing viral titers of SARS-CoV-2, compared to those without detectable virus at the time of sample collection, in both nasal and lung samples (**Fig. 3.4B-C**).

In nasal samples, the group with a higher viral load displayed a reduction in *Corynebacterium* and *Staphylococcus* and an increase in *Proteus, Enterobacteriaceae*, and *Escherichia-Shigella* (**Fig. 3.4B**). Conversely, the group with a lower viral load demonstrated an increase in *Corynebacterium* and *Enterobacteriaceae*, and a decrease in *Streptococcus* (**Fig. 3.4B**). In cases with no detectable SARS-CoV-2 in nasal samples, a reduction in *Acinetobacter* and *Prevotella*, and an increase in *Corynebacterium* and *Haemophilus* were noted (**Fig. 3.4B**).

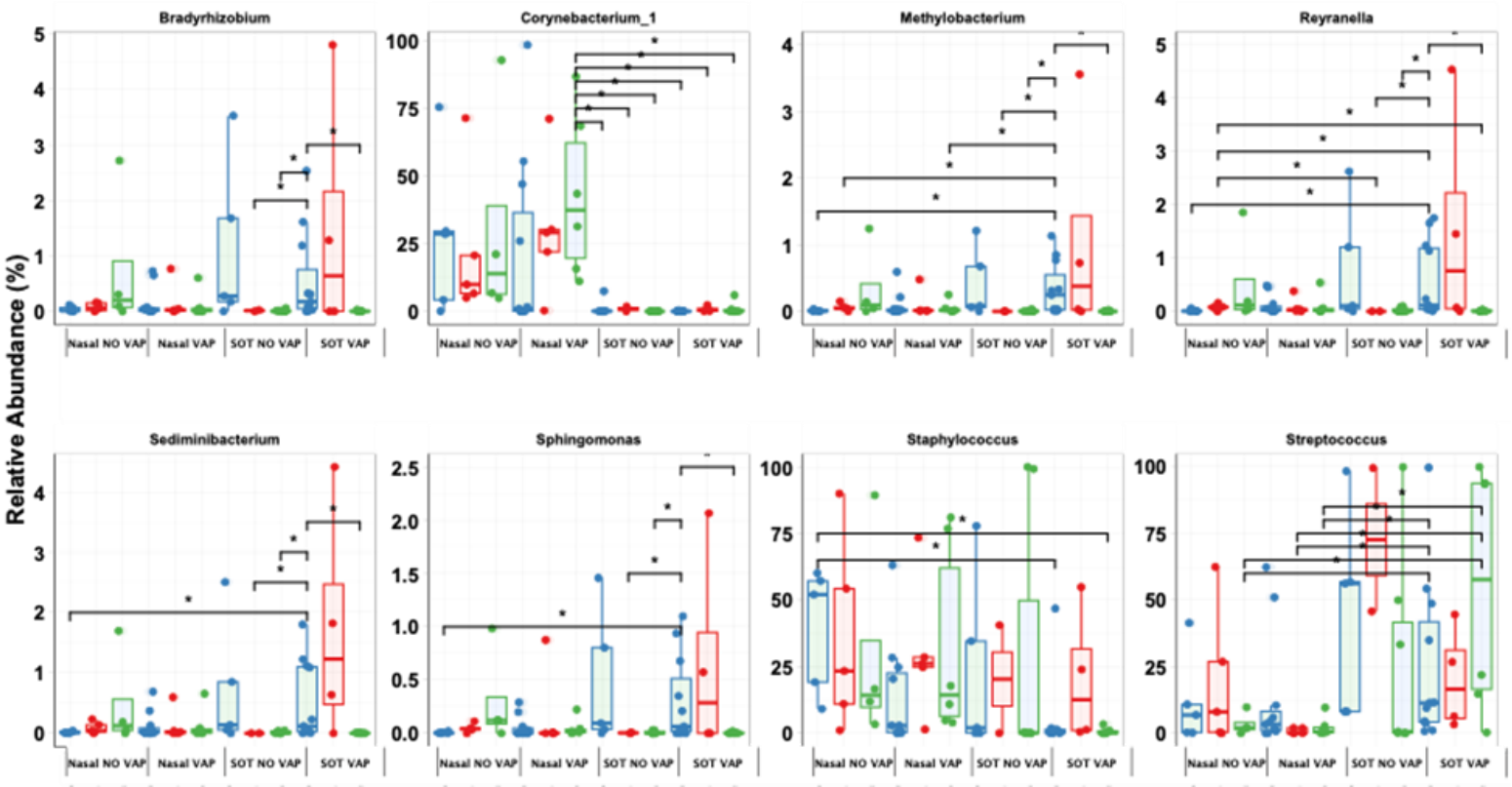
Regarding pulmonary samples, the high viral load group also exhibited a decrease in *Corynebacterium* and *Staphylococcus* and an increase in *Acinetobacter, Enterobacteriaceae*, and *Haemophilus* (**Fig. 3.4B**). In contrast, the lower viral load group showed an increase in *Acinetobacter, Neisseria*, and *Haemophilus*, and a decrease in *Streptococcus* (**Fig. 3.4B**). For

pulmonary samples with undetectable SARS-CoV-2, a reduction in *Enterobacteriaceae* and *Staphylococcus* and an elevation in *Streptococcus* and *Haemophilus* were observed (**Fig. 3.4B**).

When analyzing all samples collectively, statistical differences in the relative abundance of *Bradyrhizobium, Methylobacterium, Reyranella, Sediminibacterium,* and *Sphingomonas* were also noted (**Supplemental Figure 3.3**). In summary, the data suggest that viral titers are linked with a diminution in commensal bacteria and an escalation in Gram-negative pathogenic bacteria, potentially contributing to the development of VAP in patients under mechanical ventilation.



11. Figure 3.4: Differential abundance of SARS-CoV-2 modulates the nasal and lung microbiome. A. Log copies per-mL of SARS-CoV-2 were tested via quantitative RT-PCR. **B.** Percentage of relative abundance of the top 20 most abundant microbes in the nasal cavity and the lungs. n Distribution of relative abundance for selected bacterial genera in nasal and solid organ transplant samples, focusing on association with VAP presence. Viral load levels are differentiated.

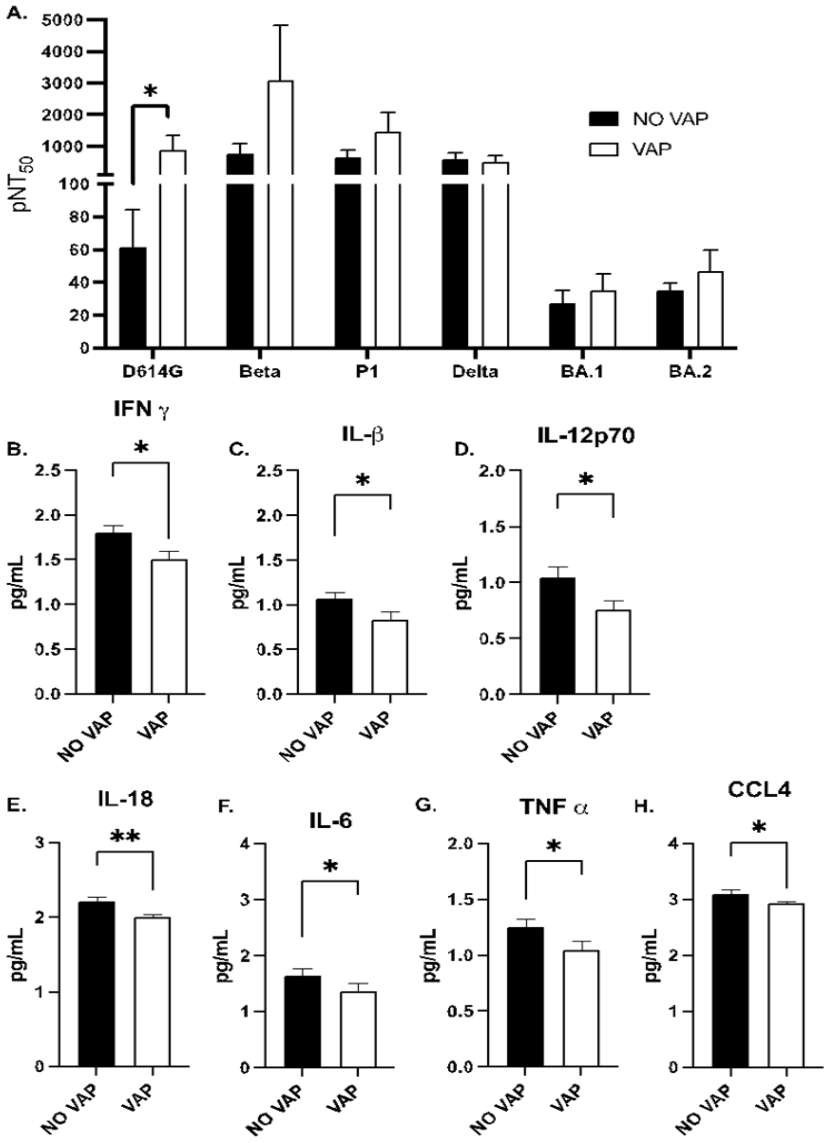


12. Figure 3.3 Supplemental: Differential changes in microbial genus associated with SARS-Cov-2titers.

4. COVID-19 patients who developed VAP showed increased SARS-CoV-2 neutralizing antibodies and decreased inflammatory cytokines and chemokines.

Spike-specific neutralizing antibodies are widely acknowledged as key indicators of the immune response against viruses and bacteria. Given that all patients in the study were diagnosed with COVID-19, the research aimed to ascertain if notable differences existed in neutralization titers between the groups with and without VAP. The analysis revealed no significant disparities in the capacity to neutralize pseudoviruses from variants of concern, namely Beta, Gamma, Delta, and Omicron subvariants BA.1 and BA.2. However, a marked elevation in the neutralization of D614 (closest to the original strain from 2019-2020) was discerned in the VAP group (**Fig. 3.5A**).

Additionally, plasma samples were procured from the 36 COVID-19 patients to quantify cytokines and chemokines. A significant reduction was observed in IFN-δ (p=0.01; **Fig. 3.5B**), IL-1β (p=0.04; **Fig. 3.5C**), IL-12p70 (p=0.01; **Fig. 3.5D**), IL-18 (p=<0.01; **Fig. 3.5E**), IL-6 (p=0.04; **Fig. 3.5F**), TNF-α (p=0.04; **Fig. 3.5G**), and CCL4 (MIP-1) (p=0.0479; **Fig. 3.5H**) in patients who developed VAP compared to those who did not. These findings suggest that in the VAP group, at the time of ICU admission, both a pronounced efficacy of neutralizing antibody activity and a decrease in inflammatory cytokines and chemokines are implicated in an antiviral response that might diminish host effectiveness against bacterial infections. Furthermore, the data indicates that the development of VAP was not linked to any specific viral variant of concern.



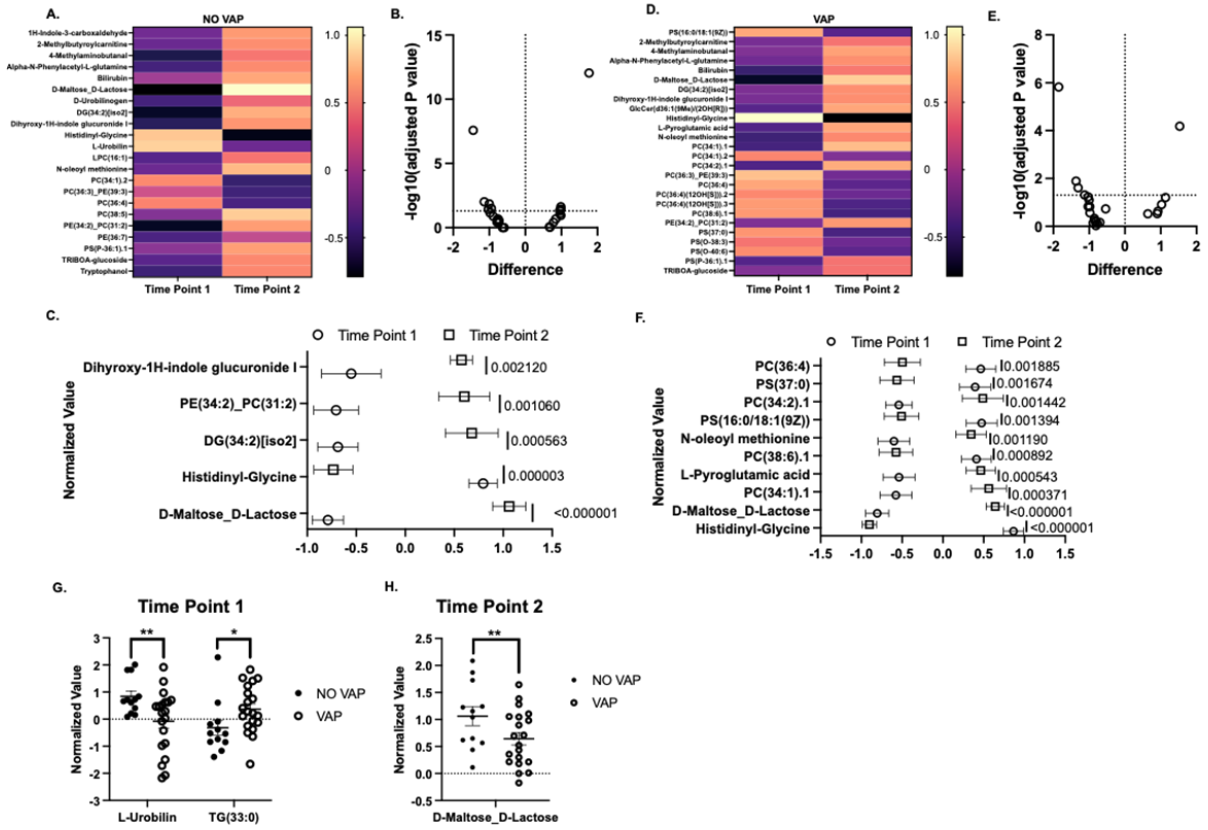
13. Figure 3.5: Plasma-neutralizing antibody titers and inflammatory effectors are differentially regulated during COVID-19 associated VAP. A. pNT50 values against the four SARS-CoV-2 pseudo-viral variants Beta, Gamma, Delta, and Omicron BA.1 and BA.2 and the control early 2020 strain with the D614 mutation. They were measured in samples collected from COVID-19 patients who developed VAP and those who did not (NO VAP). **B-H.** Cytokine and chemokine changes in serum (pg per mL). Mann-Whitney test was used to calculate the p-value. Asterisks denote the level of significance observed: * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.

5. Differential metabolomic changes occur in COVID-19 patients who develop VAP.

To test the effects of metabolic changes in COVID-19 patients who do or do not develop VAP, we carried out global metabolomics in plasma at two different time points, upon mechanical ventilation (baseline) and after 72 hours (follow-up). In patients without VAP, we observed significant changes in 22 metabolites (**Fig 3.6A**). After setting up a threshold of -log10 of 1.3 of the adjusted p value (**Fig 3.6B**). The most notably altered metabolites in this group were identified as D-Maltose-Lactose, Histidinyl-Glycine, Diacylglycerol with 34 carbons and 2 double bonds (DG 34:2), Phosphatidylethanolamine and Phosphatidylcholine combination with 34 carbons and 2 double bonds in total (PE 34:2_PC 31:2), Dihydroxy-1H-indole, and Glucuronide I (**Fig. 3.6C**). For the patients who developed VAP, we observed that 27 metabolites had significant changes between the two-time points (**Fig. 3.6D**). Upon evaluating the adjusted p-values of metabolites surpassing the -log10 threshold of 1.3 (**Fig. 3.6E**), it was discerned that the most significantly altered metabolites in the VAP cohort included Histidinyl-Glycine, a combination of Maltose and Lactose, Phosphatidylcholine with a total of 34 carbons and 1 double bond (PC 34:1), Pyroglutamic acid, Phosphatidylcholine with a total of 38 carbons and 6 double bonds (PC 38:6), a derivative of oleoyl methionine, Phosphatidylserine with 34 carbons and 1 double bond (PS 16:0/18:1), Phosphatidylcholine with 34 carbons and 2 double bonds (PC 34:2), Phosphatidylserine with 37 carbons (PS 37:0), and Phosphatidylcholine with 36 carbons and 4 double bonds (PC 36:4) (**Fig, 3.6F**).

When contrasting the NO VAP and VAP groups at the baseline, only Urobilin and Triglyceride with a total of 33 carbons differed significantly between the groups (**Fig. 3.6G**).

At the follow-up, the comparison revealed a significant difference in the levels of the maltose-lactose combination (**Fig. 3.6H**). Urobilin and maltose-lactose exhibited higher concentrations in the NO VAP group compared to the VAP group. The fluctuations in Urobilin might be linked to hepatic involvement, either as a direct consequence of the disease or due to certain medications administered. Alterations in the maltose-lactose combination are associated with shifts in the gut microbiome and a decrease in Short-Chain Fatty Acid (SCFA) producing bacteria (PMC7002114). SCFAs are crucial in modulating bacterial pathogen load and the level of inflammation (PMC8370681). Notably, Triglyceride with 33 carbons, also referred to as TG 17:0/8:0/8:0, was significantly elevated in patients who developed VAP (**Fig. 3.6G**). This triglyceride variant has been implicated in inflammation modulation and lipid metabolism, corroborating the findings presented in Figure 5. Collectively, this data suggests that changes in specific metabolites might serve as a mechanism predisposing COVID-19 patients to VAP.



14. Figure 3.6: Metabolomic changes in serum are observed during COVID-19-associated VAP. A. Heat map of the significantly changed metabolites when comparing NO VAP group at baseline and follow-up time point. **B.** Volcano plot for significant metabolites that pass the threshold of 1.3 -log10 (adjusted p-value). **C.** Most changed metabolites from the NO VAP group at baseline and follow-up time points. **D.** Heat map of the significant metabolite changes when comparing the baseline and follow-up time points of the VAP group. **E.** Volcano plot for significant metabolites that pass the threshold of 1.3 -log10 (adjusted p-value). **F.** Most changed metabolites from the VAP group at baseline and follow-up time points. **G.** Changes in L-Urobilin and TG normalized values (33:0) in VAP vs. No VAP at time point 1. **H.** Changes in normalized values of D-Maltose_D-Lactose in VAP vs. No VAP at time point 2. Student's t-test was used to calculate the p-value. Asterisks denote the level of significance observed: * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.

D. DISCUSSION

The microbiomes of the upper respiratory tract (URT) and lower respiratory tract (LRT) play a pivotal role in maintaining respiratory health by exerting influence over the severity of respiratory viruses, such as SARS-CoV-2, and potentially shaping acute immune responses (65, 135, 136). Given that the URT serves as the primary entry point for the COVID-19 virus, it is imperative to gain a thorough understanding of how the URT microbiome may impact the severity and outcomes of COVID-19 (137, 138). In our study, we observed notable disparities in the abundance of nasal microbiomes between COVID-19 patients who developed VAP and those who did not. While there were no significant alterations in the overall microbial diversity, discernible differences emerged in specific microbial abundances, particularly within the bacterial genera *Staphylococcus* and *Enterobacteriaceae*. These findings suggest a potential shift in nasal microbial colonization patterns that may contribute to an elevated susceptibility to VAP in afflicted patients. This observation aligns with prior studies, which have demonstrated overlap in the composition of the upper respiratory tract and lung microbiomes, indicating a role in overall pulmonary health (139). Furthermore, it is consistent with existing literature implicating opportunistic pathogens, such as Staphylococcus, in the severity of respiratory viral infections (140).

In ETA samples collected at the time of intubation and during a follow-up assessment, we observed a heightened microbial diversity and evenness in patients with VAP. Shifts in microbial abundance patterns indicated a reduction in *Staphylococcus* and an increase in *Enterobacteriaceae*, particularly *Escherichia spp*. These findings align with reports from other studies where *S. aureus* and *E. coli* were identified as the most common causative

microorganisms of VAP in COVID-19 patients (141-143). Additionally, less abundant bacteria, typically undetectable through conventional culture methods for VAP diagnosis, such as *Parvimonas, Anaerococcus, Psychrobacter, Prevotella, and Enterococcus*, also exhibited significant increases in patients who developed VAP. Similar trends were observed in the follow-up samples, indicating altered nasal and pulmonary microbiomes in VAP patients. Furthermore, an initial higher abundance of *Streptococcus* was observed in the baseline samples, followed by a subsequent decrease in the follow-up samples, irrespective of their classification as VAP or non-VAP cases.

Dysbiosis in the microbiome can foster an inflammatory milieu that facilitates the invasion and replication of the coronavirus, thereby constituting a risk factor for disease severity (144, 145). Our study conducted a comparative analysis of immune responses in COVID-19 patients with and without VAP. We discovered that spike-specific neutralizing antibodies exhibited similar efficacy against various SARS-CoV-2 variants in both groups, except for a notable increase in neutralization against the D614 variant within the VAP group. Furthermore, we assessed cytokine levels and observed diminished concentrations of pivotal cytokines in the VAP cohort, indicative of a subdued inflammatory response. These findings imply that individuals with VAP mount a robust neutralizing antibody response while concurrently exhibiting a reduced inflammatory cytokine profile (IFN-δ (p=0.01), IL-1β (p=0.04), IL-12p70 (p=0.01), IL-18 (p=<0.01), IL-6 (p=0.04), TNF-α (p=0.04), and CCL4 (MIP-1) (p=0.0479) upon admission to the ICU. This phenomenon may influence their immune defenses against bacterial infections, although no specific viral variant association with VAP was established.

This metabolomic analysis of COVID-19 patients unveils distinct metabolic profiles, sharply differentiating those with VAP from those without. The study identified 47 metabolites across various chemical classes and metabolic pathways, significantly altering phospholipid, sphingolipid, and glutathione metabolism in VAP patients. These changes, affecting cell membrane integrity and oxidative stress, could play a crucial role in VAP's pathogenesis, potentially enhancing bacterial adhesion and destabilizing immune responses (146). Additionally, imbalances in metabolites critical for glutathione and sphingolipid synthesis may exacerbate these effects, underlining their importance in VAP's complex pathophysiology. Conversely, patients without VAP exhibited variations in glycerophospholipids, glucuronides, and indole compounds, suggesting robust immune and metabolic responses. Glycerophospholipids indicate an optimal cellular membrane composition, vital for immune efficiency and cellular integrity, while variations in dihydroxyl, a glucuronide and indole compound, highlight its role in liver detoxification (147). Elevated urobilin levels in non-VAP patients point to preserved hepatic function, crucial for processing heme byproducts, contrasting with lower levels in VAP patients, possibly indicating liver impairment due to disease severity or medication side effects.

A primary limitation of this study is its relatively small sample size. Nevertheless, a comprehensive and multifaceted methodology was adopted to enhance the understanding of pulmonary microbiota dynamics in COVID-19 patients, focusing on developing secondary infections. These findings underscore the complex impact of COVID-19 and emphasize the critical importance of a holistic research approach. Such an approach deepens the understanding of the disease's complexities and opens new avenues for prevention and

treatment strategies, thereby making substantial contributions to the advancement of COVID-19 and respiratory infection management and patient care.

E. CONCLUSION

Our study elucidates the intricate interplay between the respiratory microbiota and COVID-19, emphasizing the significance of microbiome variations in patients with and without VAP. Employing advanced 16S RNA gene sequencing on samples from COVID-19 patients, we identified distinct microbial compositions correlated with disease severity. These findings reveal a critical link between microbial dysbiosis and the severity of COVID-19, suggesting that specific alterations in microbiota, alongside patient immunology, and metabolites, may influence both viral and bacterial pathogenesis. Despite the constraints of a small sample size, this research substantially contributes to the COVID-19 field, advocating for a holistic approach to treatment strategies and patient care. It also foregrounds the necessity for further exploration into the microbiome's role in respiratory diseases, particularly in severe viral infections, highlighting the imperative for a comprehensive understanding of these complex interactions to enhance patient outcomes amid ongoing global health challenges.

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IV. COMPARATIVE ANALYSIS OF LUNG MICROBIOME, CYTOKINES, AND METABOLOMIC PROFILES IN MECHANICALLY VENTILATED PATIENTS.

A. Abstract

1. Rationale: Understanding the relationship between the lung microbiome and ventilator-associated pneumonia (VAP) requires comprehensive research beyond metataxonomy.
2. Objectives: This study aims to explore the interplay among the pulmonary microbiome, immune responses, and metabolomic profiles to uncover factors driving the pathogenesis of VAP.
3. Methods: The study included patients needing invasive mechanical ventilation due to non-infectious respiratory failure lasting ≥ 48 hours. VAP diagnosis adhered to current clinical guidelines. Bronchoalveolar lavage samples were obtained on intubation day (baseline) and upon VAP diagnosis completion or after 72 hours for non-VAP patients. Subsequently, DNA isolation, PCR amplification, sequencing, cytokine quantification, and metabolomics analysis were conducted.
4. Results: The study involved 80 patients, with 41 (51%) developing Ventilator-associated pneumonia (VAP). Microbial diversity analysis showed slight differences between VAP and non-VAP groups initially, implying non-microbial factors' role in VAP development. Both groups maintained stable microbial abundances and similar bacterial communities over time. Cytokine analysis highlighted significant changes in TNFα and IL-1b levels between admission and follow-up in VAP patients.

Metabolomic analysis unveiled shifts in metabolic pathways, notably anaerobic glycolysis, and stress response, during VAP progression.

5. Conclusions: We found a resilient bacterial community in the microbiome, significant inflammatory alterations in cytokine profiles, and identified potential biomarkers through metabolomic analysis. These findings contribute to a deeper understanding of VAP's molecular intricacies and pave the way for future research and therapeutic advancements.
6. Keywords: Microbiota, Host Microbial Interaction, Metabolomics, Pneumonia, Ventilator-Associated

B. Introduction

Ventilator-associated pneumonia (VAP) stands as the most prevalent nosocomial infection in intensive care units (ICUs), entailing increased mortality rates of 20% to over 50%, prolonged hospital stays, elevated antibiotic usage, and higher treatment costs compared to patients without VAP (30, 148, 149). The global incidence of VAP, ranging from 9 to 18 cases per 1,000 ventilator days, varies based on hospital-specific prevention strategies and diagnostic criteria (28, 102, 150). Despite its multifactorial etiology involving bacteria, viruses, and fungi, precise microbial identification remains challenging due to limited sensitivity in conventional diagnostic methods (37, 38, 101).

Recent research utilizing 16S ribosomal RNA (rRNA) gene sequencing has significantly advanced our comprehension of microbial metataxonomy and bacterial communities in both healthy and afflicted individuals, including atypical microorganisms (41, 60, 61, 63). Metrics such as alpha and beta diversity provide insights into lung

microbiome composition (23, 148). Reduced alpha diversity within 48 hours post-tracheal intubation correlates with prolonged ventilation, although not always with VAP or antibiotic use (31, 46). Certain taxa like *Streptococcus, Lactobacillares*, and *Prevotella* exhibit decreased abundance in subsequent VAP development, contrasting with more prevalent taxa like *Haemophilus* and *Staphylococcus aureus* (25, 60, 64-66). Patients with positive respiratory microbiological cultures or categorized as microbiota manifest lower alpha diversity, highlighting microbial community roles in modulating virulence and antibiotic resistance. However, alpha diversity or beta diversity alone may not reliably diagnose lower respiratory tract infections, emphasizing the complex microbial dynamics and disease progression in VAP (25, 64, 66).

The intricate interplay between the lung microbiome and the pulmonary immune system underscores their symbiotic relationship in maintaining pulmonary homeostasis and mounting effective immune responses against pathogens (24, 75, 76). Pulmonary epithelial cells, alveolar macrophages, neutrophils, innate lymphocytes, and cytokines/chemokines collectively orchestrate innate and adaptive immunity, while lung microbial communities modulate these responses (16, 67-69). Dysbiosis can disrupt dendritic cell processes, impair T-cell responses, and alter cytokine balance, mediated by pattern recognition receptors (PRRs), shaping immune responses (5, 75-77). Dysbiosis-induced inflammation worsens pulmonary dysbiosis. Moreover, bacterial metabolites and products of bacterial metabolism can further affect inflammatory cytokine levels in the airways, establishing a bidirectional cycle of inflammation and dysbiosis (73, 80, 82, 84).

Recent research has significantly enhanced our understanding of VAP, unveiling a complex relationship among the pulmonary microbiome, host lung cells, and metabolites. This comprehension blurs the traditional boundary between commensal microorganisms and pathogens, indicating that VAP development is influenced by various factors. Despite the progress made in our knowledge, the limitations inherent in our current understanding underscore the necessity for further research to address challenges and achieve a comprehensive understanding of microbial dynamics. Therefore, this study aims to comparatively analyze the lung microbiome, cytokines, and metabolomic profiles in intubated patients, to deepen our understanding of these interactions and their implications in VAP pathogenesis.

C. Materials and Methods

1. Study Protocol and Population

The observational study at Clinica Universidad de la Sabana, Chia, Colombia (Jan 2021 - Jul 2022), ethically approved (codes: 20190903, 468), adhered to the Declaration of Helsinki. It assessed patients undergoing invasive mechanical ventilation for ≥48 hours due to non-infectious respiratory failure. Inclusion criteria comprised age >18, Critical Care Unit admission, and informed consent. Exclusions included pregnancy, breastfeeding, >24-hour pre-enrollment ventilation, prior antibiotics, acute pulmonary infection within 48 hours, or chronic lung disease. Written informed consent was obtained, with stringent risk mitigation.

2. Diagnosis Criteria for VAP and Sample Collection

VAP diagnosis adheres to the guidelines set forth by the Infectious Diseases Society of America and the American Thoracic Society, requiring ≥48 hours of ventilation. Criteria encompass radiographic infiltrate deterioration and ≥2 clinical indicators: fever (>38°C), purulent tracheal discharge, or significant change in leukocyte count (<4,000/μL or >10,000/μL). Confirmation involves isolating a pneumonia-associated pathogen. BAL and blood samples were collected within 12 hours and on day 5 of intubation, with extra samples taken at VAP diagnosis if necessary. Samples were stored at -80 °C. Data were collected until extubation, with post-discharge records reviewed in REDCap for accuracy.

3. DNA Isolation and 16S rRNA Gene PCR Amplification

DNA extraction from a 500 µl BAL fluid aliquot began without centrifugation, using the DNeasy® Blood & Tissue Kit by QIAGEN. DNA purity and concentration were assessed with the NanoDrop™ One spectrophotometer. AccuPrime High-Fidelity Taq replaced AccuPrime Pfx SuperMix for 16S rRNA gene PCR amplification targeting the V4 region. Sequencing on the Illumina MiSeq platform followed standard protocols with SOP modifications. Quantification of 16S rRNA gene copy number will be detailed in the supplement.

4. Inflammatory profiling

We quantified IL-1β, IL-6, and TNF-α cytokines in BAL. To enhance accuracy, 100 µL BAL was mixed with 100 µL sputolysin. The Milliplex® assay (Millipore Corp.) was used following the manufacturer's protocol. (High Sensitivity Human Cytokine kit; Millipore Corp., St. Charles, MO, USA, HCYTA-60K).

5. Metabolomic profiles

BAL samples were centrifuged, and metabolites were extracted using methanol: chloroform. GC-TOF-MS analyzed plasma samples post-derivatization. An Agilent GC system coupled to a QTOF 7250 was used. Data processing included deconvolution, alignment, integration, and normalization. Metabolite identification utilized a metabolomics library.

6 Data analysis

Sequencing data were processed using Mothur v.1.42.3 software by the Schloss Standard Operating Procedure. OTU classification utilized the Mothur implementation of the Ribosomal Database Project (RDP) Classifier with the RDP taxonomy training set. Operational taxonomic units (OTUs) were categorized at 97% identity. Principal component and regression analyses in R version 4.3.1 focused on OTUs present >0.5% within the sample population. Data analyses were performed using R version 4.3.1.

7 Statistical analysis

Descriptive analysis examined central tendency/dispersion with mean/median, SD/IQR. Qualitative variables were described with frequencies/percentages. Group heterogeneity was assessed with chi-square, Fisher's exact tests for categorical, and Student's T-test or Mann-Whitney U test for continuous variables. Microbial diversity was analyzed using the vegan package in R, employing PCA on normalized OTU tables. Alpha diversity was compared between groups via the Wilcoxon rank-sum test, while beta diversity was measured using Bray-Curtis dissimilarity and PERMANOVA. MVA/UVA analyses utilized SIMCA 16.0 and MatLab (R2019b), with OPLS-DA distinguishing between groups and

UVA determining significance with FDR correction. Cross-validation and permutation tests ensured model predictability and validity.

D. Results

1. Demographic Data

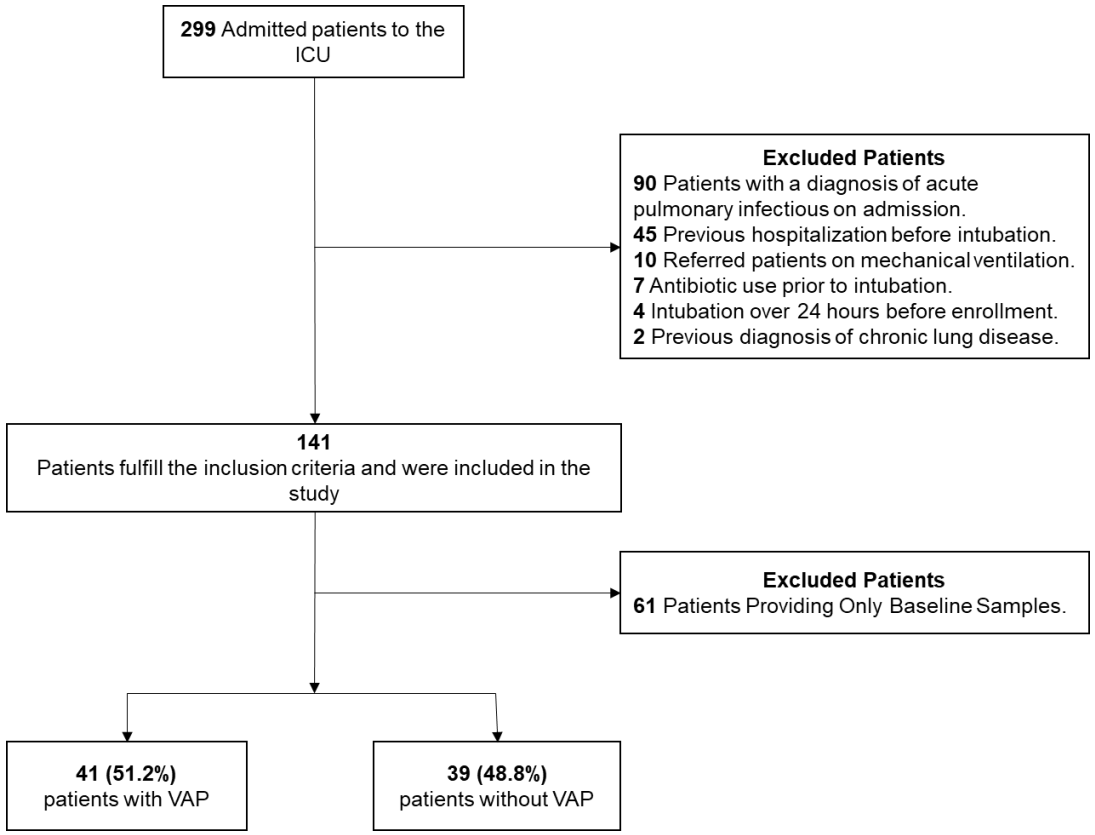
In this study, a total of 80 individuals were enrolled. The demographic characteristics, physiological data, laboratory results, and scores are presented in **Table 4.1** and **Table 4.2**. Most participants were male, accounting for 68.8% [55/80], with a median [IQR] age of 51.5 years (34.0-69.2). The most frequent comorbidities were arterial hypertension (33.89% [27/80]), diabetes mellitus (8.8% [7/80], and smoking history (7.5% [6/80]). A significant portion of the cohort, 73 patients (91.2%), received antibiotic therapy continuously or intermittently throughout the ICU admission. Among the 80 patients in the study, 41 were clinically diagnosed with VAP (**Fig. 4.1**).

Characteristic	All n = 80	VAP n = 41	No-VAP n = 39	p-value
Demographic				
Male. n (%)	55 (68.8)	23 (56.1)	32 (82.1)	**0.02**
Age. median (IQR)	51.5 (34.0-69.2)	48.0 (34.0-67.0)	53.0 (35.0-70.0)	0.47
Comorbid conditions. n (%)				
Alcohol	4 (5.0)	1 (2.4)	3 (7.7)	0.57
Stroke	3 (3.8)	1 (2.4)	2 (5.1)	0.96
Cancer	3 (3.8)	2 (4.9)	1 (2.6)	1
Diabetes mellitus	7 (8.8)	2 (4.9)	5 (12.8)	0.38
Coronary disease	2 (2.5)	1 (2.4)	1 (2.6)	1
Mental Illness	1 (1.3)	0 (0.0)	1 (2.6)	0.97
Chronic kidney disease	6 (7.5)	2 (4.9)	4 (10.3)	0.62
COPD	3 (3.8)	0 (0.0)	3 (7.7)	0.22
Cardiac failure	2 (2.5)	1 (2.4)	1 (2.6)	1
Hemodialysis	2 (2.5)	0 (0.0)	2 (5.1)	0.45
Arterial hypertension	27 (33.8)	11 (26.8)	16 (41.0)	0.26
Obesity	5 (6.3)	3 (7.3)	2 (5.1)	1
Smoking	6 (7.5)	2 (4.9)	4 (10.3)	0.62
No background	40 (50.0)	23 (56.1)	17 (43.6)	0.37
Outcomes				
Length of stay in ICU, days (IQR)	13.0 (7.7-16.0)	15.0 (11.0-24.0)	10.0 (6.0-13.5)	**0.01**
Length of stay in the hospital, days (IQR)	18.0 (11.0-38.2)	29.0 (12.0-49.0)	15.0 (10.5-22.5)	**<0.01**
Intubation time, days (IQR)	8.0 (5.0-11.0)	10.0 (7.0-15.0)	6.0 (5.0-8.5)	**<0.01**
Hospital Mortality (%)	23 (28.8)	12 (29.3)	11 (28.2)	1
Mortality 28d (%)	26 (32.5)	13 (31.7)	13 (33.3)	1
Mortality 90d (%)	28 (35.0)	15 (36.6)	13 (33.3)	0.94

4. Table 4.1: Demographic Data and Outcomes of Enrolled Patients.

Characteristic	All n = 80	VAP Baseline n = 36	No-VAP Baseline n = 39	p-value	VAP follow-up n = 41	No VAP follow-up n = 41	p-value
Physiological Variables at Admission and Subsequent Follow-Up: Median and Interquartile Range (IQR)							
Heart rate. BPM	80.0 (65.2-96.5)	77.0 (70.0-92.0)	88.0 (73.5-102.2)	0.06	78.0 (69.0-89.0)	78.0 (66.0-90.0)	0.76
Respiratory rate. RPM	18.0 (16.0-20.0)	18.5 (17.0-20.0)	18.0 (16.0-20.0)	0.59	20.0 (18.0-22.0)	20.0(18.0-20.0)	0.31
Temperature. °C	36.5 (36.2-36.9)	36.6 (36.3-37.0)	36.4 (36.2-36.8)	0.23	37.0 (36.9-37.7)	36.9 (36.5-37.0)	**0.03**
SBP. mmHg	119.0 (104.8-133.0)	123.0 (108.0-132.0)	118.0 (101.0-138.5)	0.90	125.0 (119.0-138.0)	129.0 (120.0-146.0)	0.37
DBP. mmHg	69.0 (57.0-79.0)	68.0 (57.0-76.0)	70.5 (58.0-84.5)	0.29	68.0 (62.0-74.0)	68.0 (58.0-74.0)	0.87
PAM. mmHg	10.2.1 (87.5-115.0)	103.3 (88.3-111.0)	101.3 (85.2-123.5)	0.72	105.6 (99.6-118.0)	108.3 (100.0-122.3)	0.46
SPO2. (%)	93.0 (91.0-95.0)	92.0 (92.0-96.0)	93.0 (91.0-94.0)	0.59	93.0 (91.0-94.0)	94.0 (92.0-96.0)	0.09
Glasgow	7.0 (6.0-10.0)	7.0 (6.0-8.0)	8.0 (6.0-13.0)	**0.04**	7.0 (6.0-9.0)	7.0 (6.0-10.0)	0.27
Laboratory variables at admission and Subsequent Follow-up: Median and Interquartile Range (IQR)							
WBC, cell x 103	13.1 (9.7-16.0)	13.3 (11.1-15.9)	11.8 (9.0-16.2)	0.34	10.0 (8.5-13.5)	9.95 (7.85-13.0)	0.44
Neutrophiles, (%)	81.0 (70.7-87.0)	81.0 (71.0-87.0)	81.0 (71.7-87.2)	0.86	79.5 (75.0-84.0)	78.0 (76.0-83.0)	0.82
Hemoglobin, g/dL	13.0 (11.6-14.0)	13.3 (11.8-14.5)	12.5 (11.3-13.4)	0.09	9.7(8.5-11.1)	9.7 (8.6-10.9)	0.99
Platelet, cell x 103	195.0 (157.2-270.0)	190.0 (160.0-270.0)	205.0 (160.0-270.0)	0.95	165.0 (127.5-190.0)	130.0 (120.0-172.5)	**0.04**
Creatinine, mg/dL	1.0 (0.8-1.3)	1.0 (0.8-1.3)	0.9 (0.7-1.2)	0.31	0.8 (0.6-1.1)	0.8 (0.7-1.4)	0.33
BUN, mg/dL	16.0 (12.0-21.5)	15.0 (12.0-19.2)	17.5 (13.7-26.0)	0.11	19.0 (12.0-32.0)	18.0 (12.0-27.2)	0.63
Blood glucose, mg/dL	130.0 (120.0-160.0)	130.0 (120.0-160.0)	140.0 (120.0-165.0)	0.83	140.0 (130.0-165.0)	130.0 (117.5-150.0)	**0.03**
Sodium, mEq/L	140.0 (138.0-142.0)	140.0 (138.0-142.2)	140.0 (138.0-141.5)	0.86	143.0 (139.0-145.0)	141.0 (139.0-147.5)	0.62
Potassium, mEq/L	4.0 (3.6-4.5)	4.0 (3.5-4.5)	4.0 (3.7-4.5)	0.54	3.8 (3.6-4.2)	4.0 (3.7-4.4)	0.22
pH	7.3 (7.2-7.4)	7.3 (7.2-7.3)	7.3 (7.2-7.4)	0.17	7.4 (7.4-7.4)	7.4 (7.4-7.4)	0.76
PCO2, mmHg	36.0 (33.0-42.0)	36.0 (33.0-39.2)	37.0 (33.0-44.0)	0.27	35.5 (32.0-40.0)	37.5 (32.0-42.0)	0.46
PaO2, mmHg	78.0(69.7-94.0)	76.0 (69.0-88.0)	84.0 (74.0-108.0)	0.07	75.0 (71.0-81.7)	81.5 (70.5-90.7)	0.21
FiO2	35.0 (28.0-41.2)	32.0 (28.0-36.1)	35.0 (28.0-45.0)	0.71	30.0 (28.0-32.0)	31.0 (28.0-35.0)	0.14
HCO3, mmol/L	20.0 (17.0-23.0)	20.0 (18.0-23.0)	20.5 (16.7-23.0)	0.73	25.0 (21.2-26.0)	24.0(22.0-26.2)	0.87
Acid lactic, mmol/L	1.9 (1.2-3.2)	1.5 (0.9-3.2)	2.2 /(1.4-3.1)	0.17	1.1 (0.9-1.4)	1.1 (0.9-1.3)	0.62
PT, seconds	11.0 (10.0-13.0)	11.0 (10.0-12.0)	12.0 (10.5-13.5)	0.14	11.0 (10.0-12.5)	15.0 (12.5-15.7)	**0.05**
PTT, seconds	24.0 (23.0-27.0)	24.0 (22.0-26.0)	25.0 (23.0-28.0)	0.42	29.0 (28.0 - 34.0)	26.0 (26.0-27.0)	**0.01**
Scores at admission and Subsequent Follow-up: Median and Interquartile Range (IQR)							
SOFA	8.0 (7.0-9.0)	8.0 (6.0-9.0)	8.0 (7.0-9.0)	0.18	9.0 (7.0-10.0)	9.0 (7.0-10.0)	0.82
APACHE	16.0 (10.5-19.5)	15.5 (9.0-19.2)	17.0(13.0-21.7)	0.16	14.0 (11.0-20.0)	17.0 (14.0-19.0)	0.15
CPIS	2.0 (1.0-3.0)	2.0 (1.0-4.2)	2.0 (1.0-3.0)	0.54	2.0 (1.0-2.0)	3.0 (1.75-4.0)	**<0.01**

5. Table 4.2: Physiological, Laboratory, and Scoring Data at Admission and During Follow-Up of Enrolled Patients.

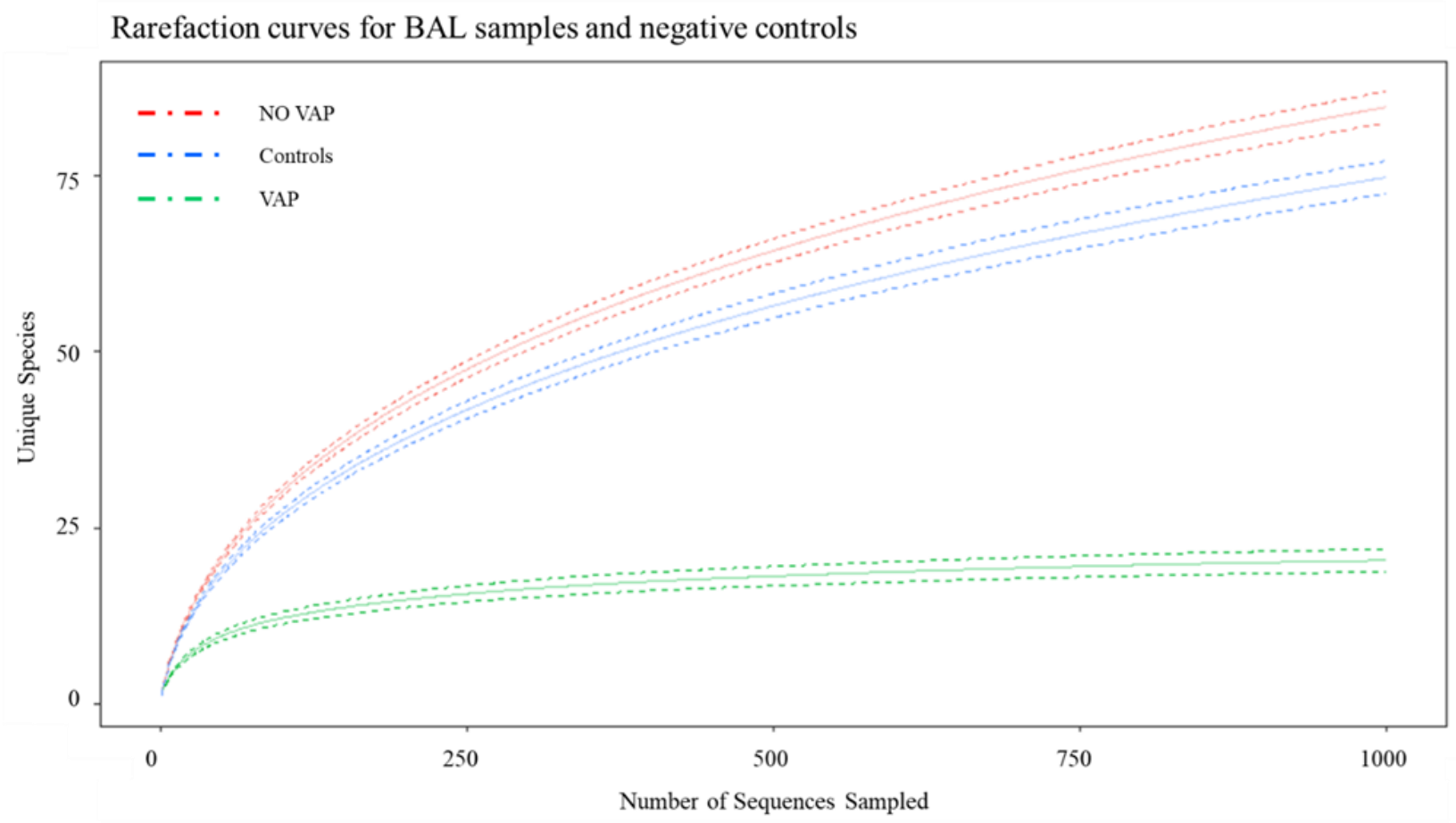


15. Figure 4.1: Study flow Chart. Patients in the intensive care unit who underwent bronchoalveolar lavage (BAL) collection.

When comparing the two groups, it was observed that the No-VAP patients were slightly older (No-VAP: 53 years [35.0-70.0] vs VAP: 48 years [34.0-67.0], p= 0.47). The ICU length of stay (LOS) was significantly higher among VAP patients (No-VAP: 10.0 days [6.0-13.5] vs VAP: 15.0 days [11.0-24.0], p=0.01). Similarly, the overall hospital LOS was notably longer in VAP patients (No-VAP: 15.0 days [10.5-22.5] vs VAP: 29.0 days [12.0-49.0], p<0.01). Furthermore, the duration of intubation was also longer in VAP patients (No-VAP: 6.0 days [5.0-8.5] vs VAP: 10.0 days [7.0-15.0], p=0.01).]. The median (IQR) physiological variables, such as heart rate (No-VAP: 88.0 bpm [73.5-102.2] vs. VAP: 77.0 bpm [70.0-92.0], p=0.06) and respiratory rate (No-VAP: 18.0 rpm [16.0-20.0] vs VAP: 18.5 rpm [17.0-20.0], p=0.59), were similar for both groups within the first 12 hours of intubation. However, Glasgow Coma Scale scores were significantly higher among No-VAP patients (No-VAP: 8.0 [6.0-13.0] vs VAP: 7.0 [6.0-8.0], p=0.04). Additionally, although PaO2 did not show a significant difference between the groups, No-VAP patients exhibited higher levels (No-VAP: 84.0 [74.0-108.0] vs VAP: 76.0 [69.0-88.0], p=0.07).

2. Taxonomy distribution and Quality control of the 16S rRNA sequence

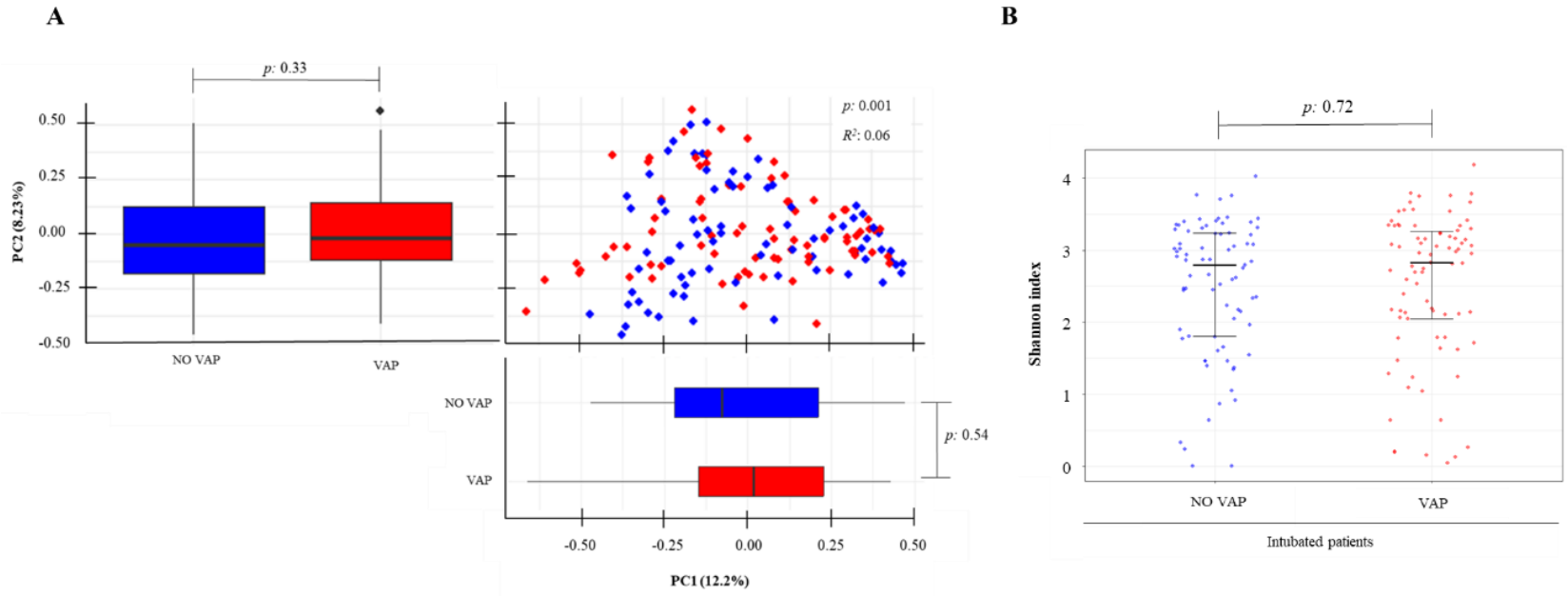
A total of 20,808,915 reads and 18,475 operational taxonomic units (OTUs) were obtained, resulting in an average of 130,873 reads and 116.1 OTUs per BAL sample. Subsequently, the OTUs were refined to 2,096, significantly reduced from the initial count of 18,475 OTUs. Sequences were classified to the lowest taxonomic designation possible, most at the genus level. The rarefaction curves illustrate that the sequencing depth was adequate, likely yielding a precise representation of the microbial population present in the BAL samples (**Fig.4.2**.).



16. Figure 4.2:. Rarefaction curves: The microbial diversity in bronchoalveolar lavage (BAL) samples from VAP patients, NO VAP patients, and negative controls using 16S rRNA gene sequencing.

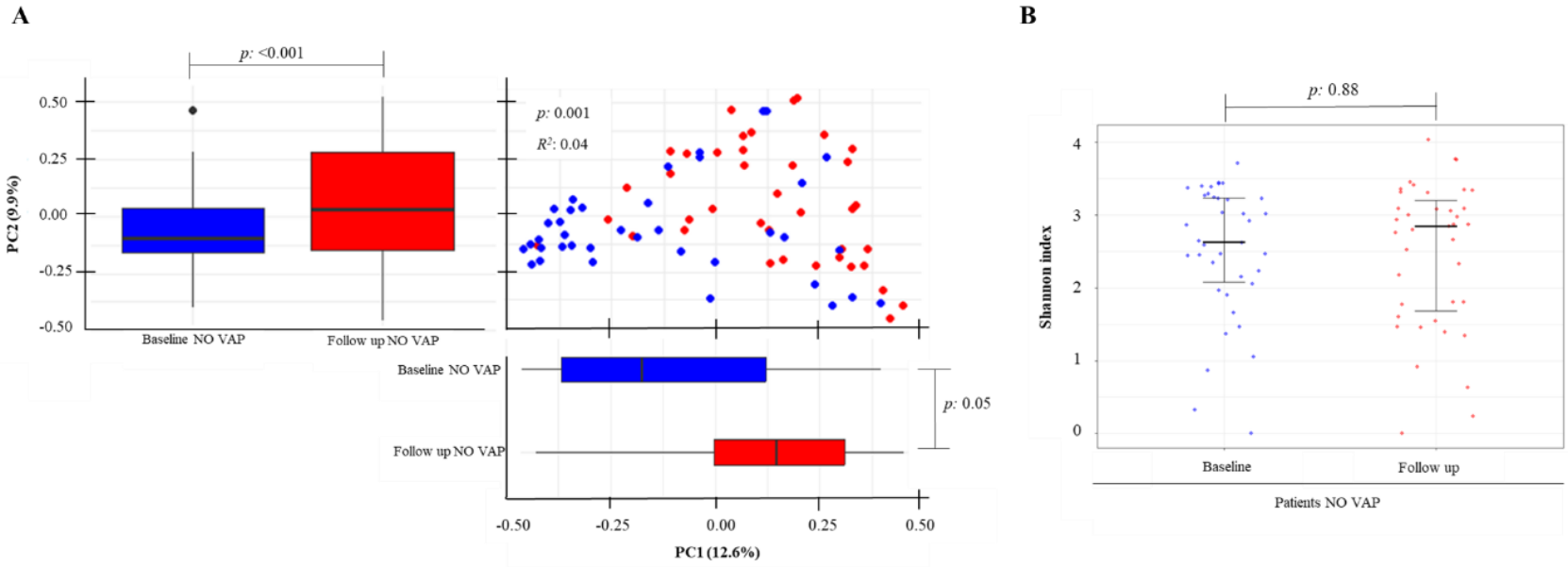
3. Shannon and Beta Diversity Analysis

The analysis employed Principal Coordinate Analysis (PCoA) based on Bray-Curtis distances, revealing a statistically significant, though modest, differentiation between the two patient groups ($p = 0.001$), with the primary components accounting for 6% of the data variance ($R^2 = 0.06$) (**Fig. 4 3a**). This indicates the presence of additional factors influencing the microbial diversity. Moreover, the Shannon diversity index assessment showed no significant diversity difference between the VAP and non-VAP groups ($p = 0.72$), suggesting that VAP status does not significantly alter the bacterial community's diversity within this patient cohort during the intubation period (**Fig. 4 3b**).

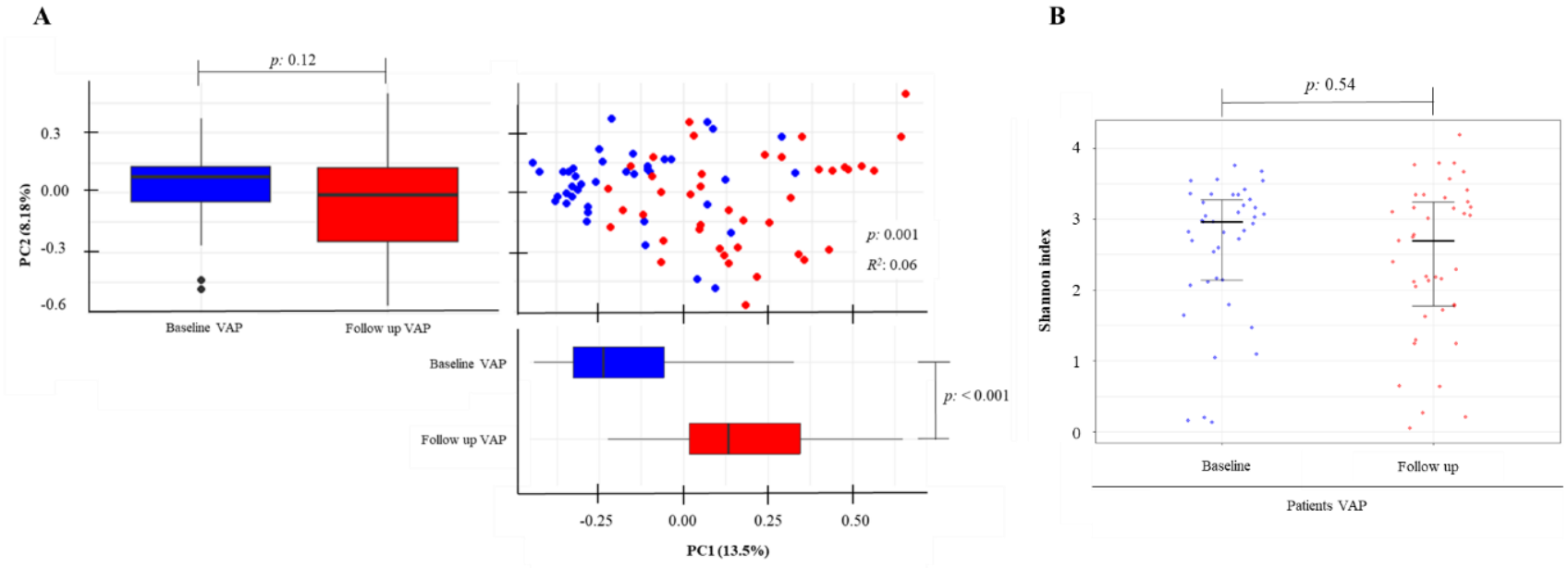


17. Figure. 4.3: Alpha and Beta Diversity Comparisons in the Respiratory Microbiomes of BAL Samples from ICU Patients with and without VAP. A. Beta diversity of BAL samples from ICU patients is depicted using a PCoA scatter plot based on Bray-Curtis distances, highlighting a significant, yet modest, distinction between patients with VAP and those without (p = 0.001, R^2 = 0.06). The variance captured by each principal coordinate is indicated in the axis titles (p = 0.001 for PC1 and p = 0.335 for PC2). **B.** Alpha diversity, as measured by the Shannon index, shows no significant disparity in microbial diversity between the VAP and NO VAP groups (p = 0.72).

A comparative analysis was also conducted on patients who did not develop VAP, examining samples taken at the time of intubation (baseline) or during follow-up, either at 72 hours or on the fifth day of intubation. Significant statistical alterations were observed in comparing baseline and follow-up samples among patients who did not develop VAP (NO VAP). PCA revealed noteworthy distinctions in PCA1 ($p = 0.05$) and even more pronounced disparities in PCA2 ($p = 0.001$). These findings imply that shifts in the bacterial community structure can occur during intubation and become evident during follow-up. PCoA similarly exhibited a significant dissimilarity with a p-value of 0.01 (**Fig. 4.4a**). Nevertheless, the Shannon diversity index, with a p-value of 0.88, indicated that the overall bacterial diversity remained constant from baseline to follow-up within the 'NO VAP' patient cohort, despite the observed alterations in the community structure (**Fig. 4.4b**). Among patients who developed VAP, this result aligns with the observations in the 'NO VAP' group, where a statistically significant change in community structure was evident, yet Shannon diversity remained unchanged (**Fig. 4.5a, Fig. 4.5b**).



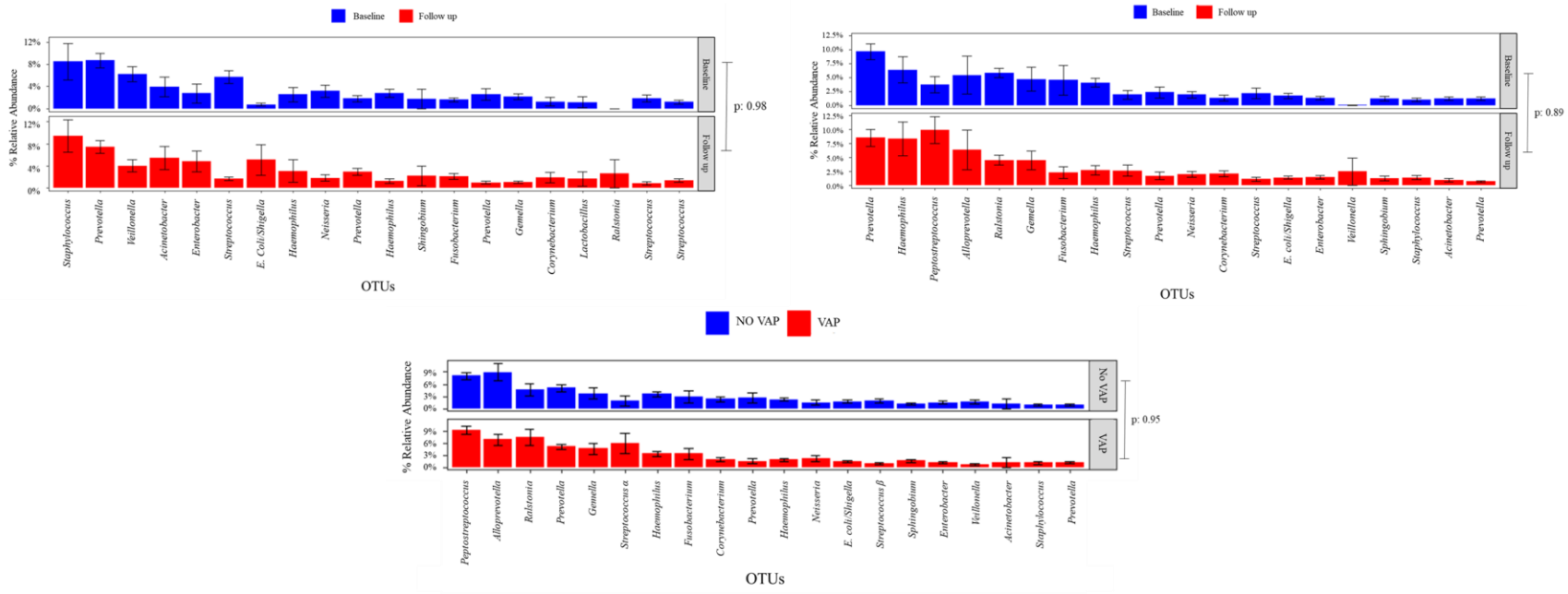
18. Figure 4.4: Alpha and Beta Diversity Comparisons in the Respiratory Microbiomes of ICU Patients without VAP between Two Time Points (Baseline and follow-up).A. Beta diversity is described using a PCoA scatter plot based on Bray-Curtis distances, highlighting a significant, distinction between baseline and follow-up samples in patients without VAP (p = 0.001, R^2 = 0.04). The variance captured by each principal coordinate is indicated in the axis titles (p = <0.001 for PC1 and p = 0.05 for PC2). **B.** Alpha diversity, as measured by the Shannon index, reveals no significant difference in microbial diversity between baseline and follow-up samples in patients without VAP (p = 0.88).



19. Figure 4.5. Alpha and Beta Diversity Comparisons in the Respiratory Microbiomes of ICU Patients with VAP between Two Time Points (Baseline and follow-up). A. Beta diversity is described using a PCoA scatter plot based on Bray-Curtis distances, highlighting a significant, distinction between baseline and follow-up samples in patients without VAP (p = 0.001, R^2 = 0.06). The variance captured by each principal coordinate is indicated in the axis titles (p = 0.12 for PC1 and p = <0.001 for PC2). **B.** Alpha diversity, as measured by the Shannon index, reveals no significant difference in microbial diversity between baseline and follow-up samples in patients without VAP (p = 0.54).

4. Variations in Genus Abundance Among Patients With and Without VAP

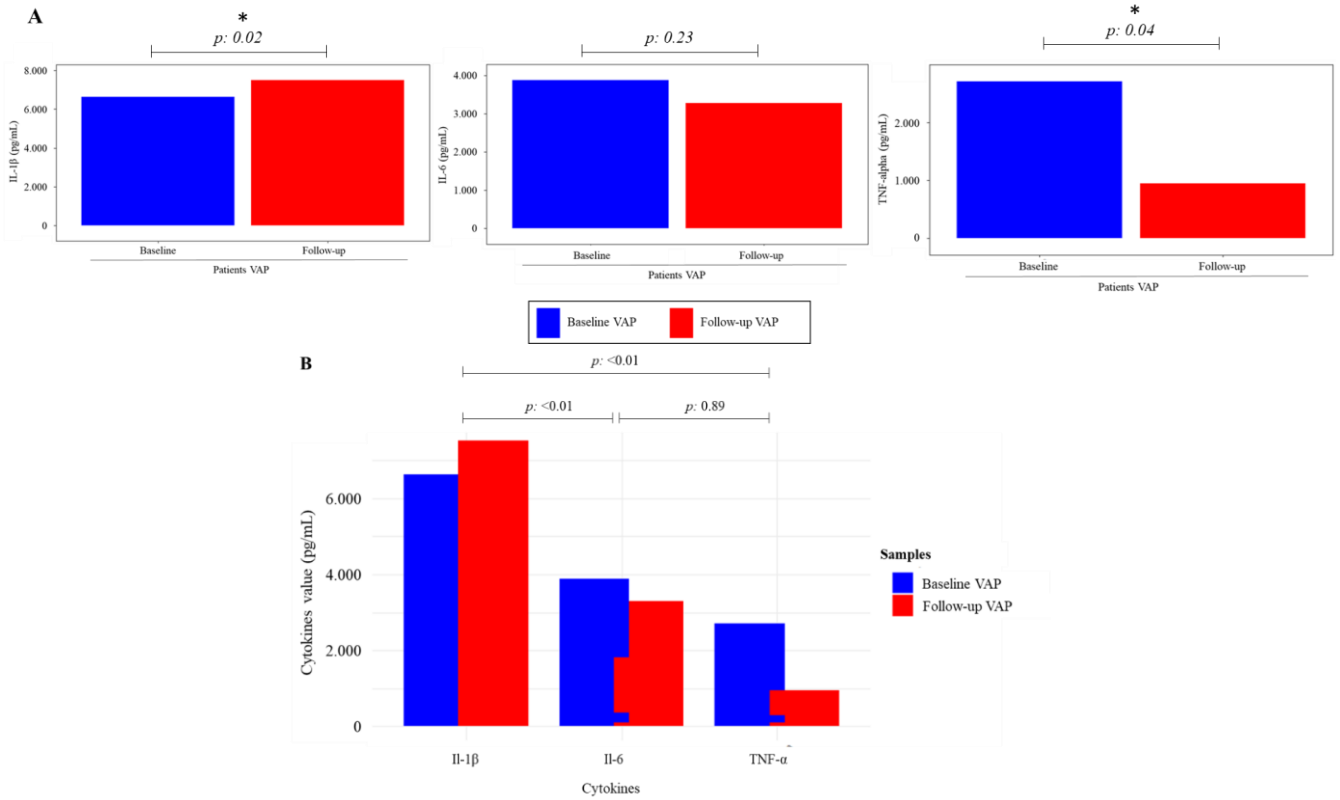
A comparative analysis of BAL samples was performed to evaluate possible temporal variations in microbial abundance with the 20 most representative OTUS. The results indicated remarkable stability in the relative abundances of bacterial OTUs between baseline and follow-up samples for both the group of patients who did not develop VAP (p-value of 0.98) and the group of patients who developed VAP (p-value of 0.89). This suggests that microbial abundance remains relatively constant in these patients over time. Furthermore, when comparing the microbial profiles between NON-VAP and VAP patients, a p-value of 0.95 was obtained, indicating similarities in the initial bacterial communities of both groups. These findings provide valuable information on the dynamics of microbial populations in intubated patients, highlighting the stability in the NO VAP group and suggesting possible temporal changes in the VAP group, although without statistical significance (**Fig. 4.6a, 4.6b, 4.6c**).



20. Figure 4.6:. Relative Abundance. A. Comparison between baseline and follow-up samples in the non-VAP patient cohort (p = 0.98). **B**. Comparison between baseline and follow-up samples in the VAP patient cohort (p = 0.89). **C.** Comparison between non-VAP and VAP patients (p = 0.95). The p-values underscore a significant constancy in microbial composition over time within patients regardless of VAP development and across both patient cohorts.

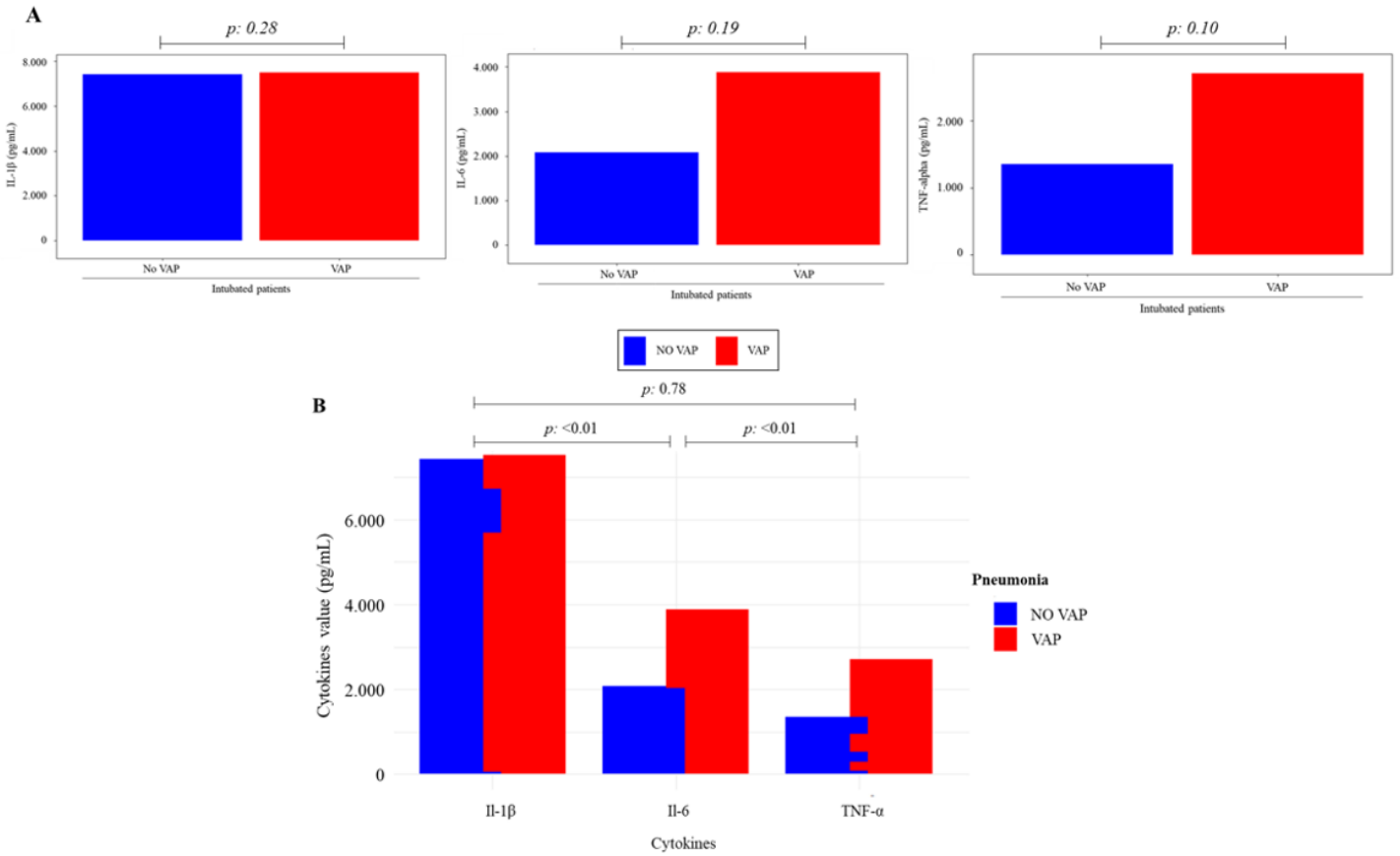
5. Cytokines analysis

In the investigation of cytokine dynamics in patients diagnosed with VAP, distinct patterns emerged from the analysis of BAL samples collected at the onset of intubation and during subsequent follow-up. Notably, there was a significant reduction in TNFα levels at follow-up, with a *p-value* of 0.04, while IL-1β levels exhibited a significant increase, evidenced by a *p-value* of 0.02. In contrast, IL-6 concentrations remained unchanged, indicating no significant variation between the initial and follow-up time points. The application of a two-factor ANOVA revealed significant variations in cytokine levels over time ($p = 0.03$) and across different types of interleukins ($p < 0.01$), with a noteworthy interaction effect between these two factors ($p = 0.03$). (**Fig. 4.7**).



21. Figure 4.7: Temporal and Type-Specific Cytokine Variability in VAP Patients. **A**. BAL sample analysis in VAP patients reveals significant cytokine level changes, with TNFα decrease and IL-1b increase at follow-up. **B.** Two-fact or ANOVA indicates significant variations by sampling time ($p = 0.03$) and interleukin type ($p < 0.01$), with notable interactions ($p = 0.03$).

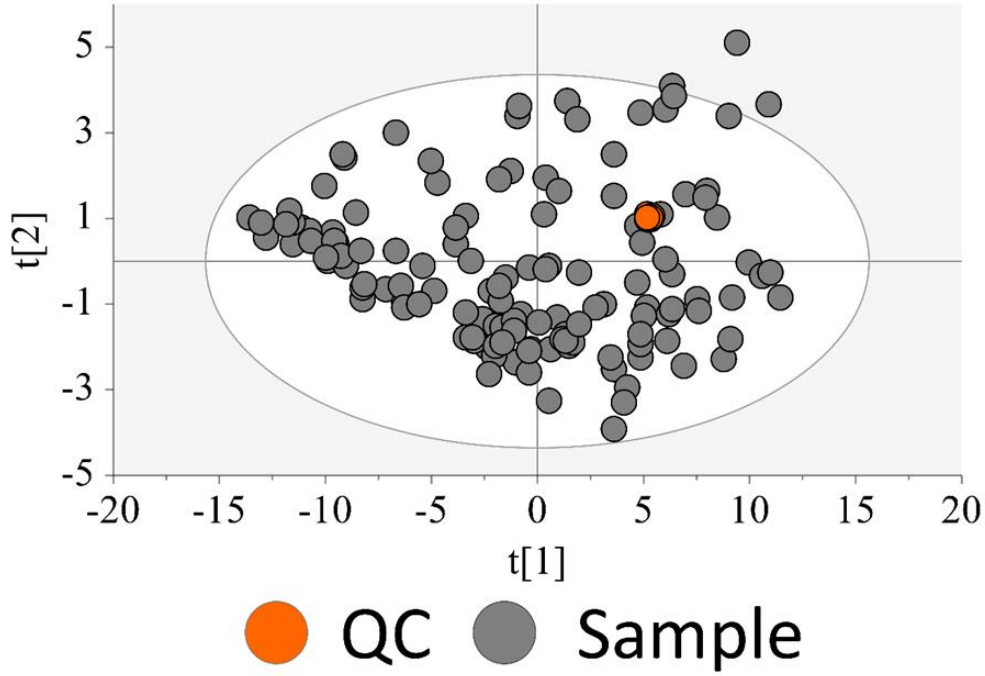
Conversely, when comparing cytokine levels in patients with and without VAP, the two-way ANOVA analysis identified significant variability in interleukin levels based on type ($p < 0.01$) but not about the presence of VAP ($p = 0.338$). Additionally, there was no significant interaction between VAP and interleukin type ($p = 0.674$) (**Fig 4.8**).



22. Figure 4.8: Temporal and Type-Specific Cytokine Variability between NO VAP and VAP Patients. **A**. Analysis of BAL samples in both NO VAP and VAP patients did not reveal significant changes in cytokine levels. **B**. Two-factor ANOVA showed significant interleukin level variability ($p < 0.01$), but no significant differences between VAP and non-VAP patient groups ($p = 0.338$), nor a significant interaction between pneumonia status and interleukin type ($p = 0.674$).

5 Untargeted Metabolomic differences between No-VAP and VAP patients

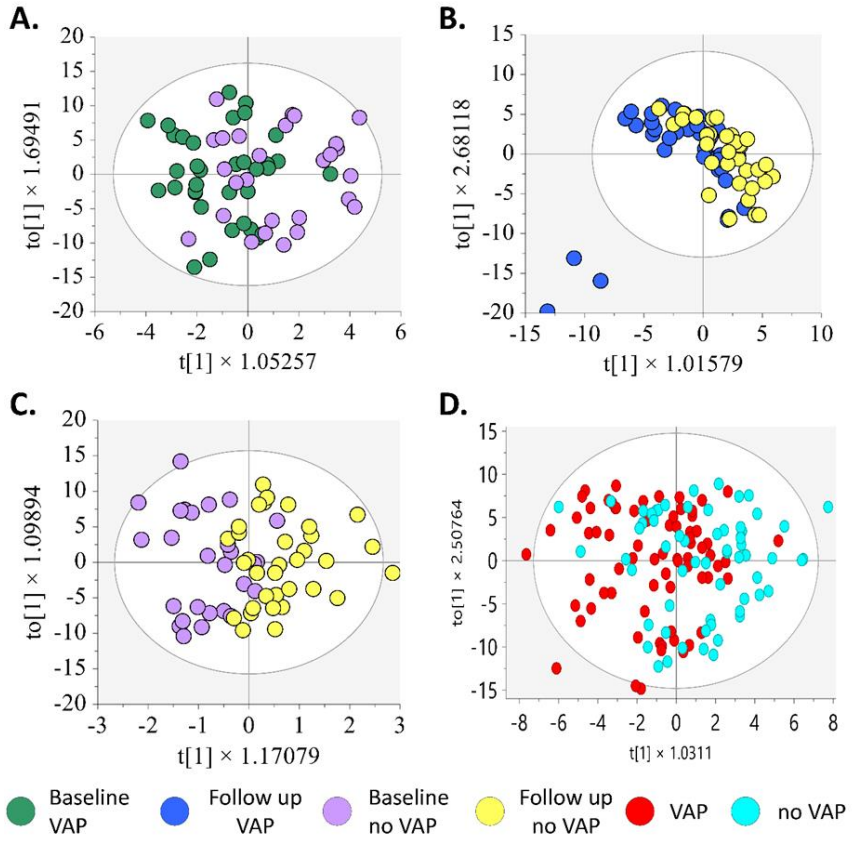
The PCA confirmed the reliability of spectrometric data (GC-QTOF-MS) by showing clustering of Quality Controls (QC) within the PCA confidence ellipse (**Figure 4.1 Supplemental**), indicating stable and reproducible data quality over time. Subsequent multivariate analysis using Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) revealed low predictive capacity ($Q2 \leq 0.0204$) when comparing patients with and without VAP, as well as their subgroups at baseline and follow-up.



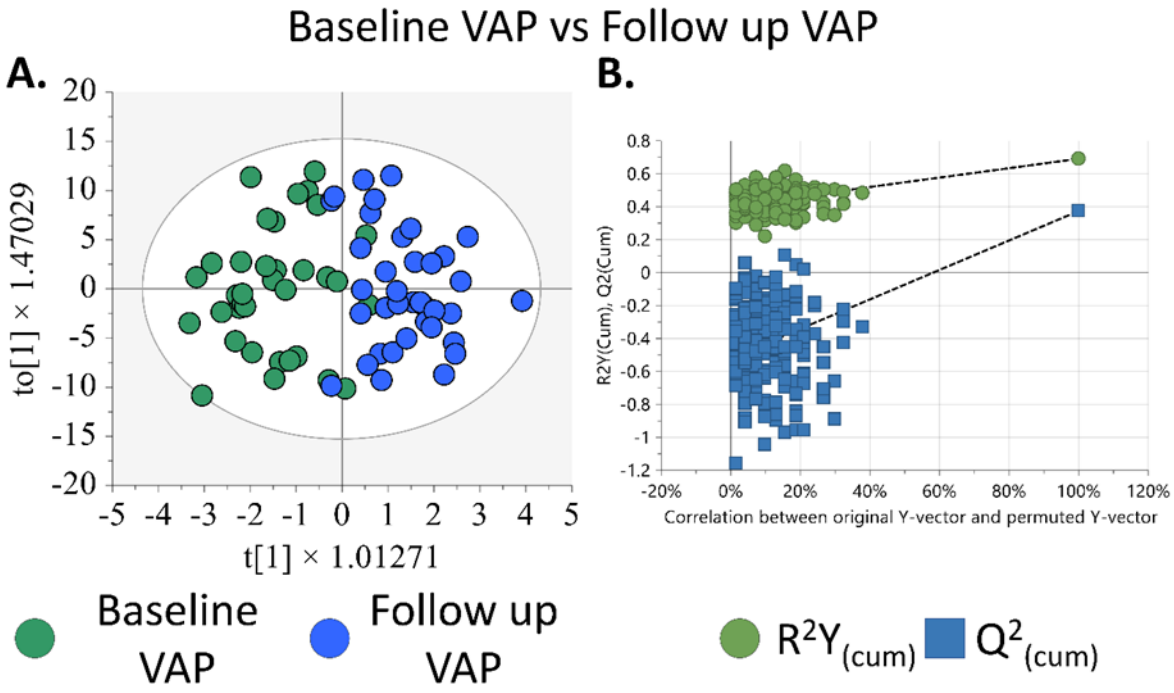
23. Figure 4.1 Supplemental: PCA score plots sample and QC. A. Metabolomics by LC-MS-QTOF: $R^2_{(cum)}$: 0.825, $Q^2_{(cum)}$: 0.775.

The models were evaluated through K-Fold cross-validation to validate their predictive capacity. The obtained results revealed pcv-anova values >0.05, which may indicate potential overfitting of the models (**Fig. 4.2 supplemental**). However, the model demonstrated acceptable predictive values when comparing the Baseline VAP group with the Follow-up VAP group (Q2=0.374). To further substantiate these findings, K-fold cross-validation was employed, resulting in a pcv-anova <0.05 (**Fig. 4.9A**). To enhance the model's validation, a permutation plot was generated. This analysis revealed that the Q2 values derived from permutations were lower than the original Q2 values. Additionally, the regression line intersected at the negative y-axis (**Fig. 4.9B**). These observations collectively suggest a low probability of model overfitting.

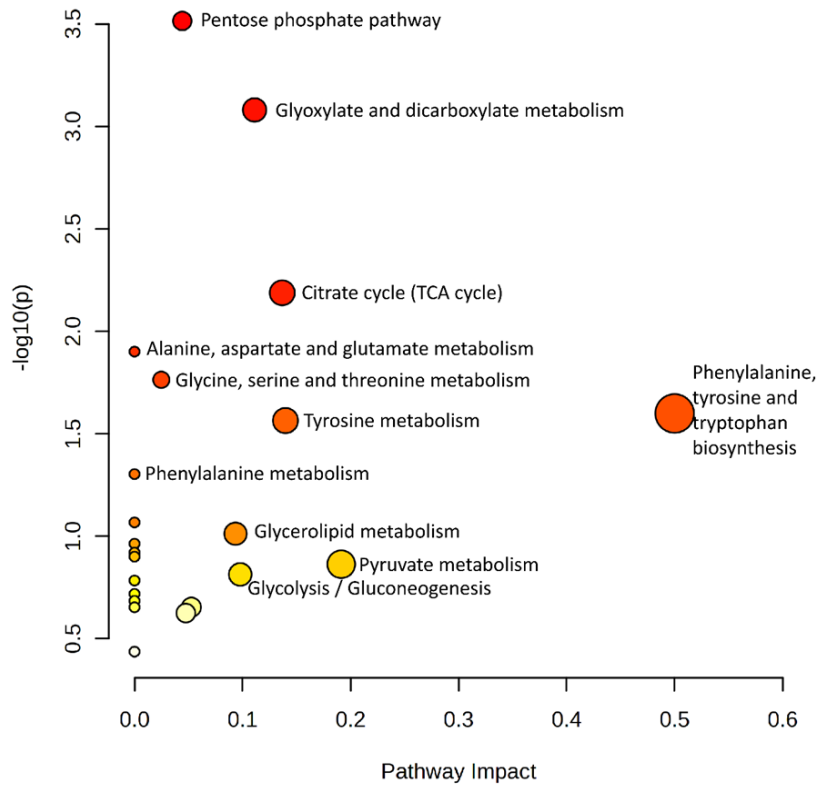
Accordingly, we employed univariate and multivariate analyses where we identified a total of 14 metabolites that met our criteria, exhibiting a p-value < 0.05 or VIP > 1. These metabolites were predominantly increased in patients with follow-up VAP compared to baseline values, as detailed in **Table 4.3**. The metabolic pathway graph, illustrated in **Figure 4.10**, effectively visualizes the relative importance of these metabolites. The circles in the graph vary in size and color intensity, reflecting their importance in the analysis. Our observations suggest that the metabolites primarily influence several key metabolic pathways: the pentose phosphate pathway, the citric acid cycle, and alanine, aspartate, and glutamate metabolism. In addition, they influence the biosynthesis of phenylalanine, tyrosine, and tryptophan, as well as the pyruvate, glycerolipids, and glycolysis/gluconeogenesis pathways.



24. Figure 4.2 supplemental: OPLS-DA score plots for: A. the HMD and LMD group. (Green dots, LMD; pink dots, HMD) **A.** Baseline VAP vs Baseline no VAP: $R^2_{(cum)}$: 0.289, $Q^2_{(cum)}$: -0.073, $p_{cv\text{-}anova}$: 1; **B.** Follow up VAP vs Follow up no VAP: $R^2_{(cum)}$: 0.252, $Q^2_{(cum)}$: -0.057, $p_{cv\text{-}anova}$: 1; **C.** Baseline no VAP vs Follow up no VAP: $R^2_{(cum)}$: 0.506, $Q^2_{(cum)}$: 0.007, $p_{cv\text{-}anova}$: 0.999 and D. VAP vs no VAP: $R^2_{(cum)}$: 0.199, $Q^2_{(cum)}$: 0.020, $p_{cv\text{-}anova}$: 0.62.



25.Figure 4.9: OPLS-DA Score Plots. (Green dots, Baseline VAP; blue dots, Follow-up VAP), Metabolomics by GC -QTOF-MS: R2(cum): 0.691, Q2 (cum): 0.374, p cv-anova: 1.47e-04, **B**. Permutation plot (n=200). green dots, R2; blue squares Q2.Accordingly, we employed univariate and multivariate analyses where we identified a total of 14 metabolites that met our criteria, exhibiting a p-value < 0.05 or VIP > 1.



26. Figure 4.10: Analysis of altered metabolic pathways. Node color is based on p-value, and node radius is determined based on pathway impact values.

	Baseline VAP vs Follow up VAP			Baseline VAP vs Follow up no VAP			Follow up VAP vs Baseline no VAP		
Compound	**[b]Fold Change**	**[c]VIP**	**[d]*p* value**	**[b]Fold Change**	**[c]VIP**	**[d]*p* value**	**[b]Fold Change**	**[c]VIP**	**[d]*p* value**
Amino acids, peptides, and analogues									
Isoleucine	0,65	1,08	-	-	-	-	2,47	-	2,86E-02
Phenylalanine	0,79	1,08	-	-	-	-	2,70	-	4.40E-02*
Aspartic acid	-	-	-	1,69	1,20	4,79E-02	4,35	-	4,51E-02
Aminovaleric acid	-	-	-	3,32	2,08	1,49E-02	-	-	-
threonine	-	-	-	2,29	1,07	-	-	-	-
Aminobutyric acid	-	-	-	3,45	1,17	-	-	-	-
Valine	-	-	-	-	-	-	1,88	-	1,60E-02
Leucine	-	-	-	-	-	-	2,19	-	1,37E-02
Proline	-	-	-	-	-	-	1,66	-	3,33E-02
Serine	-	-	-	-	-	-	1,82	-	1,66E-02
Amino levulinic acid	-	-	-	-	-	-	2,50	-	2,40E-02
Methionine	-	-	-	-	-	-	3,01	-	4.40E-02*
Glutamic acid	-	-	-	-	-	-	1,40	-	3,95E-02
Alanylleucine	0,67	1,10	-	-	-	-	7,52	-	3,21E-02
Tyrosine	0,77	1,14	-	-	-	-	2,80	-	1,12E-02
Aspartic acid	-	-	-	2,71	1,35	-	-	-	-
Tryptophan	-	-	-	2,83	1,13	-	3,23	-	2,89E-02
Oxoproline	-	-	-	-	-	-	1,42	-	3,00E-02
Benzene and substituted derivatives									
Phenyllactic acid	0,78	1,13	2,82E-02	-	-	-	2,13	-	4.40E-02*
hydroxybenzoicacid	-	-	-	-	-	-	4,30	-	4.67E-02*

Carboxylic acids and derivatives									
Citric acid	0,32	1,22	4,57E-03	-	-	-	2,39	-	4.40E-02*
Pyruvic acid	1,24	-	8,10E-03	-	-	-	1,27	-	4.40E-02*
Fumaric acid	-	-	-	-	-	-	1,63	-	2,24E-02
Diazines									
Uracil	1,07	1,15	-	-	-	-	1,33	-	4.40E-02*
Fatty Acyls									
Itaconic acid	-	-	-	2,04	1,15	3,25E-02	-	-	-
Linoleic acid	-	-	-	-	-	-	3,10	-	1,60E-02
Methylglutamic acid	0,35	1,21	4,32E-02	-	-	-	10,67	-	4.40E-02*
Oleic acid	-	-	-	-	-	-	2,60	-	4.40E-02*
Glycerolipids									
2-Palmitoylglycerol	-	-	-	0,69	-	1,35E-02	-	-	-
Hydroxy acids and derivatives									
Malic acid	-	-	-	-	-	-	1,23	-	2,50E-02
Glycolic acid	0,72	-	1,53E-02	-	-	-	1,49	-	4.40E-02*
Hydroxybutyric acid	-	-	-	2,34	-	4,79E-02	-	-	-
Organic carbonic acids and derivatives									
Urea	0,09	2,50	1.479E-07*	0,10	2,69	9,62E-07	1,62	-	4.40E-02*
Organonitrogen compounds									
Putrescine	-	-	-	4,47	1,70	4,26E-02	-	-	-
Carbohydrates and carbohydrate conjugates									
Gluconic acid	0,46	1,71	3.12E-02*	0,61	1,21	-	1,45	-	4.40E-02*
Acetyl-mannosamine	0,78	1,44	1,03E-02	-	-	-	2,02	-	3,44E-02

Gluconic acid lactone	0,90	1,09	-	5,54	1,07	-	2,15	-	2,16E-02
Digitoxose	1,17	1,13	-	-	-	-	0,82	-	2,42E-02
Glyceric acid	0,81	-	1,58E-02	-	-	-	-	-	-
D (+) galactose	-	-	-	2,74	1,16	-	-	-	-
Purines and purine derivatives									
Xanthine	-	-	-	-	-	-	1,41	-	1,31E-02
Pyrimidine nucleosides									
Cytidine	-	-	-	-	-	-	1,47	-	4,51E-02

[a]CV, coefficient of variation in the metabolites in the QC samples; [b]Change,fold change in the abundance of the specified comparison calculated as (case/control), where the sign indicates the direction of change in the case group; [c]VIP, variable importance in projection; [d]p value * corresponding to the p values calculated by the Benjamini-Hochberg false discovery rate post hoc correction (FDR < 0.05). GM: global metabolomics, GL: Global lipidomics, HILIC: hydrophilic interaction chromatography, LC: liquid chromatography, QTOF-MS: quadrupole time-of-flight mass spectrometer.

6. Table 4.3. Identification of Significant Metabolites in Intubed Patients: A Comparative Study of Baseline and Follow-up Levels.

E. Discussion

In this investigation comprising 80 participants, we conducted a thorough examination of the microbial composition, metabolomics, and cytokine dynamics associated with VAP. Our exploration of the microbiome revealed a consistent bacterial community structure, characterized by a lack of significant shifts in diversity or abundance, suggestive of resilience against the progression of VAP. The cytokine analysis illuminated significant inflammatory alterations in VAP patients, particularly evidenced by reduced levels of TNFα and elevated levels of IL-1β during follow-up assessments. Furthermore, our metabolomic inquiry identified 14 metabolites displaying notable concentration variations in VAP patients during follow-up, thus implicating critical metabolic pathways such as the pentose phosphate pathway and the citric acid cycle. These identified metabolites hold promise as potential biomarkers for monitoring VAP progression and offer valuable insights into its metabolic implications. This comprehensive examination provides profound insights into the molecular intricacies underlying VAP, thereby laying the groundwork for future research endeavors and therapeutic innovations.

The observed stability in the microbiota of patients without VAP indicates a resilient pulmonary microbiome over time, potentially reflecting robust health or effective immune responses that deter pathological transitions. In contrast, among VAP patients, although explicit p-values are lacking, visual analysis suggests microbial disturbances possibly attributable to VAP itself or its treatment. Particularly noteworthy is the similarity in microbial profiles between VAP and non-VAP patients, challenging traditional notions linking microbial composition directly to disease states. This discovery aligns with recent

research emphasizing the significance of host factors, clinical interventions, and environmental influences in determining susceptibility to VAP, rather than solely relying on microbiota composition. Our findings echo those of prior studies and underscore the necessity for a nuanced comprehension of the intricate interplay among the microbiome, host, and environmental factors in the context of VAP. This underscores the imperative for a multifaceted approach in future investigations aimed at unraveling the complex dynamics of pulmonary health and disease.

Our investigation into cytokine dynamics in VAP patients provides insights into the intricate and evolving immune response characteristic of this condition. Notable patterns emerged in cytokine profiles over the disease course, revealing a significant decrease in TNFα levels alongside a simultaneous increase in IL-1β levels from admission to follow-up. These findings align with existing literature, indicating an initial inflammatory response succeeded by a regulatory and resolving phase (151, 152). The variability in cytokine levels between admission and follow-up is notably influenced by the specific interleukin type, underscoring the precise adaptation of the immune response over time. Despite both IL-1β and TNFα being proinflammatory cytokines, their distinct functions and regulations may explain the observed differences (152). The decline in TNFα levels in VAP patients could be associated with immune cell apoptosis, particularly in monocytic cells, a significant source of this cytokine (153). Moreover, bacterial pathogenesis mechanisms in VAP may contribute to these cytokine dynamics. It is imperative to acknowledge the dynamic nature of the immune response, which can vary across different infection stages and in response to specific treatments.

We conducted an in-depth exploration of the molecular aspects of Ventilator-Associated Pneumonia (VAP) using metabolomics, revealing 14 metabolites with significant concentration changes in VAP patients during follow-up (154-156). These alterations point to critical metabolic pathways, notably the pentose phosphate pathway and the citric acid cycle. Analysis of baseline bronchoalveolar lavage (BAL) samples indicated a shift from aerobic to anaerobic metabolism, as evidenced by increased pyruvic acid and decreased citric acid levels. This shift suggests a response to oxidative stress, potentially induced by mechanical ventilation, leading to a preference for anaerobic glycolysis, likely mediated through the activation of the Nrf2 pathway and suppression of the NLRP3 inflammasome. Follow-up BAL samples displayed significant changes, particularly in amino acids such as Alanilleucine and Tyrosine, suggestive of an intensified tissue repair response to VAP-induced damage. Moreover, elevated urea levels hint at altered nitrogen metabolism or changes in alveolocapillary membrane permeability in response to lung pathology (157-159). These metabolic signatures, including increased levels of alanine, leucine, and tyrosine, imply an adaptive response involving protein synthesis for repair and altered protein catabolism under stress, possibly due to neutrophil degranulation and tissue damage. This comprehensive analysis sheds light on the metabolic adaptations occurring in the lungs during VAP, highlighting the intricate interplay between tissue repair processes and stress responses. However, further research is needed to fully elucidate the direct correlation between these metabolic changes and the pathogenesis and progression of VAP, as data on metabolic pathways in VAP patients remain limited.

The study's strengths lie in its comprehensive approach, combining microbiome, cytokine, and metabolomics analyses to offer a global view of VAP dynamics. A longitudinal sampling at baseline and follow-up enhances disease progression insights while using BAL specimens ensures clinical relevance. Nevertheless, limitations include small patient sample size and reliance on a single follow-up sample, potentially affecting generalizability and capturing temporal variations. Despite these drawbacks, the study yields valuable insights into VAP's mechanisms, highlighting the need for future research with larger cohorts for a more comprehensive understanding.

In conclusion, our study offers a comprehensive understanding of ventilator-associated pneumonia (VAP) by exploring its microbial, cytokine, and metabolomic dynamics. Through meticulous analyses, we unveil the intricate interplay of factors contributing to VAP's pathogenesis and progression. Our findings underscore the significance of considering host factors, clinical interventions, and environmental influences in assessing VAP susceptibility and severity, pointing towards promising avenues for future research. Furthermore, the identification of potential biomarkers and metabolic pathways presents opportunities for innovative therapeutic strategies in VAP management. By advancing our knowledge of VAP mechanisms, our study advocates for further exploration in larger cohorts to enhance understanding and optimize clinical outcomes for affected patients.

The final version of this chapter is pending approval from the co-authors before submission to the American Journal of Respiratory and Critical Care Medicine.

V. DISCUSSION GENERAL

Over the past decade, there has been increasing recognition of the healthy lung as a complex polymicrobial system, characterized by dynamic and transient features, and undergoing constant renewal. Everyone possesses a unique pulmonary microbiome, which is sparse yet highly diverse (14). Initial research primarily focused on exploratory studies comparing microbial communities in the respiratory tracts of healthy individuals to those with specific pulmonary diseases. However, it has become evident that merely identifying differences in microbial ecology is inadequate for establishing causality or understanding the impact of the pulmonary microbiome on respiratory diseases (6). Instead, it is essential to comprehend the microbiome within the context of mixed-species communities, complex microbial interconnections, and unique microbiome network patterns, characterized by interactions between kingdoms and biochemical signaling mechanisms (160).

The diversity of the pulmonary microbiome is remarkable, with approximately 100 different taxa implicated in various biological processes, such as resistance to pathogen invasion, dissemination, and associations with host metabolic, inflammatory, and immune homeostasis (17). Dominant phyla include Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria, with prevalent genera including *Streptococcus, Prevotella, Fusobacteria,* and *Pseudomonas* (15). Additionally, fungi such as *Aspergillus* and *Candida*, as well as eukaryotic viruses like Anelloviridae and Redondoviridae, have been identified in the pulmonary virome (161).

Several factors influence pulmonary microbiome diversity, including microbial migration from the upper respiratory tract, microbial elimination within the lung, and relative rates of microbial reproduction, all of which are influenced by factors such as temperature, pH, and oxygen availability (16). Alterations in microbial structure or abundance, referred to as dysbiosis, can contribute to the development of respiratory diseases, including pneumonia (162).

Pneumonia, characterized by microbial invasion and overgrowth in the pulmonary parenchyma, disrupts pulmonary microbiome diversity, facilitating the emergence of pathogens (22) VAP is a common complication in intensive care unit patients, associated with a high incidence and mortality rate (163). Mechanical ventilation disrupts pulmonary homeostasis, increasing secretion aspiration and susceptibility to pneumonia (13). Studies have demonstrated an association between VAP and changes in the pulmonary microbiota, with a decrease in microbial diversity observed in patients who develop the disease (67).

Although correlations between the pulmonary microbiome and VAP have been identified, understanding the underlying mechanisms, and establishing causal relationships remain ongoing challenges (97). However, continued research on the pulmonary microbiome may provide new insights into the pathophysiology of pulmonary diseases, including VAP (164).

Recent advancements in research on the pulmonary microbiota and its relation to VAP underscore the critical need to understand how alterations in the pulmonary microbiome can influence the development of this complication in patients undergoing

mechanical ventilation. Groundbreaking studies by Kelly *et al.* in 2016 and Zakharkina *et al.* in 2017 revealed that changes in the dynamics of the pulmonary microbiome during mechanical ventilation led to more pronounced dysbiosis in patients with VAP (46, 65). These findings were further complemented by the observation that prolonged mechanical ventilation is linked to reduced microbiome diversity, detrimentally impacting pulmonary health. Moreover, a significant correlation was identified between the presence of specific pathogens, such as Burkholderia, Bacillales, and Pseudomonadales, and the development of VAP, underscoring the critical role of these microorganisms in the disease's pathogenesis.

Subsequent research, including the works of Fenn *et al.* in 2022, Fromentin *et al.* in 2022, and Harrigan *et al.* in 2022, has bolstered these initial findings, highlighting significant differences in the composition of the pulmonary microbiota and a greater prevalence of pathogenic bacteria in patients with VAP or lower respiratory tract infections (25, 165, 166). Concurrently, recent studies conducted by researchers like Emonet *et al.* in 2019, Kitsios *et al.* between 2018 and 2023, and Woo *et al.* and Baek *et al.* in 2020, have explored specific microbial profiles and their association with VAP (68, 102, 148, 167, 168). These studies have discovered distinctive patterns in the microbiota of patients who develop the disease, indicating that a greater abundance of certain types of oral bacteria and Enterococcus in the lungs at the onset of ventilation may influence the risk of developing VAP. These investigations emphasize the importance of the respiratory microbiome as a determinant factor in the clinical evolution of critically ill patients, associating the decrease of anaerobic bacteria with poorer clinical outcomes.

Despite the progress, significant challenges remain in the research of the pulmonary microbiome, particularly in its clinical application as a biomarker to predict the outcomes of mechanical ventilation and patient survival. While researchers like Emonet *et al.* and Kitsios *et al.* have suggested the potential use of microbial composition as such a biomarker, they emphasize the need for more large-scale studies to confirm the efficacy of these indicators (102, 148). Interestingly, Emonet *et al.* did not observe significant differences in the evolution of the pulmonary microbiota between patients with and without VAP, though tracheal aspirates from patients with VAP showed a higher presence of Gammaproteobacteria, including *Pseudomonas spp*. and *Enterobacteriaceae*, before the diagnosis of VAP. This underscores the complexity of the role of the pulmonary microbiome in VAP and the need for further research to validate these findings and deepen our understanding of how these microbial communities affect pulmonary health in critical care settings.

The study presented here, conducted in two phases amidst the pandemic spanning primarily 2020 and 2021, initially examined the dynamics of the pulmonary microbiome using bronchoalveolar lavage (BAL) samples. The first sample collection coincided with patient intubation, while the second, a follow-up sample, was obtained upon VAP diagnosis for those affected. For patients without VAP, follow-up samples were taken either 72 hours post-intubation or on the fifth day, depending on intubation duration. This approach, encompassing COVID-19 patients and those without initial respiratory complications, aims to shed light on the pulmonary microbiota's role in VAP development.

The lung is an essential organ, not only for gas exchange but also as a complex ecosystem that hosts a diverse microbiota, challenging the traditional view of the lungs as sterile environments. The pulmonary microbiota plays a pivotal role in maintaining respiratory health, influencing immune responses, and protecting against pathogenic colonization. The optimal function of the lungs depends on a delicate balance between the host and its microbiota, where disruptions can lead to diseases. Healthy lungs maintain a controlled level of microbial presence and immune response, ensuring the integrity of the pulmonary tissue and its function. Thus, alterations in the lung microbiome, whether by infection, inflammation, or environmental stressors, can be detrimental to the respiratory epithelium and overall lung function. Researchers are increasingly recognizing that factors such as microbial dysbiosis, host genetics, age, and environmental exposures could play crucial roles in the development of respiratory conditions and lung diseases (68, 102, 148, 167, 168).

In our investigation, we aimed to clarify the intricate relationship between positive pressure mechanical ventilation and its impact on the diversity of the pulmonary microbiome. Our foundational hypothesis proposed that patients developing VAP would exhibit significant alterations in both alpha and beta diversity, alongside a reduction in microbial diversity in follow-up samples compared to baseline samples. This hypothesis guided our analysis of the lung microbiome, involving bronchoalveolar lavage fluid and rRNA sequencing from intubated patients without prior pulmonary conditions. Our methodology focused on a comprehensive evaluation of microbiota changes through alpha

and beta diversity analysis, categorizing patients based on VAP development to understand the microbial dynamics associated with VAP onset.

Our findings emphasize the crucial role of microbiomes in both the upper and lower respiratory tracts for maintaining respiratory health, particularly in the context of respiratory viruses like SARS-CoV-2 (169, 170). Despite no significant changes in overall microbial diversity, observed differences in nasal microbiome abundances between COVID-19 patients who developed VAP and those who did not suggest potential shifts in nasal microbial colonization patterns. Furthermore, our analysis revealed distinct variations in specific microbial abundances, notably within the bacterial genera Staphylococcus and Enterobacteriaceae. The observed decrease in Staphylococcus coupled with an increase in Enterobacteriaceae, particularly Escherichia spp., among VAP patients, aligns with previous literature identifying these bacteria as common causative agents of VAP in COVID-19 patients (140, 171).

In the context of VAP development in intubated patients, our investigation focused on microbial dynamics. Taxonomic analysis of 16S rRNA sequences in bronchoalveolar lavage samples yielded insights despite a reduction in operational taxonomic units (OTUs). Principal coordinate analysis (PCoA) revealed modest yet significant differentiation between VAP and non-VAP groups, influenced by additional factors impacting diversity. Shannon's diversity index indicated consistent bacterial diversity during intubation. Patients without VAP exhibited notable structural changes over time while maintaining overall diversity. Conversely, patients with VAP displayed similar changes without diversity alteration. Temporal microbial abundance analysis demonstrated stability in both VAP and

non-VAP groups, with no significant initial bacterial differences. These findings offer insights into microbial dynamics in intubated patients, suggesting potential temporal changes associated with VAP, albeit lacking statistical significance.

The correlation between the serum viral load of SARS-CoV-2 and the dynamic shifts in the nasal and pulmonary microbiome in VAP patients highlights the intricate interplay between viral infections and bacterial communities in the respiratory tract. Elevated levels of SARS-CoV-2 in patients who subsequently develop VAP upon hospital admission suggest a potential association between the initial viral load and the onset of VAP, underscoring the importance of early monitoring and intervention (172). Significant changes in bacterial abundance were observed in both nasal and pulmonary samples among patients with varying viral loads. Those with higher viral loads exhibited reductions in commensal bacteria such as Corynebacterium and Staphylococcus, coupled with increases in potentially pathogenic bacteria like Proteus, Enterobacteriaceae, and Escherichia-Shigella. Conversely, patients with lower viral loads demonstrated different shifts in bacterial composition, implying distinct microbial dynamics associated with varying levels of viral infection. The observed decrease in commensal bacteria and simultaneous rise in Gram-negative pathogenic bacteria among patients with higher viral titers suggest a potential mechanism for VAP development. These findings underscore the significance of maintaining a balanced respiratory microbiome to mitigate secondary bacterial infections in individuals with viral respiratory illnesses (173).

Overall, our research contributes to a deeper understanding of microbial dynamics in intubated patients and highlights the need to maintain a balanced respiratory microbiome

to support respiratory health and prevent adverse outcomes associated with mechanical ventilation, regardless of the cause of the intubation. More research is needed in this area to develop more effective strategies to manage and prevent VAP, ultimately improving patient outcomes in critical care settings.

The exploration of cytokine dynamics in COVID-19 patients who develop VAP unveils significant insights into the intricate immune responses elicited by this condition. Our research indicates that while spike-specific neutralizing antibodies demonstrate comparable efficacies against various SARS-CoV-2 variants in both cohorts of patients, those afflicted with VAP manifest a pronounced increase in neutralization capacity against the D614 variant. This suggests an augmented antibody response in VAP patients, intriguingly coinciding with a subdued inflammatory cytokine profile. Essential cytokines, such as IFN-δ, IL-1β, IL-12p70, IL-18, IL-6, TNF-α, and CCL4, were identified at reduced concentrations in the VAP cohort upon ICU admission, suggesting a potentially attenuated inflammatory response. These findings pose compelling questions regarding the equilibrium between effective viral neutralization and the modulation of inflammatory responses in VAP patients.

The pronounced neutralizing antibody response, compared with a diminished inflammatory cytokine profile, intimates a distinctive immune modulation in VAP patients, potentially influencing their defense mechanisms against secondary bacterial infections. Considering the outcomes of the investigation on patients without prior pulmonary conditions who either developed or did not develop VAP, similarly intriguing patterns emerge. Our discoveries reveal dynamic alterations in cytokine profiles throughout the

evolution of VAP, with distinct modifications in TNFα and IL-1β levels. The noted decrease in TNFα levels may signify a regulatory response to inflammation, whereas the elevation in IL-1β levels suggests continued immune activation. Nonetheless, the unaltered IL-6 concentrations imply a distinct regulatory mechanism or a less significant role in the progression of VAP.

The dichotomy of increased neutralizing antibody responses alongside reduced pro-inflammatory cytokine levels raises important considerations about immune balance in VAP. Neutralizing antibodies play a crucial role in viral clearance by blocking virus entry into host cells, a mechanism well documented in the literature (174-176). For instance, a study by Yu *et al* demonstrated that neutralizing antibodies against SARS-CoV-2 are critical for controlling viral replication and promoting viral clearance (177). However, the regulation of inflammatory responses is equally important, excessive activation of pattern recognition receptor (PRR) signaling, involving cytokines like IL-1β, IL-6, TNF, and type I interferon, can lead to diseases (178, 179).

The observed reduction in TNFα levels in patients with VAP could be indicative of an immune-regulatory mechanism aiming to temper inflammation and prevent tissue damage. TNFα is a key cytokine involved in initiating and sustaining inflammatory responses, and its dysregulation has been associated with pathological inflammation and damage in various diseases (180, 181). A study by Parameswaran *et al.* highlights the dual role of TNFα in immunity and pathology, emphasizing its critical function in host defense and its potential for contributing to inflammatory disease when not properly regulated (182).

Conversely, the increase in IL-1β levels suggests ongoing immune activation, possibly in response to bacterial invasion. IL-1β is a potent pro-inflammatory cytokine that plays a central role in the host defense against infections by promoting inflammation, fever, and the recruitment of immune cells to infection sites (183, 184). The role of IL-1β in bacterial infections and its impact on the pathogenesis of pneumonia have been extensively studied, with findings suggesting that IL-1β is crucial for controlling bacterial growth but can also contribute to lung injury if not regulated (185). A study by Dinarello *et al* in 2018 details the mechanisms by which IL-1β mediates its effects, including the activation of T cells and the production of secondary cytokines that amplify the immune response (186, 187).

The multifaceted role of interleukin-6 (IL-6) in respiratory infections underscores its critical functions in both amplifying and dampening immune responses, which are particularly evident in conditions such as VAP. IL-6 is distinguished by its dual pro-inflammatory and anti-inflammatory capabilities, making it a key player in the body's defense against pathogens and in the pathology of lung diseases. This cytokine's regulatory capacity is vital for managing the immune system's response to infections, striving to protect the host while preventing excessive tissue damage.

Various studies have delved into the multifaceted actions of IL-6, exploring its role beyond traditional signaling pathways and its impact on clinical outcomes. Mullberg *et al.* have particularly illuminated IL-6 receptor-independent mechanisms, broadening our comprehension of its diverse biological functions (10799718) (188). Additionally, Dienz *et al.* elucidated IL-6's vital role in combating H1N1 influenza infection by ensuring

neutrophil survival, highlighting its protective effects in viral challenges (189). IL-6 has emerged as a critical regulator of the equilibrium between Th17 cells and regulatory T cells (Tregs), pivotal for immune homeostasis and inflammatory response, with implications for the pathogenesis and treatment of autoimmune and inflammatory diseases, showcasing IL-6's therapeutic potential (190). Moreover, IL-6 employs multiple signaling pathways, including classic signaling via the membrane-bound IL-6 receptor and trans-signaling via the soluble IL-6 receptor, with implications for various diseases and conditions, highlighting the intricate nature of IL-6's role in the immune system (191).

The metabolomic analysis conducted in this study sheds light on distinct metabolic alterations in COVID-19 patients with and without VAP, showed the identification of 47 metabolites across various chemical classes and metabolic pathways, with significant alterations in phospholipid, sphingolipid, and glutathione metabolism in VAP patients, points to the profound impact of metabolic changes on cell membrane integrity and oxidative stress responses. These metabolic shifts may enhance bacterial adhesion and immune response destabilization, playing a pivotal role in the development of VAP.

Furthermore, the study delves into the metabolic disparities between patients with and without VAP, revealing intriguing variations in glycerophospholipids, glucuronides, and indole compounds among non-VAP patients. This suggests a potentially stronger immune and metabolic response in non-VAP patients, possibly contributing to their resilience against VAP development. Moreover, the identification of metabolomic signatures, particularly alterations in key metabolic pathways such as the pentose phosphate

pathway and the citric acid cycle, offers valuable insights into the metabolic adaptations and challenges encountered by the lungs in the context of VAP.

Upon intubation initiation, bronchoalveolar lavage (BAL) samples unveil metabolic modifications indicative of VAP development, characterized by elevated metabolites like pyruvic acid and decreased citric acid levels, signifying a deviation from aerobic metabolism and disruptions in tricarboxylic acid (TCA) cycle and pyruvate metabolism. These alterations, attributed to underlying medical conditions and inflammation induced by intubation, prompt a shift towards anaerobic glycolysis, potentially exacerbated by oxidative stress from mechanical ventilation. Such metabolic adaptations, reflected in the observed profile, not only mirror the physiological consequences of mechanical ventilation but also heighten susceptibility to VAP.

In follow-up BAL samples corresponding with VAP diagnosis, pronounced metabolic disparities emerge, notably elevated levels of functional amino acids like Alanilleucine and Tyrosine, indicating intensified regenerative responses to tissue damage. Concurrently, increased urea concentration suggests potential changes in alveolocapillary membrane permeability or altered nitrogen metabolism in response to the lung condition. These findings reflect dynamic shifts in pulmonary metabolism during VAP progression, highlighting the potential of metabolic profiles to elucidate specific pathophysiological mechanisms, including adaptive responses involving protein synthesis for repair and altered protein catabolism under stress conditions. Overall, these metabolic adaptations offer insights into the complex interplay of tissue repair processes and stress responses during VAP, contributing to a deeper understanding of its pathogenesis (154-159). However, the

direct correlation between these metabolic changes and VAP's pathogenesis requires further elucidation, pointing to the need for additional research to fully understand the metabolic underpinnings of VAP in COVID-19 patients.

In our comprehensive investigation, we have elucidated the intricate interplay between positive pressure mechanical ventilation, the pulmonary microbiome, cytokine dynamics, and metabolomic profiles in patients with VAP. Our study revealed significant alterations in microbial dynamics associated with the onset of VAP, underscoring the pivotal role of microbiomes in maintaining respiratory health, particularly amidst viral respiratory diseases like COVID-19. Notably, we observed distinct variations in microbial abundances, bearing implications for the development and progression of VAP. Furthermore, our cytokine dynamics analysis unveiled a distinctive immunomodulation in VAP patients, characterized by an augmented neutralizing antibody response alongside reduced levels of proinflammatory cytokines. These findings highlight the delicate balance between effective viral neutralization and inflammatory responses in VAP. Additionally, our metabolomic analysis provided insights into metabolic adaptations during VAP progression, offering a deeper understanding of tissue repair processes and stress responses. Overall, our research contributes valuable insights into the pathogenesis of VAP and underscores the importance of maintaining a balanced respiratory microbiome and immune response to mitigate adverse outcomes associated with mechanical ventilation and VAP development. Further studies are warranted to fully elucidate the underlying mechanisms and potential therapeutic targets for improving patient outcomes in critical care settings.

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