

The Respiratory Mycobiome in Critical Illness

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In critically ill patients, variation in the host inflammatory responses has been associated with poor outcomes. However, the biological mechanisms underlying this heterogeneity have not yet been defined. We investigated whether variation in fungal communities (mycobiome) in the respiratory tract are associated with host inflammation and innate immune system activation and clinical and patient-centered outcomes. We collected endotracheal aspirates (ETA) and plasma samples from 226 critically ill patients. We used extracted DNA from ETAs and performed fungal (ITS) and bacterial (16S) rRNA gene sequencing on the Illumina MiSeq platform to characterize the lower respiratory tract microbiome. We derived diversity metrics to classify patients into strata based on the alpha diversity of the mycobiome and examined associations between these diversity metrics and clinical and patient-centered outcomes. We performed network analyses using probabilistic graphical models to identify associations between bacterial and fungal taxa, clinical features, biomarkers and patient-centered outcomes. Fungal communities had very low alpha diversity overall. Low alpha diversity was associated with a higher likelihood of ARDS diagnosis and increased severity of disease. Patients with lower alpha diversity in the mycobiome of the lung had higher levels of plasma biomarkers associated with inflammation and immune system activation as well as host-response to infection. Critically ill patients with low diversity of fungal communities in the lung had longer ICU stays, longer time to liberation from mechanical ventilation, and fewer ventilator free days. Our findings suggest that the mycobiome of the lung may play a role in accentuated host inflammation and adverse clinical outcomes in critical illness

through interaction with other microbiota and the host immune system. While the lung mycobiome has been understudied, our findings represent a significant contribution to public health as they suggest that the lung mycobiome may be an important source of biological heterogeneity and clinical variation among critically ill patients and may represent a potential therapeutic target for the prevention and treatment of lung injury and ARDS. Continued investigation of the respiratory microbiome with culture-independent approaches and in vitro models of microbiome-mycobiome interaction will allow for further delineation of microbiome-mycobiome-host interactions in the respiratory tract of mechanically ventilated patients.

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Preface

I would like to thank all members of the research team of the Acute Lung Injury Registry (ALIR) and Biospecimen Repository at the University of Pittsburgh, the medical and nursing staff in the Medical Intensive Care Unit at the University of Pittsburgh Medical Center, and all patients and their families for participating in this research project. We are grateful for their involvement in this work.

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1.0 Introduction

1.1 Acute Respiratory Distress Syndrome

1.1.1 Clinical Description

Acute respiratory distress syndrome (ARDS) is a severe form of respiratory failure that is often life-threatening. First characterized by Ashbaugh and Petty over five decades ago, we now know ARDS to be an acute inflammatory process leading to protein-rich non-hydrostatic pulmonary edema that results in hypoxemia and increased lung stiffness and impaired carbon dioxide clearance.^{1,2} ARDS is a clinical syndrome and the diagnosis of ARDS is made by using the Berlin Definition and is stratified by severity using the PaO₂/FiO₂ ratio (mild 200-300 mmHg, moderate 100-200 mmHg, severe <100 mmHg) .³ ARDS is characterized by dyspnea, tachypnea, hypoxic respiratory failure despite oxygen administration and noncardiogenic pulmonary edema. Symptoms of ARDS are caused by the rapid onset of inflammation and alveolar atelectasis. The primary treatment is mechanical ventilation and treatments directed at resolving the illness provoking ARDS. If mechanical ventilation fails to resolve the hypoxia, the patient may be placed on extracorporeal membrane oxygenation (ECMO).^{4,5} Supportive care remains the only therapeutic option for patients with ARDS.^{6,7} ARDS occurs insult resulting in epithelial and endothelial damage to the lung resulting in an inflammatory cascade that results in flooding of the alveoli, severely limiting capillary gas exchange.^{3,4} These insults can be both indirect, or extra-pulmonary, or an insult directly impacting the lungs, such as pneumonia, aspiration or inhalational injury.⁸ (Table 1.1) Critical illnesses associated with the provocation of ARDS include sepsis,

trauma, pancreatitis, and pneumonia. These critical illnesses act as an injurious exposure causing inflammation which creates epithelial and endothelial damage to the lung, resulting in an inflammatory cascade, capillary leakage and clinical manifestations of the syndrome.⁶ Endothelial dysfunction allows for inflammatory cells and exudate to enter the alveoli, prohibiting gas exchange leading to hypoxia, increased work of breathing and permanent damage to the alveoli. It is widely known that an inappropriate inflammatory response leads to the devastating lung damage seen in ARDS; however, the mechanisms behind this aberrant immune response are not well understood.

Table 1.1: Clinical Disorders Considered Risk Factors for ARDS

Indirect Insult: Extra-pulmonary Etiology	Direct Insult: Pulmonary Etiology
Trauma	Pneumonia
Shock	Aspiration
Extra-pulmonary sepsis	Inhalation injury
Acute pancreatitis	Pulmonary contusion
Cardiopulmonary bypass	Fat emboli
Disseminated intravascular coagulation	Amniotic emboli
Burns	Near-drowning
Head injury	Reperfusion injury
Transfusion related	
Drug Overdose	

1.1.2 Heterogeneity in ARDS

While the need for a broadly efficacious treatment for ARDS is indisputable, biological and clinical heterogeneity in critical illness with acute lung injury and ARDS resulting in differing responses to potential treatments have made it difficult to determine which patients will benefit from investigational therapies. As such, recent research has focused on identifying subphenotypes of patients within ARDS who have differential responses to treatments or who have increased risk

of worse disease related outcomes.⁹⁻¹² Identification of these differences could lead to the development of a precision medicine approach to research in critical care.¹²⁻¹⁴

Recent research focused on identifying differences in patients with ARDS to determine subphenotypes within the syndrome have consistently identified two distinct group of patients: hyper- and hypoinflammatory subgroups.¹³ These subphenotypes were initially identified through unsupervised investigations of clinical trial populations using latent class analysis including biomarkers and clinical variables.¹⁴⁻¹⁶ Increased inflammation in the lung has been a recognized feature of ARDS for decades and elevated levels of biomarkers associated with inflammation (interleukin 1B [IL-1B], interleukin 6 [IL-6], interleukin 8 [IL-8], interleukin 10 [IL-10], and tumor necrosis factor receptor 1 [TNFR-1]), endothelial (angiopoietin 2 [ANG-2]) and epithelial injury (receptor for advanced glycation endproducts [RAGE]) have been associated with worse outcomes in patients with ARDS and with ventilation strategies found to be injurious.¹⁷⁻²¹ Biomarkers consistent with a hyperinflammatory state in ARDS had higher mortality as well as differential responses to treatment with statins, fluid management and positive end-expiratory pressure levels.

^{14-16,22,23}

With the intention of applying these subphenotypes in a clinical setting, a more parsimonious clinical model for prediction of subphenotype assignment has been proposed and includes biomarkers TNFR-1, IL-8 and bicarbonate.²² Kitsios et al have utilized latent class analysis to apply the hyper- and hypoinflammatory subphenotype framework more broadly to include critically ill patients at risk for ARDS and identified additional biomarkers associated with the subphenotypes.²⁴ In an extension of their examination of differences between the subphenotypes beyond clinical features and biomarkers of host response, Kitsios et al characterized the microbiome in critically ill mechanically ventilated patients and identified that

low alpha diversity and enrichment of bacteria known to be pathogenic in endotracheal aspirate samples was independently associated with a hyperinflammatory subphenotype and was associated with elevated biomarkers and worse clinical outcomes.²⁵ This broadening of the applicability of subphenotyping to critically ill patients at risk of developing ARDS and to the inclusion of respiratory microbiota as a potential contributor to the heterogeneity in critical illness will likely lead to a better understanding of the biological mechanisms underlying heterogeneity in clinical characteristics and outcomes in critical illness and ARDS.

1.1.3 Epidemiology

Globally, about 3 million patients per year are diagnosed with ARDS and 35-50% of those do not survive. Of those who do, morbidity is severe and survivors often have a significantly impaired quality of life.⁴ Globally, patients with ARDS make up 10-14% of ICU patients and 23-30% of mechanically ventilated patients meet the diagnostic criteria for ARDS.^{26,27}

In the U.S, the crude incidence of ARDS has been estimated to be 58.7 per 100,000 person years and the estimated age-adjusted incidence was 64.0 per 100,000 person years. The in-hospital mortality rate was estimated to be approximately 40% across many major observational studies and that about one in three patients diagnosed with ARDS will survive 60 days.^{5,26,28,29} Annually, there are an estimated 141,500 cases of ARDS resulting in 59,000 deaths. These patients required an estimated 2.7 million hospital days and 1.6 million ICU days.²⁹

Rates of critical illness with acute lung injury requiring mechanical ventilation have also been estimated in the U.S, with a crude incidence rate of 78.9 per 100,000 person years. Age adjusted incidence was 86.2 per 100,000 years with an in-hospital mortality rate of 38.5%. Annually, there are an estimated 190,600 cases of critical illness with acute lung injury in the U.S.

resulting in 74,500 deaths. Patients with acute lung injury required over 3.6 million hospital days and nearly 2.2 million ICU days.²⁹ Approximately one third (34%) of the estimated 100,000 patients who survive their acute illness are able to be discharged directly home, emphasizing the significant morbidity and long term health impacts of ARDS and critical illness including cognitive abnormalities, physical weakness, anxiety and depression, post-traumatic stress disorder and pulmonary disease.^{27,29-32} These estimates pre-date the COVID-19 pandemic which has only increased the incidence of ARDS.³³

1.2 The Mycobiome

The microbiome in total is made up of bacteria, fungi, viruses and archaea and has been demonstrated to be essential in human physiology, including immune development and function, metabolism and energy acquisition, vitamin-cofactor availability and metabolism of xenobiotics.³⁴⁻³⁶ The term mycobiome refers to the “fungal microbiome” and is used to describe the diverse array of fungi (mycobiota) and their genetic material in an environment. The mycobiome has been recognized as essential for the maintenance of the overall microbial community structure, immune priming and as an important contributor in immune modulation.³⁷⁻³⁹ Mutualism between fungi and humans is not yet well understood and there remains much to be learned about how fungi interact with the other non-fungal members of the microbiome.

1.2.1 Fungi-Host Relationship

1.2.1.1 Unique structural features of fungi impact the interaction between fungi and the human host

Fungi are heterotrophic eukaryotes with a unique biology, which in part, allows them to control their interactions with the human host. Fungi are able to assess their surroundings, to detect changes in their environment, compete for nutrients and to respond to and exploit host defense mechanisms to their advantage.⁴⁰ The fungal cell wall can vary widely in structure depending on the morphotype, growth stage and environment of the fungi. Distinct features of the cell wall, called pathogen-associated molecular patterns (PAMPs) allow the host immune system to discriminate between pathogenic and non-pathogenic fungi using pattern recognition receptors (PRRs) expressed on cell surfaces to activate the inflammatory pathways that lead to cytokine release and initiation of the innate immune system.^{41,42} Pattern recognition receptors are either intracellular, bound to the cell surface or soluble. Soluble PRRs include collectins, ficolins, complement components, and pentraxins.⁴³ Found in all fungi pathogenic to humans, β -glucans (which are glucose polymers) chitin (a polymer of *N*-acetylglucosamine); and mannans (which are chains of several hundred mannose molecules) are major components of the fungal cell wall that are recognized by PRRs. These PRRs include toll-like receptors, C-type lectin receptors, mannose receptors, and galectin proteins.⁴¹ Activation of pattern recognition receptors initiates intracellular pathways that promote immune system activation through the production of defensins, chemokines, cytokines, reactive oxygen species and specific host defense mechanisms directed at the clearance of fungi.^{41,42} The initial immune response is phagocyte driven and is focused directly on killing the fungal pathogen. The recognition of fungi by dendritic cells; however, promotes the differentiation of naïve T cells into effector T helper (T_H) subtypes that activate the inflammatory

pathways that lead to cytokine release and initiation of the innate immune system.^{41,42} However, activation of the innate immune system may paradoxically cause tissue damage and promote infection. Given the propensity of fungi to trigger an inflammatory cascade, the role of the mycobiome may be important in influencing the immune response of the respiratory tract and contribute to the development of lung damage. It is not clear the role of commensal fungi of the mycobiome play in immune system activation and tissue damage.

1.2.1.2 Fungi are able to both provoke and evade the host immune system

There is evidence that many fungal species have co-evolved with their mammalian hosts over time, suggesting that there exists both an intricate immune surveillance system in the host and complex fungal strategies to provoke and evade that immunity.^{41,44} Using a genome-wide expression profiling approach, Brown et al have shown that changes in *Candida* gene expression in the setting of infection represent a response to changes in the host-environment and reflect fungi's ability to counteract different host-defense mechanisms.⁴⁵ This ability to adapt to the host-environment extends to an ability to subvert host detection systems. In *Candida albicans*, β -glucans are masked during the hyphae-stage, allowing the fungi to avoid recognition. In *Blastomyces dermatitidis*, *Histoplasma capsulatum* and *Paracoccidioides brasiliensis*, the production of α -(1,3)-glucans blocks PRRs that recognize β -glucans, contributing to the virulence of those pathogens.⁴⁶ *Aspergillus fumigatus* conidia produce hydrophobins and melanin that subvert the immune surveillance system.⁴⁷ *Pneumocystis jirovecii* prevents detection by changing the expression glycoproteins on the cellular surface.⁴⁸ A common fungal evasion tactic takes advantage of complement receptors that recognize β -glucans to reduce the inflammatory response.⁴¹ This ability to change in response to the host-environment may play a significant role

in the composition of the mycobiome and the relationship between the mycobiome and the host immune response; however, this relationship remains unknown.

1.2.1.3 The relationship between the host and fungi is dynamic and delicately balanced.

Fungi can have a variety of relationships with their human host, with dynamics ranging from symbiotic and commensal to latent or pathogenic. While more is known about the pathogenic relationship demonstrated fungal infection, the vast majority of relationships between fungi and the host are positive or neutral. The host-fungus relationship is maintained on a delicate balance between both pro- and anti-inflammatory host signals. Disruption of this relationship can result in pathological consequences.⁴¹ In a murine model, McAleer et al found both significant decreases in IL-17, a pro-inflammatory cytokine associated with autoimmune disorders, and IL-22, a tissue modulating cytokine upregulated in many inflammatory diseases, along with elevated IL-4, an anti-inflammatory cytokine, in their lungs after exposure to *Aspergillus fumigatus*.⁴⁹⁻⁵² The relationship between the mycobiome and the pro- and anti-inflammatory responses of the host is essential to understanding the role of the mycobiome in disease and tissue damage; however, this relationship is not well understood.

1.2.1.4 The co-occurrence of and interactions between fungi and bacteria have an important impact on host immune response and inflammatory processes.

Fungi and bacteria interact directly with one another or indirectly via the host response impacting health and disease. By altering the host defense response, colonization with bacterial and fungal pathogens in concert can induce different inflammatory responses than colonization with either microbe alone and can vastly alter the outcome of the disease. Bacteria and fungi secrete molecules that influence their environment. These molecules directly influence other

microbial species by promoting or inhibiting their growth. Roux et al. showed colonization of rat respiratory tract with *Candida albicans* increases the risk of development of subsequent bacterial pneumonia by inducing an immune response and increasing pro-inflammatory cytokines.⁵³ In a murine model, infection with *Candida parapsilosis* and *Staphylococcus aureus* was associated with increased organ damage and higher mortality compared to single-pathogen infection as well as higher levels of circulating inflammatory cytokines.⁵⁴

The paucity of research into the respiratory mycobiome has limited the ability to understand the bacteriome-mycobiome co-occurrence and the impact on respiratory health. In culture-based studies, Mowat et al describe an antagonistic interaction between *Pseudomonas aeruginosa* and *Aspergillus fumigatus* in the lungs of patients with cystic fibrosis that impacts the formation of biofilms.⁵⁵ In a comparison of co-occurrence networks in the lungs of patients with asthma and healthy controls, Sharma et al found significantly different topologies between asthmatic patients and healthy controls.⁵⁶ This association in atopic lung disease supports the hypothesis that bacteriome-mycobiome co-occurrence patterns may play a role in respiratory disease; however, there is a limited understanding of fungal-bacterial interactions and their function in respiratory health and disease.

1.2.2 The Lung Mycobiome

The respiratory tract represents the main portal of entry for microorganisms. The respiratory tract provides the primary entry site for the numerous microorganisms that are spread as airborne particles. As the human body's second-largest mucosal surface area, roughly the same size as a tennis court, human lungs are constantly exposed to fungi through inhalation.⁵⁷⁻⁵⁹ Airborne fungi are especially prevalent, comprising up to 50,000 spores per cubic meter of air, in

exposing the respiratory tract to a wide variety of fungi.⁶⁰ In recent studies of both rural and urban areas, fungal spores make up to 11% of fine particle material ($\leq 2.5 \mu\text{m}$).⁵⁸ Fungi have also been detected in clouds, fog, and precipitation.⁶¹ Inhaled fungi can be extremely diverse and include species known to be pathogenic as well as those believed to be commensal or harmless in humans. In a DNA-based analysis of diversity of aerosol fungal species, Fröhlich-Nowoisky et al found that the diversity of fungi was much higher than had been previously demonstrated and included fungi known to be pathogenic in humans.⁵⁸ In a study of indoor air quality and dust, Pitkäranta et al found extremely high fungal diversity represented by nearly 400 different taxa in an office building.⁶² In a study of dust samples collected from 928 U.S. homes, Grantham et al identified 40,000 different fungal taxa present, illustrating the wide range of fungi the respiratory tract is exposed to daily.⁶³ The lungs are exposed to a large number of diverse fungi daily; however, the impact of this exposure remains widely unexplored.

Fungi are present in the respiratory tract in states of both health and disease. Until very recently, it was commonly believed that healthy lungs were a sterile environment.⁶⁴ Historically, low levels of fungi in the lungs of healthy people paired with the low sensitivity of culture-based techniques allowed for the assumption that healthy lungs contain no fungi.⁶⁵ Despite the low relative abundance ($< 0.1\%$) of fungi to bacteria, the mycobiome is a fundamental part of the human microbiome. There is increasing evidence that the lung microbiome varies in health and disease states.⁶⁶⁻⁶⁹ Fungal communities are present in the respiratory tract in both healthy individuals and those with pulmonary disease. There is significant variation in the taxonomic composition of these communities between individuals, with those with pulmonary disease trending toward lower fungal diversity.⁷⁰

1.2.2.1 The Lung Mycobiome in Healthy Individuals

The mycobiome of the lung in healthy people is comprised primarily of fungi normally found in water, plants and soil samples. The most common fungi in the lungs of healthy study participants belong to the phyla Ascomycota and Basidiomycota and have included *Davidiellaceae*, *Hyphodontia*, *Cladosporium*, *Eurotium*, *Penicillium*, *Kluyveromyces* and *Eremothecium*.⁷¹⁻⁷³ Other fungi found in healthy individuals include those known to be pathogenic including *Candida*, *Neosartorya*, *Malassezia* and *Pneumocystis*.⁷¹⁻⁷³ Composition and stability of mycobiota are specific to each individual and is likely dependent on the unique host immune system.⁷⁴⁻⁷⁶ The role of the mycobiome in maintaining microbial community structure, metabolic function, and immune priming and modulation are unexplored.

1.2.2.2 The Lung Mycobiome in Respiratory Disease

Exposure to airborne fungi can lead to respiratory disease. In the lungs, mucosal epithelium covers the surface of the lung and act as both a physical and immunological barrier to inhaled particles.⁵⁹ Inhalation studies in animals have shown that the majority of inhaled fungal spores were retained in the mucosa and alveolar epithelium.^{77,78} Many of these fungal spores are not hazardous, but some have the ability to cause lung disease.⁷⁹ Healthy individuals are usually able to avoid colonization by fungal spores through mucociliary clearance, innate immune response and other pulmonary defense mechanisms such as cough.⁸⁰ In patients with critical illness, spores can bypass these defense mechanisms and evade the lung immune response, allowing them to persist in the airways.⁸¹ Evidence from human and animal studies supports an association between fungal exposure and non-infectious lung diseases.⁷⁹ Airborne fungi are capable of causing infectious respiratory disease, but the role of fungi in non-infectious respiratory disease is not well understood.

The respiratory mycobiome has only been studied in a small number of pulmonary diseases. Fungi have been associated with the development of atopic disease, allergic respiratory disorders, and asthma.^{82,83} Pulmonary diseases with known inflammatory and immune components of pathogenesis have been associated with fungal dysbiosis.^{56,84} Decreased respiratory function has been associated with lower fungal diversity in cystic fibrosis, asthma, lung transplant and chronic obstructive pulmonary disease (COPD).

The respiratory mycobiome has been most studied in cystic fibrosis, a systemic inherited metabolic disorder that changes the quality and quantity of mucus, predisposing patients to microbial colonization and subsequent lethal pulmonary damage and respiratory failure. Despite the high rate of fungal infections in patients with cystic fibrosis, significant work has been done describing the colonization of the lungs by bacteria; however, there has been less of a focus on the mycobiome and the impact of fungal-bacterial co-occurrence patterns. In a longitudinal study of sputum samples from patients with cystic fibrosis, Kim et al did not identify associations between mycobiome dysbiosis and clinical characteristics of patients. However, during the sampling period, many participants were prescribed several different antifungal drugs, potentially confounding the results.⁸⁵ Delhaes et al found lower fungal diversity to be associated with decreased lung function and worse clinical status when comparing sputum samples from a small number of patients with cystic fibrosis. Changes in pulmonary physiology likely play a role in this dysbiosis.⁸⁶ Changes in the nutrients available to fungi in the lungs, such as in the setting of respiratory infection, impaired mucociliary clearance or a change in the quality or quantity of mucus secreted can selectively impact fungal growth.^{80,87}

Bronchiectasis is a chronic inflammatory lung disease characterized by pathogenic widening of the airways causing a productive cough, hemoptysis and exacerbations frequently

caused by infection. Patients often experience pooling of mucus in the airways, selectively promoting fungal growth and persistence. Fungi have been associated with worsening of disease in patients with bronchiectasis.⁸⁸In a longitudinal study of patients with bronchiectasis, Máiz et al found that persistence of *Aspergillus* and *Candida* over time as associated with production of purulent sputum, worse FEV₁, increased number of hospital-treated exacerbations and use of long-term antibiotics.⁸⁹ Aogáin et al undertook a cross-sectional NGS study of the mycobiome in 238 patients with bronchiectasis and matched controls. They found that fungal diversity was significantly lower in bronchiectasis and that the presence of *Aspergillus* in the lung was associated with increased immunoglobulin E (IgE) levels, an antibody used as a biomarker of allergic response and increased disease severity. Distinct mycobiome patterns associated with clinical phenotypes of bronchiectasis were also identified.⁹⁰ This work strongly suggests a role for fungi in bronchiectasis and inflammatory lung disease; however, the relationship between the co-occurring bacteria is unexplored.

In asthma, a chronic pulmonary condition with pathognomonic wheezing and dyspnea caused by airway hypersensitivity and inflammation, exposure to fungal spores has been associated with a decrease in lung function, worsening of asthma symptoms and even increased mortality.⁹¹ Severe asthma has been associated with fungal sensitivity including allergic bronchopulmonary mycosis, a serious airway disease with an unclear pathogenesis. In a cross-sectional study of asthmatic patients, Sharma et al demonstrated that lower mycobiome diversity was associated with a high inflammatory eosinophilic-mediated phenotype of asthma compared to a low inflammatory neutrophilic-mediated phenotype of asthma as well as with clinical parameters of respiratory disease. These results are confirmed in a small case-control study performed by van Woerden et al that found there are significantly different mycobiome patterns in patients with asthma compared

to healthy controls.⁹² In a larger study with well-characterized phenotypes of asthma as well as healthy controls, Fraczek et al reported that higher fungal burden is associated with more severe asthma phenotypes as well as treatment with corticosteroid treatment.⁹³ This association of higher fungal burden with a well-known and often immune-modulating therapy may point toward the role of the host immune system and the mycobiome in disease severity.

In COPD, a heterogeneous chronic inflammatory disease characterized by progressive airflow limitation and caused primarily by cigarette smoking, there is growing evidence that the mycobiome may play an important role in the occurrence of exacerbation and the worsening of lung function. Fungi are frequently identified in the lungs of patients with COPD, likely due in part to the extensive use of immunosuppressants and antibiotics treating frequent bacterial infections and exacerbations.⁹⁴ Several studies have shown that patients with COPD are frequently colonized with *Pneumocystis jirovecii* and that an increase in *Pneumocystis* burden is associated with increased disease severity independent of smoking status.⁹⁵⁻⁹⁷ In a murine model, exposure to cigarette smoke was associated with increased *Pneumocystis* burden in the lung as well as changes in pulmonary function and pathophysiology consistent with COPD.⁹⁸ A small study (n=6) of patients experiencing an acute exacerbation of severe COPD examined the sputum mycobiome longitudinally during the hospital stay and found heterogeneity in the fungal communities between patients as well instability of the mycobiome over time with-in individual patients.⁹⁹ This study is limited by its small sample size, but also by the use of only the ITS1 rRNA for fungal sequencing, which does not allow for the identification of *Pneumocystis*.

In critical illness, the pulmonary mycobiome represents both a source of potential infection and a driver of immune and inflammatory pathway activation. In the intensive care unit (ICU), an estimated 60% of patients have fungal colonization of their lungs.¹⁰⁰ Garnacho-Monntero et al

undertook a large multicenter cohort study to assess risk factors and outcomes in ICU patients with fungi isolated using culture-dependent methods in tracheal secretions. They found that nearly 50% of patients were colonized with *Candida* and an additional 6% had invasive candidiasis. Non-*Candida* fungal infection was identified in another 3.4% of patients.¹⁰⁰ In a study pneumonia being treated in the ICU, Bousbia et al used NGS methods to characterize the respiratory microbiota. They found fungi in 17% of bronchial alveolar lavage samples with the majority of taxa identified representing *Candida*.¹⁰¹ Given the identification of tree fungi in several lung samples in this study, there is a possibility that the samples were contaminated during the laboratory processing and may not represent the true fungal communities. The pulmonary mycobiome has been considered in only a few types of respiratory disease and the studies that have been undertaken have been small and limited by technological challenges; however, these studies have demonstrated associations between mycobiome dysbiosis and respiratory disease. Continued research into the role of the pulmonary mycobiome is needed to elucidate the role of the mycobiome in respiratory disease.

1.2.3 The Gut Mycobiome

Fungi are present in the gut in health and disease. The gastrointestinal tract is exposed to the environment and acts as a system for the absorption of nutrients and other metabolites. The gut microbiota has been well studied and their role in both health and disease has become increasingly clear. The vast majority of the surveys of gut microbiota have focused on the bacterial communities; however, there is increasing awareness of the mycobiome.^{102–104} This increasing interest is primarily driven by findings demonstrating that fungi can modulate the host immune response and, in doing so, may represent an important factor in both health and disease. Fungal communities are present in the gastrointestinal tract in both healthy individuals and those with

disease. While most of the fungi identified in the gut appears to have a commensal or mutualistic relationship with the host, the potential of the gut to act as a reservoir for pathogenic fungi cannot be underestimated. Additionally, the possible health benefits of specific fungal species in the gut including both probiotic and antibiotic mechanisms have been described.¹⁰⁵ The fungi described in the human gut have been diverse, but comprise primarily 3 phyla: Ascomycota, Basidiomycota, and Zygomycota.¹⁰³ Fungal communities are present in the gastrointestinal tract in both healthy individuals and those with disease. There is significant variation in the taxonomic composition of gut mycobiomes between individuals, with those with disease trending toward lower fungal diversity. The role of the gut mycobiome in maintaining overall microbial community structure, metabolic function, and immune priming and modulation remain unclear and represent an opportunity to expand our understanding of the role of the gut mycobiome in health and disease.

The gut mycobiome is important in the development and homeostasis of inflammatory immune responses in the host. It is well established that the gut microbiota are critical to gastrointestinal function as well as the health and disease of their human host.^{102,106} The gut microbiome plays an important role in not only the digestive metabolism but also in the development and regulation of the host inflammatory immune responses. There is increasing evidence showing that the microbiome-host interactions occurring along gastrointestinal mucosa are important contributors to the development of inflammatory and immune responses at distal sites and systemically. Bacterial gut dysbiosis has been associated with chronic and inflammatory diseases such as inflammatory bowel disease, type 2 diabetes, and cardiovascular disease.¹⁰⁷ Compared to the bacteriome, the role of the mycobiome in the gut on immune function is poorly understood. Mycobiome alteration influences both local and systemic immunity through the activation and modulation of the host immune and inflammatory systems. Mycobiome dysbiosis

in the gastrointestinal tract influences immunity at distant sites, including as the lung, and can contribute to allergic responses.¹⁰⁸ Gut inflammation and permeability of the intestinal epithelial barrier initiated by non-fungal triggers can allow for exposure to fungal antigens and to the development of systemic IgG and IgA.¹⁰⁹ Mucosal immunity to gut fungi during dysbiosis is not well understood. At mucosal surfaces, the bacteriome and mycobiome interact with each other; as such, dysbiosis is probably a feature representing the complex crosstalk between the fungi, bacteria and the host.

1.2.3.1 The Gut-Lung-Axis

The gut can impact respiratory disease but the gut mycobiome has only been studied in a small number of pulmonary diseases. The association between the gut-lung axis, a conceptual connection between the alterations in the gut and their effects on immune function and the lung, and respiratory disease is well described. There is a growing body of research supporting the role of the gut microbiota in respiratory health.^{110–118} Research focused specifically on the gut mycobiota and respiratory health and disease is lacking and is limited primarily to animal models of allergic airway disease and asthma.^{119–124} Noverr et al utilized a murine model of cefoperazone-induced gut dysbiosis resulting in increased enteric bacteria and *Candida albicans*. Dysbiotic mice and controls were exposed to *Aspergillus fumigatus* to provoke an allergic airway response. Compared to controls, dysbiotic mice showed a significant allergic airway response to the fungal provocation as demonstrated by increased levels of eosinophils, mast cells, IL-5, IL-13, and INF γ in the lungs and IgE in serum.¹²⁵ Noverr et al repeated this experiment in IL-13^{-/-} mice and controls and found that the impact of gut dysbiosis on allergic response was independent of genetic background but required IL-13 production. In a fluconazole induced model of gut mycobiome dysbiosis in mice, Li et al demonstrated that gut mycobiome dysbiosis has significant and

persistent impacts on allergic airway disease mediated through mononuclear phagocytes with fractalkine receptors.¹¹⁹ Wheeler et al identified an increase in *Aspergillus*, *Wallemia*, *Epicoccum* and a decrease in *Candida* in a murine model of antifungal-mediated dysbiosis that was associated with increased allergic airway disease exacerbations.¹⁰⁹ Skalaski et al replicated this increase of *Wallemia*, which is typically identified as a non-pathogenic commensal, in fungal dysbiosis associated with allergic airway disease.¹²⁰ Accumulated evidence of an association between allergic airway disease and the gut mycobiome demonstrates that there is an important role for the gut mycobiome in the host immune response and respiratory health; however, the role of the mycobiome in the gut-lung axis is not well understood.

1.2.3.2 Gut permeability increases in critical illness.

There is growing evidence demonstrating that in critical illness, microbes from the gut enter the lungs through translocation facilitated by increased permeability of both the gut and the alveolar capillaries.^{126,127} In ARDS, direct or indirect injury to the lungs causes the alveolar-capillary membrane to become increasingly permeable. The influx of fluid into the alveoli that is pathognomonic of ARDS is directly caused by increased permeability of the lungs.¹²⁷ It is well known that gut permeability increases during sepsis and other critical illnesses.¹¹⁴ Gut permeability has been found to be predictive of multi-system organ failure in critically ill patients¹²⁸ and of sepsis in postsurgical patients.¹²⁹ In a rat model of shock, Baker et al found that bacteria translocated from the gut to the lymph nodes, livers, and spleens within 30 minutes of the onset of the experimental shock. There was a dose-dependent relationship between critical illness and translocated bacteria with rats subjected to shock for 90 minutes exhibiting an increase in the degree of translocated bacteria as compared to those who experienced shock for 30 or 60 minutes. Injured intestinal mucosa, epithelial edema and areas of focal necrosis in the gut were identified

in the rats as well.¹³⁰It is logical to hypothesize that critically ill patients with ARDS may be at risk of microbial translocation between the gut and lung due to both increased gut and alveolar permeability. The role of the gut-lung-microbiome axis is not yet fully understood and the specific role of the mycobiome and bacterial-fungal interactions in this translocation is even more unclear.

1.3 The Microbiome in Critical Illness

Critical illness and the interventions performed as a part of clinical care are known to significantly alter the microbiome. Critical illness significantly changes the physiological status of the host, which profoundly distorts the composition and diversity of the microbiome, a state known as 'dysbiosis'. It has been proposed that in critical illness there is increased gut and alveolar-capillary permeability, allowing for the translocation of gut microbes into the lungs. Clinical care such as sedatives, endotracheal intubation, and mechanical ventilation can decrease mucociliary clearance and cough reflex leading to a decreased ability to eliminate microbes. 75% of ICU patients are treated with antibiotics during their hospital stay which is known to cause severe damage to the microbiome through indiscriminate destruction of the commensal microbes.¹³¹ This dysbiosis increases vulnerability to infection with additional pathogens. Additionally, the ICU environment, including room surfaces, medical devices or even the hands of healthcare providers may harbor microbes that can colonize vulnerable patients.¹³² While there are many potential challenges to maintaining microbiome balance in critical illness, very little is known about the temporal relationship between dysbiosis and critical illness and the role of mycobiome in critical illness.

1.4 The Microbiome in ARDS

Perturbation of the microbiome may play a role in mediating the inflammatory cascade that is responsible for the acute lung injury leading to ARDS. In healthy lungs, the alveoli do not represent a rich environment for microbes to reproduce. In ARDS, the endothelial exudate and edema in the alveoli provide a nutrient-rich medium for microbes to thrive.¹³³ The impact of the host stress response and the use of systemic antibiotics alters the ecological community in the lungs and can provide an opportunity for pathogens to flourish. Microbes migrate to the lungs through the oropharynx in healthy lungs. In ARDS, this migration is altered due to the introduction of the endotracheal tube required for mechanical ventilation. The population of commensal bacteria in the mouth changes in intubated patients to be dominated by pathogenic bacteria increasing the likelihood that pathogenic bacteria migrate and colonize the lungs.^{134,135} In addition to colonization with pathogens, clinical interventions to treat ARDS may contribute to a decreased rate of microbial clearance. Sedation during mechanical ventilation diminishes the cough reflex and both the sequelae of critical illness and the presence of the endotracheal tube impairs the mucociliary escalator.¹³⁶ Microbial elimination can be impaired by having the head of the bed raised as well.¹³⁷ Physiologic consequences of ARDS and the medical care provided to patients with ARDS increase the opportunity for microbial dysbiosis. As such, fungal dysbiosis may play a role in ARDS; however, this remains unexplored.

The microbiome and alveolar injury propel each other in a dysregulated feedback loop. Endothelial dysfunction allows for inflammatory cells and exudate to flood the alveoli. This protein-rich fluid provides a rich energy source for rapidly producing microbes and increases the concentration of host stress response chemicals, such as catecholamines and inflammatory cytokines, selectively promoting microbial growth and virulence and in turn maintain a microbial

community that perpetuates alveolar inflammation. The influx of fluid in the alveoli breaks down host defenses by creating oxygen steep oxygen gradients, inactivating antimicrobial surfactant and impairing mucociliary clearance. It is possible that the critical illness or exposures resulting from clinical care cause altered microbial communities in the lung. This dysbiosis may play a role in mediating the inflammatory cascade that is responsible for the acute lung injury leading to ARDS. However, without a clear understanding of the temporal relationship of acute lung injury and dysbiosis, it is possible that dysbiosis in the lung is a result of the pathophysiology of ARDS. The more likely assumption is that both the lung microbiome and the inflammatory cascade of lung injury perpetuate each other in a positive feedback loop responsible for non-resolving ARDS (Figure 1.1). Researchers have hypothesized that the primary therapeutic targets, host inflammation, and cellular injury are the sequelae of host-microbiome interaction.^{6,7,64} Recently, the hypothesis that ARDS and the microbiome perpetuate each other in a feedback loop has been put forward as an explanation of the relationship between the microbiome and alveolar lung injury. However, there is a paucity of research into the mycobiome and the interaction between the mycobiome and both the bacterial communities and the host immune system.

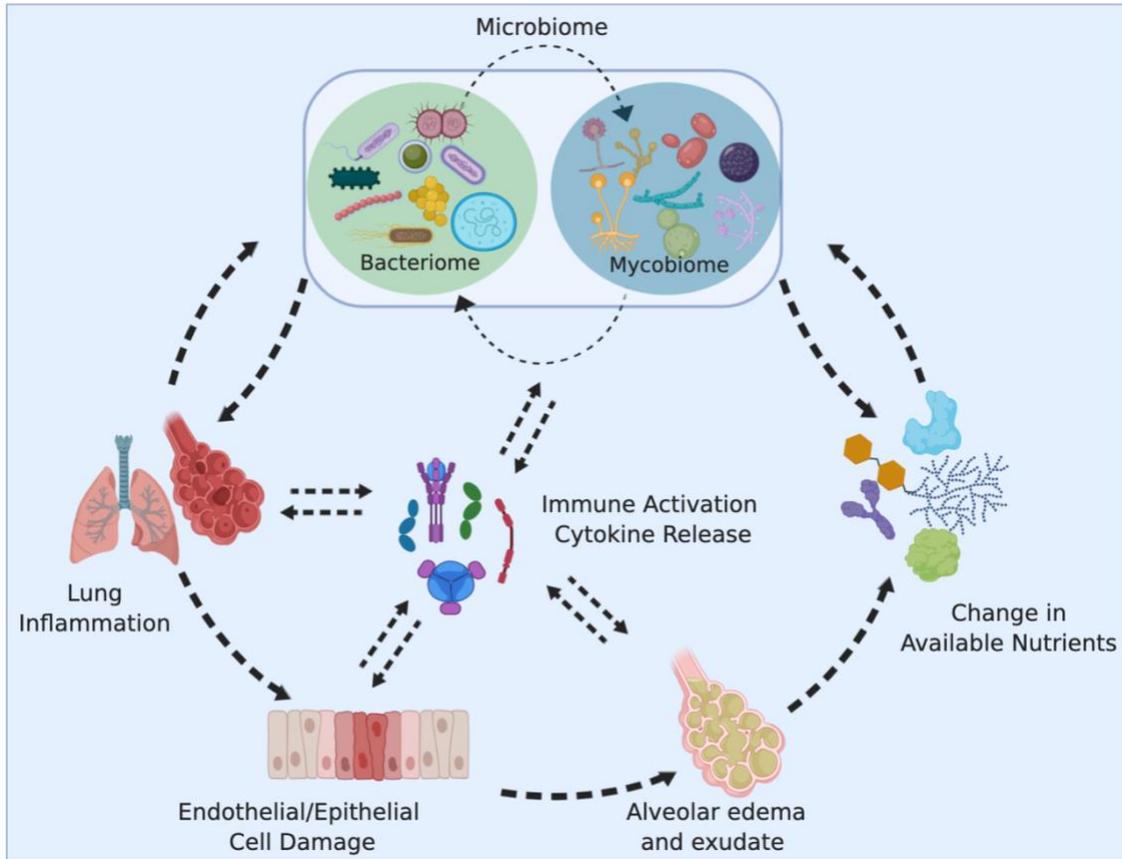


Figure 1.1: Example of a Potential Dysregulated Feedback Loop Resulting in ARDS and Dysbiosis

In ARDS, local inflammation of the lung leads to damage to the endothelial and epithelial cells lining the alveoli. The alveolar space then floods with nutrient-rich exudate, which selectively promotes the growth in the microbiome leading to dysbiosis in both the bacterial and fungal communities. This perpetuates a positive-feedback loop of inflammation, lung injury, alveolar edema, and continued dysbiosis.

1.4.1 The Mycobiome in Critical Illness

The lung mycobiome may have profound inflammatory effects that can cause or worsen lung disease. In addition to clinical infections, the lung mycobiome may be responsible for inflammatory factors that can cause or worsen pulmonary disease. The mycobiome may play an important role in the respiratory immune and inflammatory response and contribute to lung damage or the worsening of lung disease. The epithelium lining our airways are inhaled fungi's initial point of contact with the host and act as the frontline defense against fungal exposure. As

such, the lung epithelium has evolved to initiate the earliest immune events to resist fungal penetration.^{41,80} While the epithelium activates immunity to fungi, this immunity can be inappropriately triggered by innocuous fungi, causing harm to the host. Consequently, there is a fine balance between protective immunity against fungal pathogens and inflammatory disease caused by an excessive immune response.⁴¹ The significance of the epithelium as a barricade against fungal invasion is well known. However, the potent actions in fungal pathogen resistance by respiratory epithelium are not well elucidated.

1.5 Measuring the Mycobiome

1.5.1 Challenges in Measuring the Mycobiome

The majority of fungal species are not cultivatable using conventional microbial methods. The fungal kingdom, estimated to contain up to 3.8 million species, contains enormous diversity.¹³⁸ It is estimated that up to 95% of fungi are unculturable using standard clinical culture media or represent new or unknown pathogens.^{139,140} These traditional techniques have low sensitivity, are time-consuming and if the fungus is successfully grown, a further step of species identification must be taken.¹⁴¹ Following the advent of next-generation sequencing (NGS), there has been a dramatic increase in the number of new fungal species described each year with a 117% increase in the last 5 years. Scientists are now identifying approximately 1800 new fungal species each year.¹³⁸ In a study comparing fungi identified using culture-dependent and culture-independent next-generation sequencing techniques in soil, Stefani et al found that only 8.2% of fungi identified using next-generation sequencing methods were also identified using culture-dependent methods,

with only 3 out of 31 of the cultured fungi ranking in the 20 most abundant fungal taxa by culture-independent methods.¹⁴² Kim et al compared culture-dependent and culture-independent fungal identification in human sputum samples and found that only 23.5% of taxa identified by culture-independent methods were also identified using culture-based methods.⁸⁵ There are a number of fungi that are unidentifiable using traditional culture techniques, leading to a gap in our knowledge about the composition of the mycobiome and its role in health and disease.

1.5.2 Next Generation Sequencing of Mycobiome

Next-generation sequencing technologies allow the opportunity to both identify unculturable species of fungi and provide a more comprehensive characterization of fungal communities. Elucidation of the microbiome has historically been outside of the reach of scientific inquiry because culture-based methods do not allow for the sampling of hundreds of different taxa and the characterization of composition and abundances.¹⁴³ Recent advances in technology have provided powerful high-throughput sequencing and bioinformatics tools that allow us to characterize and investigate the contribution of the human microbiome.¹⁴⁴ This information has formed a tentative understanding of the relationships between specific microbiomes and health. The understanding of the relationship between microbes with each other as well as the relationships between the host immune system is an emerging field of study. Technological advances allowing for the elucidation of microbial communities have not been applied to the mycobiome in ARDS representing an area rich for discovery.

1.5.3 Measuring Biomarkers Host Response

For host-response experiments, validated biomarkers of ARDS in plasma and ETA samples were quantified (Table 1.2) including with *innate immune responses* (IL-6, IL-8, IL-10, TNFR1, suppression of tumorigenicity-2 [ST-2], fractalkine), *epithelial injury* (RAGE), *endothelial injury* (ANG-2) and *host-response to infection* (procalcitonin and pentraxin-3) to assess host response in relationship to microbial profiles.^{16,22,145} (1→3)-β-D-Glucan (BDG) from both serum and endotracheal aspirates was quantified to assess the presence of fungal cell wall products present in circulation and locally in the lung.^{146,147} As a biomarker of injury to the gut epithelium and intestinal permeability, we measured levels of fatty acid binding protein-2 (FABP-2).

Table 1.2: Host-Response Biomarkers of Interest

Host-Response Biomarkers
Innate Immune Response
IL-6
IL-8
IL-10
TNFR-1
ST-2
Fractalkine
Epithelial Injury
RAGE
FABP-2
Endothelial Injury
ANG-2
Host Response to Infection
Procalcitonin
Pentraxin-3
Fungal Translocation
BDG

1.5.3.1 Interleukin-6 (IL-6)

Various cell types produce IL-6, a cytokine that demonstrates both pro- and anti-inflammatory properties. IL-6 induces pro-inflammatory effects using trans-signaling through the soluble IL-receptor while anti-inflammatory effects are only able to be produced by cells that

express the IL-6 receptor such as macrophages, neutrophils and T cells.¹⁴⁸ Trans-signaling of IL-6 through endothelial cells has been associated with alveolar-capillary vascular permeability and may be responsible in part for the leakage of fluid into the alveolar space.¹⁴⁹ It is well known that IL-6 is an effective recruiter of inflammatory cells to the lung during acute inflammation.¹⁵⁰ Elevated during infectious, traumatic or inflammatory stress, IL-6 has been correlated with the pro-inflammatory state in ARDS and is found to be elevated during the course of ARDS. Bouros et al found that levels of IL-6 were significantly higher in patients at risk of ARDS who developed ARDS within 48 hours compared to patients who were at risk but did not develop ARDS suggesting that IL-6 is present in both the plasma and BAL before the onset of ARDS.¹⁵¹ In a meta-analysis, Terpstra et al found that IL-6 was associated with an ARDS diagnosis with an odds ratio(OR) of 2.4 (95% CI, 1.32-4.26) and that elevated plasma levels of IL-6 are independently associated with morbidity and mortality in ARDS patients (OR 3.38, 95% CI, 1.81-6.31).¹⁵² This result has been replicated reliably in patients with ARDS with elevated IL-6 also being associated with a decrease in ventilator-free days and overall poorer outcomes.^{19,153-155}

1.5.3.2 Interleukin-10 (IL-10)

IL-10 is an important anti-inflammatory immune regulator in the setting of infection and systemic inflammatory syndrome (SIRS).¹⁵⁶ Many different types of cells produce IL-10; however, IL-10 is found primarily in infected tissue. IL-10 down-regulates cell-mediated immune and pro-inflammatory responses including inhibiting the activity of Th1 cells, NK cells, and macrophages, which are both required for clearance of infection and known to cause tissue damage.¹⁵⁷ As such, IL-10 can ameliorate pathologic damage but can also impede the clearance of pathogens. In humans who were administered exogenous IL-10, Huhn et al found that levels of inflammatory cytokines, including interleukin-1 beta (IL-1 β) and TNF- α , were suppressed¹⁵⁸ and further clinical

data suggests that elevated levels of IL-10 are an indicator of poor prognosis in patients with trauma and infection, with an association with infection risk, multiple organ failure and mortality.^{159–162} In severely ill patients receiving extracorporeal membrane oxygenation (ECMO), plasma IL-10 early in the disease course has been associated with mortality and has been suggested as a prognostic factor in this population.^{163,164} In ARDS, the role of IL-10 is not entirely clear and associations with ARDS and IL-10 have been inconsistent, this could be due in part to a genetic polymorphism associated with IL-10 expression.¹⁶⁵ Armstrong and Miller identified lower levels of IL-10 in patients with ARDS as compared to critically ill patients; however, this study may be limited by the small sample size included in this study (N=26).¹⁶⁶ Parsons et al and Donnelly et al both identified high levels of plasma IL-10 and low levels of IL-10 in the BAL of patients with ARDS were associated with increased mortality.^{18,167} In an observational study of patients with ARDS receiving ECMO, Liu et al identified that plasma IL-10 at the time of cannulation for ECMO and during the first 6 hours of ECMO support were strongly correlated with mortality.¹⁵⁶

1.5.3.3 Tumor Necrosis Factor Receptor-1 (TNFR-1)

TNFR-1 is a receptor for tumor necrosis factor-alpha (TNF- α) that is ubiquitous and present on nearly every cell type. Associated primarily with inflammation and tissue degradation, TNFR-1 is responsible for reducing neutrophil infiltration and migration and for attenuating the cell death and inflammation associated with TNF- α . In the setting of inflammation, TNFR-1 decouples from the cell surface and binds to circulating TNF- α . TNF- α has been identified as a mediator of inflammation early in the disease course of ARDS^{168–170}; however, there has been inconsistency in the association of both plasma and BAL levels of TNF- α and clinical outcomes in ARDS.^{171–174} This inconsistency may be due in part to the binding of TNFR-1 to TNF- α with levels of TNFR-1 potentially representing the biological impact of TNF- α in ARDS.¹⁷⁰ Parsons et

al undertook a large multicenter study of ARDS and found plasma TNFR-1 was associated with worse clinical outcomes, decreased ventilator-free days, and mortality.¹⁷⁰ In both an animal study and randomized clinical trial in human patients, Proudfoot et al found that patients given a novel TNFR-1 antibody had lower levels of pulmonary neutrophilia, endothelial injury and significantly attenuated inflammation and lung injury.¹⁷⁵

1.5.3.4 Suppression of Tumorigenicity-2 (ST-2)

ST-2 is an interleukin-1 (IL-1) receptor that acts as a mediator of inflammation and immunity. Expressed primarily on mast cells and T helper type 2 (T_H2) cells, ST-2 plays a role in cell proliferation and T_H2 immune response.^{176,177} Elevated levels of ST-2 have been associated with cardiac disease, asthma, and idiopathic pulmonary fibrosis.^{178,179} In a murine model of acute lung injury, ST-2 was expressed in alveolar macrophages and suggested that ST-2 may modulate acute lung inflammation.¹⁷⁹ Brunner et al found that in patients with sepsis and trauma, plasma ST-2 is elevated. Hoogerwerf et al also demonstrated that ST-2 was elevated in patients with sepsis, remained elevated throughout the course of the disease and was associated with disease severity and mortality.¹⁸⁰ In trauma patients, elevated ST-2 is associated with mortality.¹⁷⁶ Bajwa et al found that patients with ARDS had significantly higher levels of ST-2 than critically ill controls and that in ARDS that could be used to discriminate between the two groups. Additionally, elevated ST-2 was independently associated with mortality on the first day of disease and over time and with unfavorable clinical outcomes including decreased ventilator-free days and longer length of ICU stay.¹⁷⁷

1.5.3.5 Fractalkine

Fractalkine is a chemokine produced primarily by endothelial cells as well as by several different cell types. Fractalkine expression at the site of inflammation is responsible for attracting and activating natural killer (NK) cells causing the lysis of endothelial cells in close proximity.¹⁸¹ Fractalkine is associated with many inflammatory disorders, such as rheumatoid arthritis, atherosclerosis, polymyositis, dermatomyositis, nephritis, and inflammatory bowel disease.¹⁸² In allergic asthma, increased levels of fractalkine have been found in BAL and in airway smooth muscle cells, lung endothelium, and epithelium.¹⁸² In a murine model of experimental sepsis, elevated fractalkine levels were associated with increased mortality.¹⁸³ In an observational study, patients with sepsis had highly elevated fractalkine levels compared to controls and fractalkine levels were associated with increased organ failure and mortality.¹⁸⁴

1.5.3.6 Receptor of Advanced Glycation End-Products (RAGE):

RAGE is a multiligand-binding cell surface molecule that can act as a transmembrane pattern recognition receptor. Accumulation of ligands, such as advanced glycation end-products (AGEs), amyloid fibrils and amphoterin, can dictate the biology of RAGE and lead to upregulation of the receptor and persistent RAGE-dependent cellular dysfunction. RAGE is not implicated in the initial pathogenesis of inflammatory disorders; it functions as a progression factor that exaggerates the host response toward tissue destruction and impedes a restitution of homeostasis. While many cells express RAGE, alveolar epithelial cells highly express RAGE and allow for the activation of pathways responsible for innate immunity and alveolar inflammation. Soluble forms of RAGE can be measured in bronchoalveolar lavage fluid (BAL) and plasma. In an observational study of patients with ARDS and critically ill controls, Nakamura et al found that patients with ARDS had higher levels of soluble RAGE and demonstrated that levels of RAGE are

independently associated with increased mortality in ARDS.¹⁸⁵ Jabaudon et al found that levels of RAGE were elevated in ARDS, but not in sepsis, suggesting that RAGE may be a unique biomarker of lung epithelial damage. Additionally, Jabaudon et al demonstrated in both an animal model and an observational human study an association between the clinical and radiographic severity of ARDS with levels of RAGE and decreasing RAGE overtime associated with clinical improvement, suggesting that levels of RAGE correlate with the degree of lung injury.¹⁸⁶⁻¹⁸⁸

1.5.3.7 Angiopoietin-2

Angiopoietin-2 (ANG-2) is a well-known pro-inflammatory biomarker of endothelial activation and permeability that is strongly associated with ARDS and clinical outcomes in ARDS. ANG-2 is a ligand for the Tie2 receptor which inhibits Tie2 phosphorylation subsequently promoting blood vessel permeability, cell death and inflammation.¹⁸⁹ Plasma ANG-2 is elevated in patients with sepsis and correlated with disease severity and with PaO₂:FIO₂ ratio less than 200.¹⁹⁰ In animal models, ANG-2 has been shown to be a significant mediator of acute lung injury. Elevated levels of plasma ANG-2 have been correlated with increased pulmonary permeability and leakage, severity of illness and mortality in patients with acute lung injury.¹⁸⁹⁻¹⁹¹ ANG-2 has been identified as a potential prognostic factor for ARDS risk as well as a possible therapeutic target.¹⁹²

1.5.3.8 Procalcitonin

Procalcitonin is a prohormone of calcitonin and has been described as an early, sensitive and specific biomarker for sepsis and severity of infectious disease due to bacterial infection.¹⁹³ Procalcitonin is more elevated in sepsis than in other inflammatory conditions and correlates strongly with clinical outcomes in sepsis.¹⁹⁴ In addition to bacterial sepsis, elevated procalcitonin

has been demonstrated in trauma, burns and major surgery.¹⁹⁵ The association between procalcitonin and ARDS has not been well studied, has been found to be inconsistent and should be explored further. In an observational trial of ARDS, elevated procalcitonin levels in plasma but not in BAL could discriminate between patients with sepsis-induced ARDS and non-sepsis induced ARDS and are associated with increasing severity of multiple organ dysfunction syndrome in patients with sepsis-induced ARDS.¹⁹⁶ Other studies have found no association between mortality and ARDS.¹⁹⁷ Procalcitonin may have a role as a biomarker predictive of ARDS; however, its role as a biomarker of bacterial infection and sepsis is clear.

1.5.3.9 Pentraxin-3

Pentraxin-3 belongs to the same family as C-reactive protein and is a proinflammatory fluid phase receptor associated with innate immunity and inflammation.¹⁹⁷ Pentraxin-3 is produced by several different cell types including neutrophils, macrophages, and lung endothelial and epithelial cells in response to TNF- α and IL-1 β .¹⁹⁸ Pentraxin-3 has been associated with innate immune and inflammatory response to fungi, specifically *A. fumigatus*.^{199,200} Pentraxin-3 levels increase significantly in inflammatory disease conditions. This increase has been demonstrated in critical illness, tuberculosis, Dengue, acute myocardial infarction, and vasculitis.^{43,201} In acute myocardial infarction, pentraxin-3 has been identified as an early marker of permanent damage and the only independent predictor of mortality within 24 hours.²⁰² In critical illness, including SIRS, sepsis and septic shock, pentraxin-3 was highly elevated and correlated with disease severity and mortality.²⁰¹ Pentraxin-3 has been found to be overexpressed in severe infection, mechanical ventilation, and ARDS.^{43,203,204} In an observational study, elevated plasma pentraxin-3 was associated with mortality in patients with ARDS and there was not a difference in pentraxin-3 levels between sepsis-induced ARDS and non-sepsis induced ARDS. Additionally, pentraxin-3

levels remained highly elevated over the course of the disease and were associated with mortality over time.⁴³ Pentraxin-3, as a mediator of inflammation, may represent a reliable early biomarker of disease severity and subsequent clinical outcomes in ARDS.

1.5.3.10 Fatty Acid Binding Protein-2 (FABP-2)

FABP-2 is a validated marker of epithelial cellular dysfunction in the gut. Present in the cytoplasm of small bowel enterocytes, FABP-2 is a small protein involved in fatty acid transport during lipid absorption and is normally undetectable in healthy patients.²⁰⁵ When gut epithelial cells are damaged, FABP-2 is expressed by enterocytes and related into circulation.²⁰⁶ As such, FABP-2 has been widely accepted as a marker of early intestinal epithelial cell damage and gut permeability.²⁰⁷ Plasma FABP-2 elevation is observed frequently in critically ill patients and has been associated with shock and inadequate organ perfusion.²⁰⁸⁻²¹⁰ Intestinal ischemia and reperfusion can induce damage to the mucosal barrier in the gut, resulting in the translocation of micro-organisms, endotoxins and inflammatory cytokines into both local and more distal body tissues.²¹¹⁻²¹⁷ This results in a self-perpetuating signaling cascade with the potential to escalate into a cycle of increasing intestinal permeability, translocation of gut microbes and other gut-derived factors resulting in a stronger inflammatory response.²¹⁸ This increased inflammatory response and the alteration of microbiota, contribute to the onset of systemic inflammatory response, sepsis, acute lung injury and ARDS. In critically ill patients, Piton et al found that FABP elevation was an independent predictor of 28-day mortality and Derikx et al reported that in a population of patients with sepsis, non-survivors had elevated levels of FABP compared to those who survived.^{207,208} Hanssen et al demonstrated that FABP was positively correlated with levels of IL-6 and 8 in plasma in critically ill patients, suggesting a link between gut wall integrity and systemic inflammation.²¹⁹ In a murine model of intestinal ischemia and reperfusion, plasma FABP

elevations were directly correlated with lung injury as well elevated levels of IL-1 β and TNF- α .²¹⁸ FABP-2, as an indicator of gut permeability, may be an important biomarker in critical illness and as well as potentially providing mechanistic pathway for the impact of the microbiome in critical illness.

1.5.3.11 (1 \rightarrow 3)- β -D-Glucan (BDG)

BDG is a polysaccharide component of the fungi cell wall, except for in *Cryptococcus neoformans* and Zygomycetes. BDG is immunogenic and activates macrophages, neutrophils, T cells and stimulates the release of pro-inflammatory cytokines such as IL-6, IL-8 and TNF- α .^{220–224} These cell wall PAMPs are shed during infection and elevated serum BG levels have been associated with fungal infection.^{220,225–227} The identification of BDG in serum has been used as a surrogate for invasive fungal infection.²²⁸ Lower BDG levels have also been detected in healthy individuals, presumably from commensal fungi sloughing off into the bloodstream.^{220,229,230} It has recently been hypothesized that BDG may be a marker of gut permeability and translocation of fungi from the gut.^{231–233} Issara-Amphorn et al demonstrated in lupus nephritis that elevated BDG was associated with spontaneous gut leakage. In a recent study of intestinal mucositis in chemotherapy patients; however, no association was found between intestinal mucosal barrier damage and BDG. This finding may be confounded by the administration of antifungal drugs to the majority of participants.²³² In both animal and human models of HIV, BDG has been associated with gut permeability and microbial translocation from the gut and has been associated with systemic inflammation, HIV progression, cardiopulmonary comorbidities, and mortality.^{234,235} We will use BDG as a marker of gut permeability and microbial translocation.

1.6 Knowledge Gaps

The majority of fungi are unidentifiable using traditional culture techniques, leading to a gap in our knowledge about the composition of the mycobiome and its role in health and disease. Recent technological advances allow us to characterize the microbial communities; however, these strategies have not been applied to fungi in critical illness. We have a developing understanding of the role of the bacteria in health and disease; however, the role of the mycobiome in maintaining microbial community structure, metabolic function, and immune priming and modulation remain primarily unexplored. Fungi activate both pro- and anti-inflammatory pathways and the environmental-sensing nature of eukaryotes allow fungi to evade detection by the host immune system, although much of what is known about the interaction between fungi and the host is focused on pathogenic fungi and fungal infection. There is very little understanding of the role of commensal fungi in respiratory health and disease. In addition to interactions between fungi and the host, bacteria and fungi also inhabit the same niches and interact. The impact of these interkingdom interactions on the immune system remains unexplored. In ARDS, it is hypothesized that interactions of the microbiome and the host immune system create a negative feedback loop that perpetuates acute lung injury; however, this relationship is not yet clear.

The vast majority of study into microbiome has focused exclusively on bacteria. This holds true in research into critical illness and ARDS where the status quo has been to consider only the bacterial component of the microbiome. It is well known that fungi are potent immunomodulators and their interaction with the host may result in dramatic inflammatory responses. Given that ARDS is the sequelae of an explosive immune response with an often unclear etiology, we will advance the field by elucidating the role of the mycobiome as a potential factor impacting the host immune response in ARDS. While recent research on the microbiome in critical illness and ARDS

has focused on bacteria, there has been very little research into the fungal component of the microbiome and even less on the interactions between the bacteria and fungi co-inhabiting the same anatomical niche. Both bacteria and fungi are known to impact patterns of abundance, growth, and virulence of the larger microbiome and to have a dramatic effect on the host immune response. These complex relationships between bacteria and fungi may impact the overall composition and abundance of different bacterial and fungal taxa as well as the host immune response in ARDS. We will explore these intricate relationships and expand our understanding of the co-occurrence of bacteria and fungi in ARDS in the context of other clinical characteristics.

1.7 Specific Aims

The goals of this dissertation are to investigate the associations of the lung mycobiome with host inflammation and clinical outcomes in critical illness and ARDS. To address these goals, I will be utilizing data from the Acute Lung Injury Registry and Biorepository (ALIR), a cohort of mechanically ventilated, critically ill adult patients. At study enrollment (within 72 hours of intubation), endotracheal aspirate samples were collected to allow for the assessment of the microbiota in the respiratory tract and plasma samples were collected to assess biomarkers associated with innate immunity and inflammation. We obtained data about patient demographic characteristics and clinical variables from the electronic medical record and followed patients prospectively for time to liberation from mechanical ventilation, duration of stay in the ICU and survival up to 90 days from intubation. We extracted and sequenced fungal DNA from the endotracheal aspirate samples to allow us to characterize the fungal communities in the respiratory tract. We derived measures of alpha diversity as well as measurements of fungal abundance to

characterize the lung mycobiome. Using this data, we can investigate the associations of the mycobiome with biomarkers of inflammation and innate immunity and clinical outcomes in critically ill patients with and without ARDS. Additionally, we extracted and sequenced bacterial DNA from the endotracheal aspirates, allowing us to identify correlations between the bacterial microbiome and the fungal mycobiome and clinical features of critical illness and ARDS. To identify potential relationships between specific bacterial and fungal taxa, we will use Probabilistic Graphical Models (PGMs). PGMs estimate and graphically represent the complex relationships of large numbers of variables that interact with each other, allowing for the discovery of direct links between variables based on their conditional dependencies. We hypothesized that (1) specific mycobiome characteristics and patterns and (2) distinct microbiome-mycobiome co-occurrence networks are associated with clinical disease course and outcomes in critical illness and ARDS.

Manuscript 1:Characterization of the Lung Mycobiome in Critical Illness

Aims: To describe the mycobiome in critical illness and ARDS.

Hypothesis: A fungal signal that is distinct from experimental controls will be observed in endotracheal samples from critically ill patients.

Manuscript 2: The Role of the Lung Mycobiome in Clinical Outcomes in Critical Illness and ARDS

Aims: To determine the associations of features of the lung mycobiome with biomarkers indicating inflammation and with clinical outcomes in critical illness and ARDS.

Hypothesis: Features of the mycobiome (diversity, single-taxa dominance, community composition) will be associated with increased biomarkers of inflammation and worse clinical outcomes (time-to-liberation from mechanical ventilation, ICU length of stay, mortality at 30 day).

Manuscript 3: Interkingdom Interaction in The Lung Microbiome in Critical Illness

Aim 3: To determine correlations between bacteria and fungi in the lung and their associations with clinical outcomes in critical illness.

Hypothesis: The co-occurrence of specific species of fungi and bacteria will be associated with clinical variables in a model accounting for the complexity of the relationship between bacteria, fungi, and clinical characteristics.

2.0 Manuscript 1: Characterization of the Lung Mycobiome in Critical Illness

2.1 Introduction

Background: Research into the pulmonary microbiome of critical illness has primarily focused on bacteria. However, research into the fungal communities (mycobiome) has been exceedingly limited. Given that the pulmonary mycobiome may represent a potential source of infection as well as a driver of immune and inflammatory pathway activation, we sought to describe the composition of the lung mycobiome in critically ill patients.

Methods: We collected endotracheal aspirates (ETA) from critically ill mechanically ventilated patients. We used extracted DNA from ETAs and negative reagent controls to perform fungal rRNA gene sequencing (internal transcribed spacer (ITS)) on the Illumina MiSeq platform. Diversity and taxonomic analyses were performed using the DADA2 and Phyloseq packages. We measured the alpha diversity and single-species dominance of each sample.

Results: 99 different fungal species were identified in the ETA samples from N=226 critically ill patients. However, most samples (51.3%) were comprised of a single species, and 96.4% of samples were dominated by one species. We observed low fungal diversity with a mean Shannon Diversity index of 0.18 and a mean Simpson's Diversity index of 0.79 and fungal richness with a Berger-Parker index of 0.92 and a mean of 1.92 observed ASVs. *Candida* was the most abundant fungal genus observed, comprising 65% of fungal abundance in ETA samples.

Conclusion: Among mechanically-ventilated critically ill patients, the lung mycobiome has overall low diversity. Although a large number of different fungal species were identified, the majority of ETA samples were dominated by a single fungal taxon. Our findings indicate the need

for an investigation into correlations between mycobiome composition and diversity and clinical outcomes and ICU outcomes.

2.2 Background

In the last decade, technological advances in culture-independent microbiology have shown that despite previously being thought to be sterile, there are unique and dynamic communities of microbes, including bacteria, fungi, and viruses in the lungs.^{66,70} There is growing evidence to suggest that the lung microbiome has an important role in immune function and clinical outcomes in both acute and chronic respiratory disease.^{25,69,236,237} Both anatomical and pathophysiologic changes in the respiratory tract in the setting of disease may distort the composition and diversity of the microbiome in the lung.^{25,237,238} The majority of research into the lung microbiota have focused on bacteria and have shown that in the lung bacteria are present in healthy individuals, are altered in both acute and chronic lung disease due in part to anatomical and pathophysiological changes, are associated with variation in immune function and have been shown to be associated with clinical and patient centered outcomes.^{25,69,126,237,239–244}

Dickson et al recently studied the bacterial component of the microbiome in mechanically ventilated critically ill patients and found that variation in the bacterial microbiota predicts outcomes in the ICU. They identified a significant correlation between bacterial burden, bacterial microbiota diversity, and composition with the number of ventilator free days.²³⁷ Kitsios et al found that bacterial communities in the lung of mechanically ventilated critically ill patients with low alpha diversity and low relative abundance of protective commensal bacteria were associated with systemic inflammatory responses, longer time to liberation from the ventilator and higher

mortality at 30 days compared to critically ill patients with higher alpha diversity and greater relative abundance of commensal bacteria.²⁵ While recent research on the microbiome in critical illness has focused on bacterial communities, there has been very little research into mycobiome in critical illness.

Despite the low relative abundance (0.1% of sequencing reads) of fungal DNA to bacterial DNA, the mycobiome is a fundamental part of the human microbiome.²⁴⁵ Fungi are present in the respiratory tract in states of both health and disease.¹²¹ Historically, undetectable levels of fungi in the lungs of healthy people paired with the low sensitivity of culture-based techniques allowed for the assumption that healthy lungs contain no fungi.⁶⁵ Next-generation sequencing technologies allow the opportunity to both identify unculturable species of fungi and provide a more comprehensive characterization of fungal communities. Elucidation of the mycobiome has historically been outside of the reach of scientific inquiry because culture-based methods do not allow for the identification of hundreds of different taxa and the characterization of composition and abundances due to the difficulty and different requirements of fungi to grow in culture as well.¹⁴³ Recent advances in technology have provided powerful high-throughput sequencing and bioinformatics tools that allow us to characterize and investigate the contribution of the human microbiome.¹⁴⁴ Compared to studies of the bacterial communities of the lung, investigations into the lung have been exceedingly few.^{70,121}

Fungi have been identified in the lung in healthy individuals and are altered in respiratory diseases including cystic fibrosis, bronchiectasis, asthma, lung transplant, chronic obstructive pulmonary disease, and fungal infection.^{39,70,81,121,246–249} The lung mycobiome may play an important role in the respiratory immune and inflammatory response and contribute to lung damage or the worsening of lung disease. The fungal cell wall contains pathogen associated

molecular patterns (PAMPs) that can activate the immune system and trigger inflammation through pattern recognition receptors (PRRs) on lymphocytes.²⁵⁰ The epithelium lining our airways are inhaled fungi's initial point of contact with the host and act as the frontline defense against fungal exposure. As such, the lung epithelium has evolved to initiate the earliest immune events to resist fungal penetration.^{41,80}

Using culture-dependent methods, an estimated 60% of ICU patients have fungal colonization of their lungs; however, there has been a paucity of research into the role of the lung mycobiome in critical illness and acute respiratory distress syndrome (ARDS).^{100,101,251} In critical illness, clinical care, such as sedatives, endotracheal intubation, and mechanical ventilation can decrease mucociliary clearance and cough reflex leading to an increase in the movement of microbes from the oral cavity to the lower respiratory tract and decreased ability to eliminate microbes from the lungs while damaged alveoli flooded with nutrient-rich edema provide an ideal environment for aberrant microbial proliferation.^{7,25,132,237} Three quarters (75%) of ICU patients are treated with antibiotics during their hospital stay which is known to cause severe damage to the microbiome through indiscriminate destruction of commensal microbes.¹³¹ This dysbiosis increases vulnerability to infection with opportunistic pathogens and may provide an opportunity for an aberrant proliferation of fungi in environmental niches left empty after bacterial death.²⁵² Additionally, the ICU environment, including room surfaces, medical devices, or even the hands of healthcare providers may harbor mycobiota that could colonize vulnerable patients.¹³²

In a 2012 study of pneumonia being treated in the ICU, Bousbia et al used NGS methods to characterize the respiratory microbiota in 185 samples from 130 ICU patients with and without pneumonia; however, not all patients were mechanically ventilated. Using the 18S rRNA from bronchial alveolar lavage (BAL) samples, they found fungi in 17% of bronchial alveolar lavage

samples from 22 different species with the majority of taxa identified representing *Candida*. In critically ill patients with pneumonia, *Candida spp.* were more frequently identified than in patients with pneumonia not critically ill and controls. Fungi from environmental sources were found more often in samples from patients with pneumonia compared to patients without pneumonia.¹⁰¹ This initial description of the fungal communities in the lungs of critically ill patients lacks descriptions of the ecological diversity of the lung mycobiome and the relative abundances of fungal species in the lung. Additionally, while no specific genetic marker will allow for the identification of every species using next-generation sequencing methods, the internal transcribed region (ITS) has been proposed as the standard barcode for fungal sequencing due to the reliable polymerase chain reaction (PCR) amplification success, power for species discrimination and ability to identify of a broad range of fungal species.²⁵³

Krause et al undertook a culture-independent investigation of *Candida* in the lungs of healthy controls (n=105) and critically ill mechanically ventilated patients (n=65) and utilized sequencing of the ITS region to characterize the lung mycobiome in critical illness. The relative abundance of *Candida* was significantly higher in ICU patients compared to healthy controls with a majority of ICU patients demonstrating *Candida* dominance of their lung microbiota, and samples from patients in the ICU had lower alpha diversity. *Candida* was found to be the most abundant genus in the mycobiome in critically ill patients with pneumonia being treated with antibiotics and comprised 73% of all fungal genera. The Krause study identified similar fungal genera in critically ill and control patients, but they utilized BAL in critically ill patients and endotracheal secretions in healthy controls which could have distorted these comparisons. This study provided a taxonomic description and characterizes the diversity and relative abundance of the lung mycobiome in critical illness; however, Krause et al conducted their analyses at the genus

level and do not provide data about the differential abundance of *Candida* species dominating the lung microbiota. While *Candida albicans* is the most commonly identified pathogenic member of the genus, there are approximately 20 other species of *Candida* that are known to be pathogenic in humans, and non-*albicans Candida* (NAC) species are responsible for between 35-55% of candidemias in ICU patients.^{254,255} Differences in immunopathogenesis have been shown between *Candida* species due in part to virulence factors associated with mannan in the fungal cell wall and speciation of *Candida* in the lung mycobiome may provide more insight into the role of *Candida* colonization in critical illness.²⁵⁶ Antifungal susceptibility varies between *Candida albicans* and different NACs as well.^{254,255} Significant differences in mortality attributed to *Candida albicans* and different NACs have been demonstrated and may account for the lack of association between *Candida* colonization and mortality at the genus level in Krause et al's study.^{251,254} Continued studies into the role of the lung mycobiome at the species level and in larger cohorts are needed to elucidate the role of the mycobiome in critical illness.

To characterize the diversity and composition of the lung mycobiome in critically ill patients, we performed a prospective, observational cohort study in critically ill patients receiving mechanical ventilation. In this manuscript, we describe key features of the lung mycobiome (fungal burden and diversity) and characterize the taxonomic composition at the species level in the intensive care unit.

2.3 Methods

This study utilizes the Acute Lung Injury Registry (ALIR) and Biospecimen Repository, an ongoing prospective observational cohort of critically ill patients at UPMC Presbyterian

Hospital, Pittsburgh Pennsylvania from April 2015– September 2019, we prospectively enrolled a convenience sample of consecutive, adult patients with acute respiratory failure, who were intubated and mechanically ventilated in the Medical or Cardiac Intensive Care Units (ICU) at UPMC. Participants were enrolled within 72 hours of initiation of mechanical ventilation. Patients were excluded based on the following criteria: i) patients with tracheostomy, due to frequent airway colonization with pathogens and its indication of a more chronic disease process, ii) expected survival <48hrs, and v) inability to obtain informed consent from a legally authorized representative. We collected ETA samples at study enrollment and abstracted clinical data from the electronic medical record. The study was approved by the University of Pittsburgh Institutional Review Board (protocol PRO10110387), and written informed consent was provided by all participants or their surrogates.

2.3.1 Clinical Sample Collection

Distal tracheal secretions were suctioned through a closed endotracheal tube suctioning system. In cases that suctioning did not return adequate (>5ml) amount of ETAs, we instilled 5cc of sterile saline through the tubing system and then repeated suctioning. We also collected the left-over saline not used for sample collection (~ 5cc) as a negative control to examine for procedural contamination of our samples. Samples were directly collected in sputum collection traps, labeled and frozen to -80°C as quickly as possible and stored at this temperature until sample processing.

2.3.2 Clinical Characteristics

For each patient, we collected prospective detailed data abstracted from the electronic medical record (EMR) that were used in downstream analyses. Demographic and medical history data were collected from the EMR on the day of enrollment.

2.3.3 Clinical Microbiology

We utilized clinical microbiologic results obtained within 48hrs of research biospecimens in order for the clinical microbiologic results to be of the same infectious process being studied by the next-generation sequencing. All clinical cultures were obtained at the discretion of the treating physicians and were not obtained specifically for research purposes. All specimen cultures were performed by the clinical microbiology laboratory at UPMC. Samples were used to inoculate agar plates: trypticase soy agar with 5% sheep blood, chocolate agar (for fastidious organisms), Columbia Naladixic Acid Agar (*CAN Agar for gram-positives*), and MacConkey (for gram-negatives). Samples are incubated for up to 24hrs and the following day, isolated colonies are identified with matrix-assisted laser desorption/ionization with time-of-flight mass spectroscopy (MALDI-TOF MS) (Bruker Biotyper) and susceptibility testing is performed with the MicroScan WalkAway platform (Beckman Coulter).

Respiratory samples (sputum, ETA, or bronchoalveolar lavage (BAL)) where pathogenic bacteria or fungi were isolated by the clinical laboratory were considered to be culture positive. When “normal respiratory flora” or no organism growth was reported for respiratory samples by the clinical laboratory, we considered samples to be culture negative. We considered blood cultures

as positive when the clinical laboratory reported the growth of organisms that were not deemed skin contaminants by the treating physicians.

2.3.4 DNA Isolation

We extracted microbial DNA directly from ETA samples using the Powersoil (MoBio) extraction kit following the manufacturer's instructions, as previously described.²⁵⁷ Due to the high viscosity of ETA samples, we pretreated them with Dithiothreitol (0.1% DTT in phosphate-buffered saline) in 1:1 dilution to dissolve the mucus and allow usability in DNA extraction columns.

2.3.5 Fungal Gene Amplification and Sequencing

We amplified extracted DNA by PCR using the method of Caporaso et al and the Q5 HS High-Fidelity polymerase (NEB) targeting the internal transcribed regions 1 and 2 of the ITS rRNA gene.^{253,258} We utilized reagent controls for each step of the process (DNA extraction and PCR amplification). We amplified four microliters per reaction of each sample with a single barcode in triplicate 25 microliter reactions. We utilized a 2-step nested PCR protocol. Initial cycle conditions were 98°C for 30 seconds, 15 cycles of 98°C for 10 seconds, 55°C for 30 seconds, and 72°C for 90 seconds followed by 72°C for 2 minutes. 5 microliters of the initial PCR reaction were added to the nested cycle and were processed using the following conditions: 98°C for 30 seconds, 15 cycles of 98°C for 10 seconds, 55°C for 30 seconds, and 72°C for 1 minute followed by 72°C for 2 minutes. We combined triplicates and purified with the AMPure XP beads (Beckman) at a 0.7:1 ratio (beads:DNA) to remove primer-dimers. We performed sample pooling on ice by

combining 30 microliters of each sample. We purified the sample pool with the MinElute PCR purification kit. The final sample pool underwent two more purifications – AMPure XP beads to 0.7:1 to remove all traces of primer dimers and a final cleanup using the Purelink PCR Purification Kit (Life Technologies). We quantitated the purified pool in triplicate on the Qubit fluorimeter prior to preparing for sequencing. The sequencing pool was prepared according to instructions by Illumina (San Diego, CA), with an added incubation at 95°C for 2 minutes immediately following the initial dilution to 20 picomolar.²⁵⁷ We then diluted the sequencing pool to a final concentration of 7 pM + 15% PhiX control. Amplicons were sequenced on the Illumina Miseq platform. We collected experimental negative control samples used to assess for possible contamination events from sample collection to DNA extraction and PCR amplification.

2.3.6 ITS Sequence Processing

ITS rRNA sequences from the pooled sequencing run were demultiplexed into individual sample/replicate fastq files. The variable-length reads were processed, trimmed and quality filtered with a quality control pipeline utilizing the DADA2 package in R.²⁵⁹ Paired sequences with forward and reverse reads passing the quality filtering and trimming steps were merged. Chimeras were removed and the Unite database will be utilized to classify reads into amplicon sequence variants (ASVs) using the naïve Bayesian classifier method.^{260,261} ASVs are inferred based on sequence data and can be defined at different levels of resolution (phylum, class, order, family, genus, and species). Results of ASV annotation were converted into per-sample taxonomic profiles represented as categorical counts in matrices of dimension (number of samples) x number of categories). The taxa table was filtered for low abundance taxa (relative abundance, <0.005%)

and singletons, and also for samples that generated fewer than 10 reads in the range of negative control samples.

2.3.7 Experimental Controls

Low biomass studies of the microbiome are susceptible to contamination by microbial DNA in the sample collection process and in reagents used in DNA extraction and amplification. Additionally, the mycobiome is orders of magnitude less abundant than bacteria in the microbiome and as such, studies of the mycobiome are vulnerable to contamination. Given the risk of contamination, we compared the fungal reads identified in clinical ETA samples that were distinct from negative experimental control samples to assess the amount of contamination impacting our sequencing results.

2.3.8 Diversity Measures

Diversity describes the variability among organisms in the same environment or sample, and it is further defined in terms of species richness and evenness as well as relative species abundance.²⁶² Species richness refers to the number of different species present in a sample and evenness compares the uniformity of the population size of each of the species present in a sample. To compare the diversity of the microbial composition of samples, a variety of bioinformatics tools have been developed. A simple measure of species richness is the number of observed species, measured as ASVs, in a sample. The Shannon and Simpson diversity indices are commonly used in microbiome diversity measurement based on ASVs that consider richness, evenness, and relative abundance of species. However, both diversity indices have specific biases. The Shannon

index emphasizes species richness, compared to the Simpson index which considers species evenness more than species richness in its measurement.²⁶³ The Shannon index increases as the number of species in the sample increases and as the distribution of individual species becomes more even.²⁶⁴⁻²⁶⁶ The Simpson index ranges from 0 to 1 and the index increases as the diversity decreases.^{263,267,268} The Berger-Parker index is used to describe the dominance of the most abundant species in a sample and is calculated as the fraction of total sampled individuals that is contributed by the most abundant species.^{269,270}

2.3.9 Statistical Methods

We performed ecological analyses of alpha diversity for this sample using the *vegan* and *phyloseq* packages in R. We calculated the Shannon diversity index, Simpson's diversity index, Berger-Parker Index, and the number of observed ASVs. We calculated descriptive statistics for the diversity measures by calculating the mean (standard deviation) and median (first and third quartile). All ASVs were included in diversity analysis. We characterized the taxonomic composition of samples by examining the relative abundance of specific taxa in each sample. Samples were normalized to the percent of total reads, and we restricted the analysis to ASVs that were present at greater than 1% of the sample population.

We utilized two-sided Wilcoxon rank sum tests for independent samples and Fisher's Exact tests to test for differences between ETA samples and experimental control samples. For continuous measures and for total and relative abundance of specific taxa, respectively P-values were adjusted for the false discovery rate to account for multiple comparisons. We calculated beta diversity metrics to identify differential clustering between ETA samples and experimental control samples by calculating a distance matrix utilizing the Manhattan distance which accounted for the

compositional nature of microbiome data, and comparisons were made using permutation analysis of variance (PERMANOVA at 1000 permutations) with a correction for the false discovery rate.

2.4 Results

2.4.1 Study Population

We analyzed data from 226 critically ill mechanically ventilated patients who were enrolled from ICUs at the UPMC Presbyterian Hospital between April 2015– September 2019.^{24,271} Demographic characteristics and medical of included patients are shown in Table 2.1. Clinical characteristics (Table 2.2), measures of severity of illness (Table 2.3) and clinical outcomes (Table 2.4) are provided as well.

2.4.2 Fungal microbiota of ETA samples from critically ill patients are distinct from those of sequencing controls

To assess the risk of contamination, we first determined whether a fungal signal could be detected in our clinical ETA samples that was distinct from negative experimental control samples. 120 control samples were used in our experimental pipeline to assess potential contamination. We included 19 negative experimental controls and 32 positive experimental controls from the DNA extraction procedure. 41 negative experimental controls and 28 positive experimental controls from the PCR amplification procedure were included in our sequencing (Figure 2.2). ETA samples from ICU patients had higher numbers of ITS reads compared to negative experimental control

samples ($p < 0.001$). ETA samples produced a median of 6846 reads (high-quality ITS sequences) whereas negative experimental controls from DNA extraction generated relatively few reads with a median of 1 read and negative experimental controls from PCR amplification generated a median of 4 sequencing reads (Table 2.2, Figure 2.3).

Rank abundance analysis showed differences in the relative abundance of taxa in negative experimental control samples and clinical samples (Figure 2.3). The dominant species in experimental control and ETA samples was *Candida albicans*, although the maximum number of sequencing reads assigned as *Candida albicans* in experimental controls was very low (median=0, maximum=31 read) and likely did not contribute to significantly to contamination as ETAs contained a much larger number of sequencing reads assigned as *Candida albicans* with a median of 1540 reads in ETA samples ($p < 0.001$)(Figure 2.3).

ETA samples were compositionally dissimilar to experimental control samples by Manhattan distances (PERMANOVA, $p = 0.01$) (Figure 2.2). Despite the relatively low fungal biomass and variability in the number of reads present in individual samples, we concluded that while the overall number of sequencing reads were low in ETA samples and were comprised of a single species, there was evidence of a distinct fungal signal in a subset our clinical ETA samples compared to our negative experimental control samples.

2.4.3 The lung mycobiome of critically ill patients has overall low microbial diversity

We observed overall low diversity of fungal communities in the lung of critically ill patients with a large number of samples being dominated by a single fungal species, with the mean number of observed ASVs of 1.92 (Figure 2.4A, Table 2.7) and mean Shannon Diversity index of 0.18 (Figure 2.4B, Table 2.7). We also observed high levels of evenness in the fungal communities

in ETA samples, in line with the high numbers of samples comprised of a single fungal species as indicated by an average Simpson's Diversity index of 0.79 (Figure 2.5A, Table 2.7) and Berger-Parker index of 0.92 (Figure 2.5B, Table 2.7).

Approximately half of the lung microbiome samples from the critically ill subjects (51.3%) were comprised of a single species and 96.4% of samples had a single species with greater than 50% relative abundance. Of those samples with a relative abundance of a single species greater than 50%, *Candida* was the predominant genus represented, with 58.4% samples dominated by *Candida albicans*, 12.3% of samples dominated by *Candida dubliniensis*, and 7.0% dominated by *Candida tropicalis* (Figure 2.6, Table 2.8).

Ninety-nine (99) different ASVs with greater than 0.005 relative abundance were identified in the ETA samples from critically ill patients (Figure 2.7). The most abundant species in the ETA samples were *Candida albicans*, *Candida dubliniensis*, *Candida tropicalis*, and *Candida parapsilosis* (Figure 2.8, Table 2.9) with the *Candida* genus comprising 88.8% relative abundance of all ETA samples (Table 2.11). The genera *Cladosporium* (2.8%), *Meyerozyma* (1.5%), *Pneumocystis* (1.4%), and *Saccharomyces* (3.5%) were also among the most abundant in ETA samples.

2.5 Discussion

We characterized the lung mycobiome in a large cohort of mechanically ventilated ICU patients. We found that the lung mycobiome was detectable and differed from that of environmental controls. Overall diversity was low as measured by alpha diversity. Although we identified a large number of different fungal species (99 unique taxa), the majority of ETA samples

were dominated by a single fungal taxon. In 51.3% of ETA samples, only one fungal species was identified after quality control filtering and over 96% of ETA samples were dominated by a single species comprising greater than 50% of the relative abundance. The majority of samples were dominated by *Candida* species. The 10 most abundant fungal species identified in ETA samples include primarily pathogenic fungi associated with infection in humans. The *Candida* genus represents the majority of taxa identified in ETA mycobiome samples with 88% relative abundance of fungi identified in ETA samples coming from the genus *Candida*. This *Candida* dominance is consistent with other culture-independent studies of the lung mycobiome in respiratory diseases including CF, lung transplant, and in critical illness.^{76,101,251,272–274}

Fungal infection and colonization represent a significant clinical challenge in critically ill patients. In a large multicenter cohort study of 1756 critically ill patients in 73 intensive care units (ICUs), Garnacho-Montero et al found that 60% of patients' lungs were colonized with fungi using culture-dependent methods.¹⁰⁰ In a second cohort of mechanically ventilated patients in the ICU, 75.3% of patients had *Candida* cultured from their respiratory secretions.²⁷⁵ Culture-dependent studies of *Candida* colonization of the lungs are significantly associated with an increase in length of hospital stay, increased severity of disease, a longer duration to liberation from the ventilator, and increased in-hospital mortality and mortality at 28 days in critically ill patients.^{276–280} Compared to healthy controls, mechanically ventilated patients with *Candida* colonization in their respiratory secretions had higher levels of procalcitonin, C-reactive protein (CRP) and interleukin-6 (IL-6) indicating increased activation of the innate immune system and inflammation that was also associated with longer ICU stay duration and higher mortality at 28 days.²⁸¹

Culture-independent studies of the respiratory mycobiome have been undertaken in only a few types of respiratory disease; however, these studies have demonstrated associations between

mycobiome alterations and respiratory disease. In studies using next-generation sequencing, alterations in the fungal communities of the lung have been associated with poor clinical outcomes in cystic fibrosis, asthma, lung transplant, and chronic obstructive pulmonary disease (COPD).^{56,72,92,249,282} Two previous studies have examined the lung mycobiome in critically ill patients. In 2012, Bousbia et al performed a study of the lung mycobiome in critically ill patients with pneumonia. *Candida* species were the most abundant fungi in the BAL samples, and there were significantly more *Candida albicans* and NACs compared to controls without pneumonia.¹⁰¹ Krause et al utilized BAL to study the lung mycobiome of 65 mechanically ventilated patients. Shannon Diversity indices were significantly lower than healthy controls, similar to our findings. *Candida* species accounted for approximately 65% of the relative abundance of the bronchial alveolar lavage samples and were increased in relative abundance compared to healthy controls.²⁵¹ Similarly, we found *Candida* species to be the most abundant in our ETA samples and found that 58% of our samples were dominated by *Candida albicans* and an additional 23% were dominated by NACs.

Candida albicans comprised about 65% of the relative abundance of all ETA samples in our study and is the most prevalent fungal species in the human microbiota.²⁸³ *Candida albicans* is a pathobiont that often asymptotically colonizes many different body sites of healthy individuals. However, in response to changes in host immunity, stress, microbiota alteration, and other factors, *Candida albicans* can become pathogenic and cause infections ranging from superficial mucosal infections to life-threatening candidiasis. Disseminated candidiasis is the fourth most common nosocomial infection and is associated with a mortality of 45-75%.^{284,285}

NACs comprised 23% of the relative abundance of our ETA samples. *Candida dubliniensis* accounted for about 15% of the relative abundance of the samples. *Candida dubliniensis* is a newly

identified species and was first reported in 1995. Although often mistaken for *Candida albicans* clinically, *Candida dubliniensis* is significantly less virulent than *Candida albicans*.^{286,287} Increasing reports of *Candida dubliniensis* causing candidemia and invasive fungal infection are sparking interest in the role of this newly discovered species as a pathobiont. In our ETA samples, we identified a relative abundance of 7.5% *Candida tropicalis*. *Candida tropicalis* is a pathobiont and is found in the mycobiomes of healthy individuals, but is responsible for both superficial and systemic infection, specifically in immunocompromised patients or those with antibiotic exposure. *Candida tropicalis* is considered one of the most virulent *Candida* species and is the second most common etiological agent of candidemia. *Candida parapsilosis* (2.6%) and *Candida metapsilosis* (0.5%) were also identified in our ETA samples. *Candida metapsilosis* is a newly identified species and is very similar to *Candida parapsilosis* both phenotypically and evolutionary and is considered to be a part of the *Candida parapsilosis* complex.²⁸⁸ The *Candida parapsilosis* complex causes an estimated 20-40% of all candidemias; however, among NACs, *Candida parapsilosis* has the lowest mortality rates and is seen primarily as a nosocomial or opportunistic infection.²⁵⁴ *Candida parapsilosis* infections have been on the rise with an increase of about 20% over the last 10 years.²⁵⁴ Additionally, 1.5% of the relative abundance of the ETA samples were comprised of *M. caribbica* which is very closely related to and phenotypically indistinguishable from a subset of *Candida* species. *M. caribbica* has been characterized as a “killer yeast” due to significant antimicrobial activity against bacteria and other fungi.^{289,290} In immunocompromised patients it can act as an opportunistic pathogen and is a frequent nosocomial yeast estimated to be responsible for more than 11% of all systemic candidiasis.^{291,292}

Interestingly, we identified *Pneumocystis jirovecii* in four of our clinical samples that were identified clinically. *Pneumocystis jirovecii* causes *Pneumocystis pneumonia* in

immunosuppressed patients, most notably patients with acquired immune deficiency syndrome (AIDS). We identified many fungi known to be primarily non-pathogenic in immunocompetent patients including *Saccharomyces cerevisiae* and species from the *Cladosporium* genera.

Our study has several limitations. These data came from a single-center study, and the generalizability of our results require external validation. However, utilizing one ICU at a single site allows an attempt to minimize issues related to the variability of the environmental microbiome contamination which can confound the results. This study utilizes samples collected at a single time-point and as such we cannot determine the temporality of mycobiome alteration with critical illness. Microbial DNA is widespread, not removed by sterilization techniques, and can even be found in laboratory reagents used in DNA extraction, amplification, and sequencing. While we were able to identify a clear fungal signal distinct from negative controls, sample contamination from the upper respiratory tract remains a significant concern. ETA samples are at risk of contamination by endotracheal tube biofilms, upper airway or oral microbiota, or environmental contaminants. The risk is similar for samples being sent for clinical culture; however, the high sensitivity of sequencing makes contamination problematic. We have a standardized collection protocol to minimize contamination and used controls from specimen collection tubes to identify microbial signal attributable to contamination during sample collection. We utilized standard reagents for microbial nucleic acid extractions, polymerase chain reactions, and library preparation and sequencing, according to the manufacturer's instructions. Due in part to both the low biomass of the lung and the low abundance of fungi in the microbiome, there is a risk that we were not able to amplify and sequence fungi that were present in ETA samples in very low abundance. This challenge faces all studies of the fungal communities of the lung.^{272,293} A face this challenge We utilized protocols optimized for fungal detection and performed amplification

in triplicate to maximize fungal detection. We ran positive controls (both single organism and mock communities or mixtures of microorganisms of known composition) from specimen collection through laboratory processing and data analysis to ensure that reagents and processes were performing within acceptable limits.

In summary, we found that lung fungal communities in mechanically-ventilated patients had very low diversity. Most ETAs were dominated by a single fungal species, and *Candida* species made up the majority of the fungi identified. Our findings prompt further investigation into correlations between mycobiome composition and diversity with clinical outcomes and ICU outcomes. As fungi are potent immunomodulators and their interaction with the host may result in dramatic inflammatory responses, understanding the biology of the mycobiome in the intensive care unit is clinically relevant. Examination of the correlations between biomarkers of inflammation and immune response with mycobiome features may expose an important relationship between the host immune system and the mycobiome in critical illness. Additionally, interactions between fungal and bacterial communities and their role in host immune response and clinical outcomes represent an intriguing line of inquiry to follow.

2.6 Tables

Table 2.1: Demographic Characteristics and Medical History

Demographics	N=226
Age, median [Q1, Q3], yrs	60.6 [45.0, 68.9]
Males, N (%)	121 (53.5)
BMI, median [Q1, Q3]	29.2 [25.1, 35.2]
Medical History	
Diabetes, N (%)	75 (33.2)
COPD, N (%)	57 (25.2)
Immunosuppression, N (%)	56 (24.8)
Chronic cardiac failure, N (%)	33 (14.6)
Chronic kidney disease, N (%)	48 (21.2)
Pulmonary Fibrosis, N (%)	18 (8.0)

Abbreviations: [Q1, Q3]the first and third quartile; BMI: body mass index; COPD: chronic obstructive pulmonary disease

Table 2.2: Clinical Characteristics and ARDS Status

Clinical Characteristics	N=226
Systemic Antibiotics, N (%)	195 (86.3)
Respiratory Culture Positive, N (%)	51 (22.6)
WBC, median [Q1, Q3], x 10 ⁹ per liter	11.9 [8.9, 17.1]
ARDS Status	
ARDS, N (%)	47 (20.8)
At Risk For ARDS, N (%)	135 (59.7)
Risk factors for ARDS	
Pneumonia, N (%)	84 (37.2)
Sepsis, N (%)	34 (15.0)
Aspiration, N (%)	34 (15.0)
LIPS score, median [Q1, Q3]	5.00 [4.00, 6.88]

Abbreviations: LIPS: lung injury prediction score; WBC: white blood cell count

Table 2.3: Severity of Illness

Severity of Illness	N=226
SOFA score, median [Q1, Q3]*	6.0 [4.0, 9.0]
PaO ₂ :FIO ₂ ratio, median [Q1, Q3], mmHg	174 [119, 221]
Plateau pressure, median [Q1, Q3], cm	19.0 [16.0, 25.0]
Shock, N (%)	91 (40.3)
Acute Kidney Injury, N (%)	141 (62.4)

Abbreviations: SOFA: sequential organ failure assessment; PaO₂: partial pressure of arterial oxygen; FiO₂: Fractional inhaled concentration of oxygen

* SOFA score calculation does not include the neurologic component of SOFA score because all patients were intubated and receiving sedative medications, impairing our ability to perform an assessment of the Glasgow Coma Scale in a consistent and reproducible fashion.

Table 2.4: Clinical Outcomes

Clinical Outcomes	N=226
Duration of mechanical ventilation, median [Q1, Q3], days	5.0 [3.0, 9.0]
ICU LOS, median [Q1, Q3], days	8.0 [5.0, 12.0]
VFD, median [Q1, Q3], days	21.0 [0.0, 24.0]
30 Day mortality, N (%)	58 (25.7)
90 Day mortality, N (%)	63 (27.9)

Abbreviations: ICU LOS: intensive care unit length of stay; VFD: ventilator-free days

Table 2.5: Total Number of Sequencing Reads by Sample Type

Sample Type	Total Fungal DNA Reads, Median [Q1, Q3]
ETA	6846 [626, 27213]
Extraction Negative	1 [0, 2]
Extraction Positive	3585 [74, 5000]
PCR Negative	4 [2, 12]
PCR Positive	4784 [763, 11706]

Table 2.6: Total Abundances of Species by Sample Type

Total Abundance	ETA N=226	Extraction Negative N=19	PCR Negative N=41
Candida albicans, median [min, max]	1540 [0, 204000]	0 [0, 2.00]	0 [0, 31.0]
Candida dubliniensis, median [min, max]	0 [0, 96800]	0 [0, 0]	0 [0, 10.0]
Candida metapsilosis, median [min, max]	0 [0, 15100]	0 [0, 0]	0 [0, 0]
Candida parapsilosis, median [min, max])	0 [0, 70800]	0 [0, 2.00]	0 [0, 0]
Candida tropicalis, median [min, max]	0 [0, 80800]	0 [0, 2.00]	0 [0, 2.00]
Cladosporium delicatulum, median [min, max]	0 [0, 7890]	0 [0, 0]	0 [0, 0]
Cladosporium sphaerospermum, median [min, max]	0 [0, 2040]	0 [0, 0]	0 [0, 0]
Meyerozyma caribbica, median [min, max]	0 [0, 6190]	0 [0, 0]	0 [0, 0]
Pneumocystis jirovecii, median [min, max]	0 [0, 11800]	0 [0, 0]	0 [0, 0]
Saccharomyces cerevisiae, median [min, max])	0 [0, 5510]	0 [0, 0]	0 [0, 0]

Table 2.7: Mycobiome Diversity of ETA Samples

Diversity Metrics, N=226	Median [Q1, Q3]	Mean (SD)
Shannon Diversity Index	0.00 [0.00, 0.26]	0.18 (0.33)
Simpson's Diversity Index	1.00 [0.54, 1.00]	0.79 (0.26)
Berger-Parker Index	1.00 [0.93, 1.00]	0.92 (0.15)
Number of Observed ASVs	1.00 [1.00, 2.00]	1.92 (1.61)

Table 2.8: Single Species Dominance of ETA Samples

Single Species Dominance	N=226
100% Dominance by a Single Species, N (%)	116 (51.3)
Single Species Relative Abundance Greater than 50%, N (%)	218 (96.4)
Relative Abundance Greater than 50%	
Candida albicans, N (%)	132 (58.4)
Candida dubliniensis, N (%)	28 (12.3)
Candida tropicalis, N (%)	16 (7.0)
Candida parapsilosis, N (%)	5 (2.2)
Cladosporium delicatulum, N (%)	4 (1.7)
Meyerozyma caribbica, N (%)	3 (1.3)
Aspergillus penicillioides, N (%)	1 (0.4)
Penicillium vanluykii, N (%)	1 (0.4)
Pneumocystis jirovecii, N (%)	3 (1.3)
Rhodotorula mucilaginosa, N (%)	1 (0.4)
Saccharomyces cerevisiae, N (%)	6 (2.6)
Schizophyllum commune, N (%)	1 (0.4)

Table 2.9: Relative Abundance by Species in ETA Samples

Relative Abundance	N=226
Candida albicans, (%)	64.9
Candida dubliniensis, (%)	15.1
Candida metapsilosis	0.5
Candida parapsilosis, (%)	2.6
Candida tropicalis, (%)	7.5
Cladosporium delicatulum, (%)	2.4
Cladosporium sphaerospermum, (%)	0.4
Meyerozyma caribbica, (%)	1.5
Pneumocystis jirovecii, (%)	1.4
Saccharomyces cerevisiae, (%)	3.5

Table 2.10: Total Abundances of Species by Sample Type

Total Abundance	ETA N=226	Extraction Negative N=19	PCR Negative N=41
Candida albicans, median [min, max]	1540 [0, 204000]	0 [0, 2.00]	0 [0, 31.0]
Candida dubliniensis, median [min, max]	0 [0, 96800]	0 [0, 0]	0 [0, 10.0]
Candida metapsilosis, median [min, max]	0 [0, 15100]	0 [0, 0]	0 [0, 0]
Candida parapsilosis, median [min, max])	0 [0, 70800]	0 [0, 2.00]	0 [0, 0]
Candida tropicalis, median [min, max]	0 [0, 80800]	0 [0, 2.00]	0 [0, 2.00]
Cladosporium delicatulum, median [min, max]	0 [0, 7890]	0 [0, 0]	0 [0, 0]
Cladosporium sphaerospermum, median [min, max]	0 [0, 2040]	0 [0, 0]	0 [0, 0]
Meyerozyma caribbica, median [min, max]	0 [0, 6190]	0 [0, 0]	0 [0, 0]
Pneumocystis jirovecii, median [min, max]	0 [0, 11800]	0 [0, 0]	0 [0, 0]
Saccharomyces cerevisiae, median [min, max])	0 [0, 5510]	0 [0, 0]	0 [0, 0]

Table 2.11: Relative Abundance by Genus in ETA Samples

Relative Abundance	N=226
Candida, (%)	88.0
Cladosporium, (%)	2.8
Meyerozyma, (%)	1.5
Pneumocystis, (%)	1.4
Saccharomyces, (%)	3.5

2.7 Figures

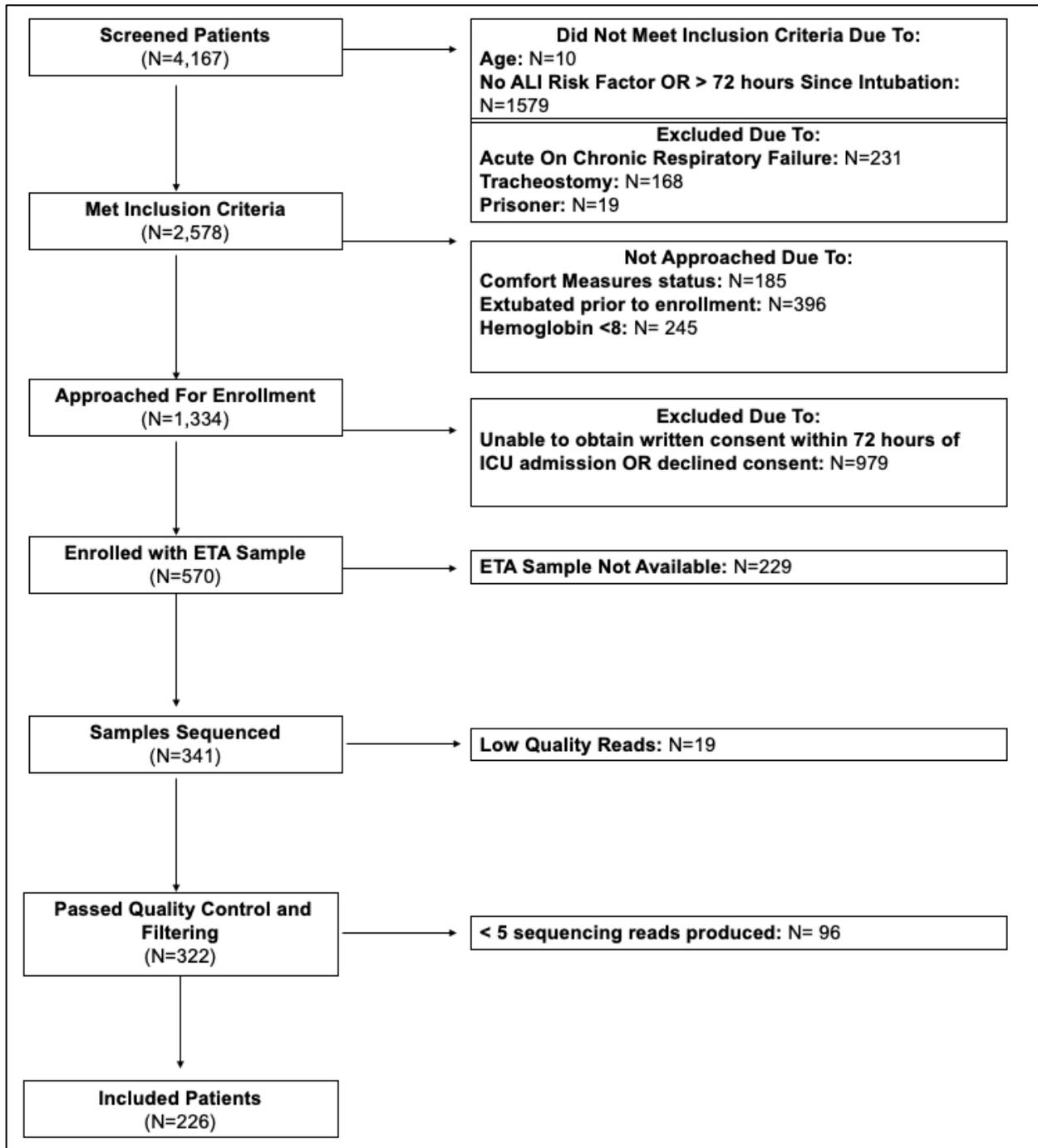


Figure 2.1: Flow Diagram of Subject Inclusion

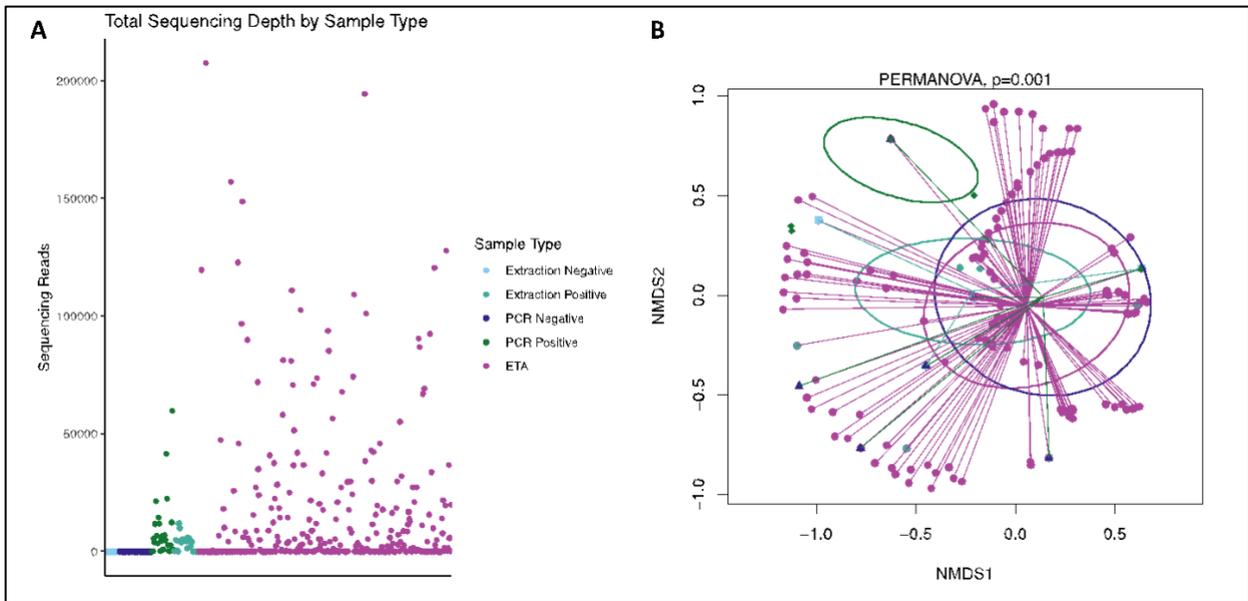


Figure 2.2: Differences in Experimental Control and ETA Samples

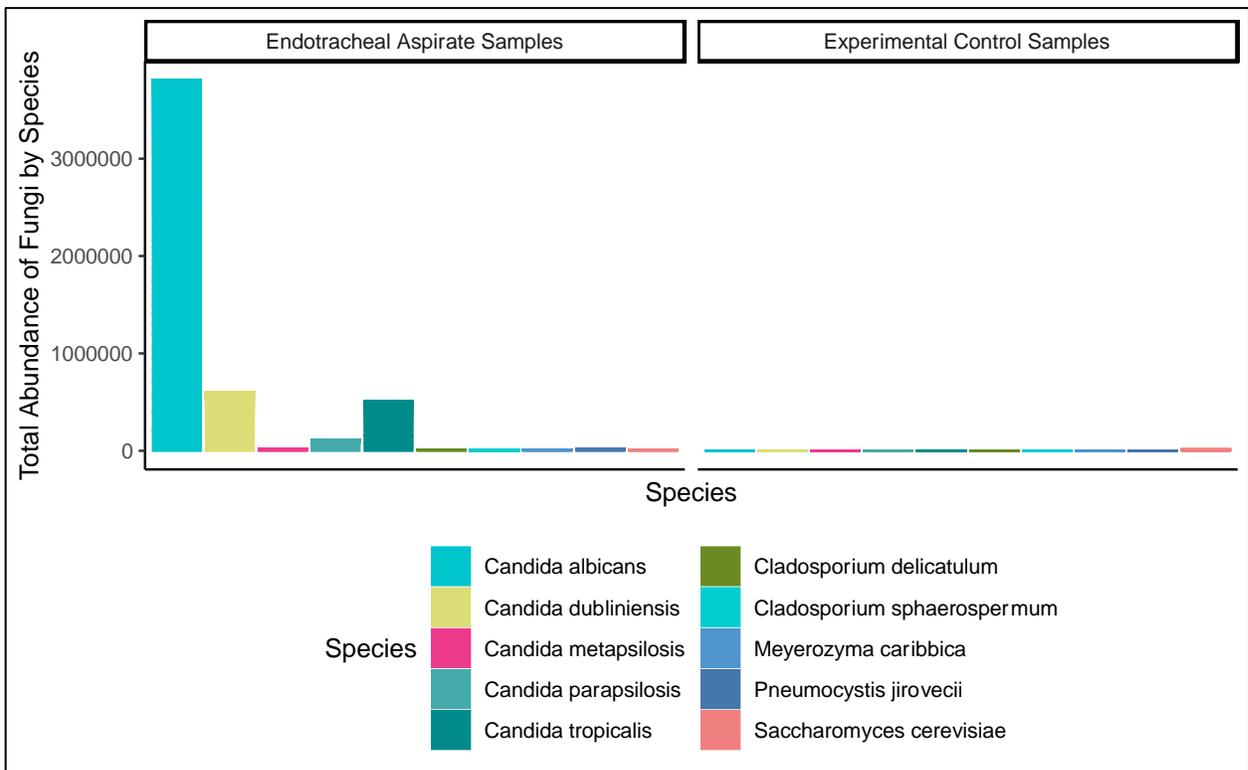


Figure 2.3: Total Abundances of Fungal Species by Sample Type

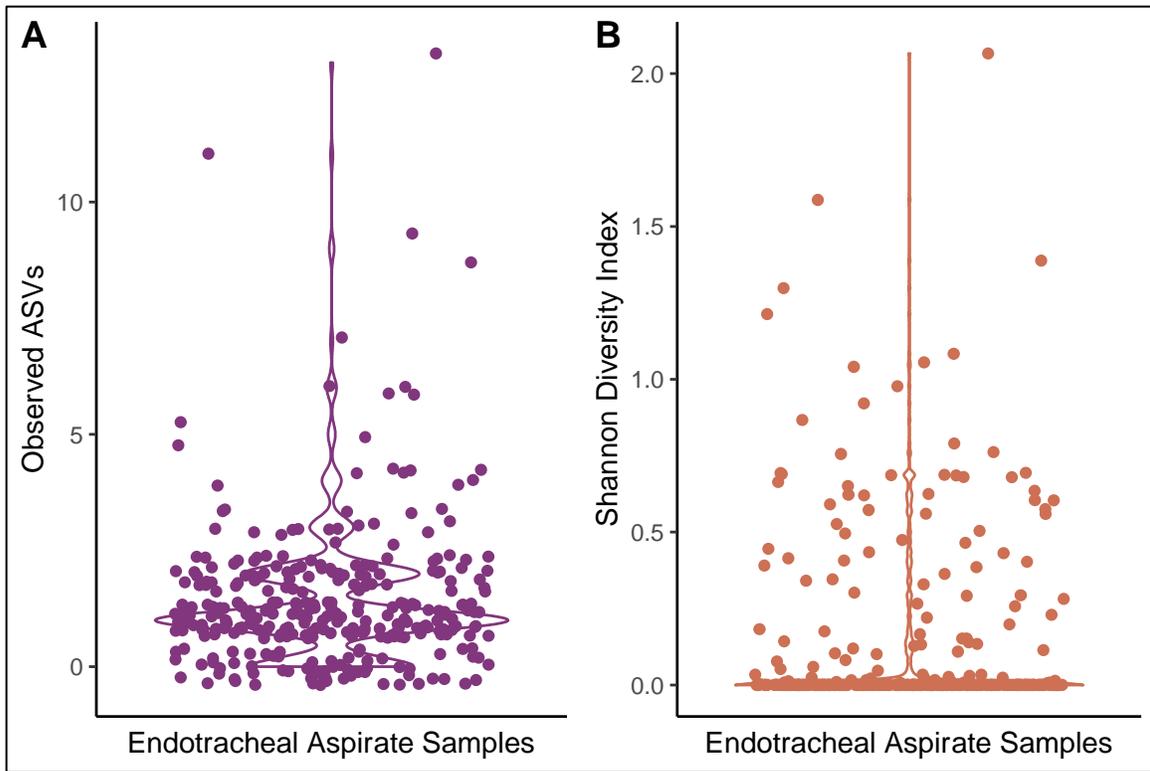


Figure 2.4: Mycobionne Richness in ETA Samples

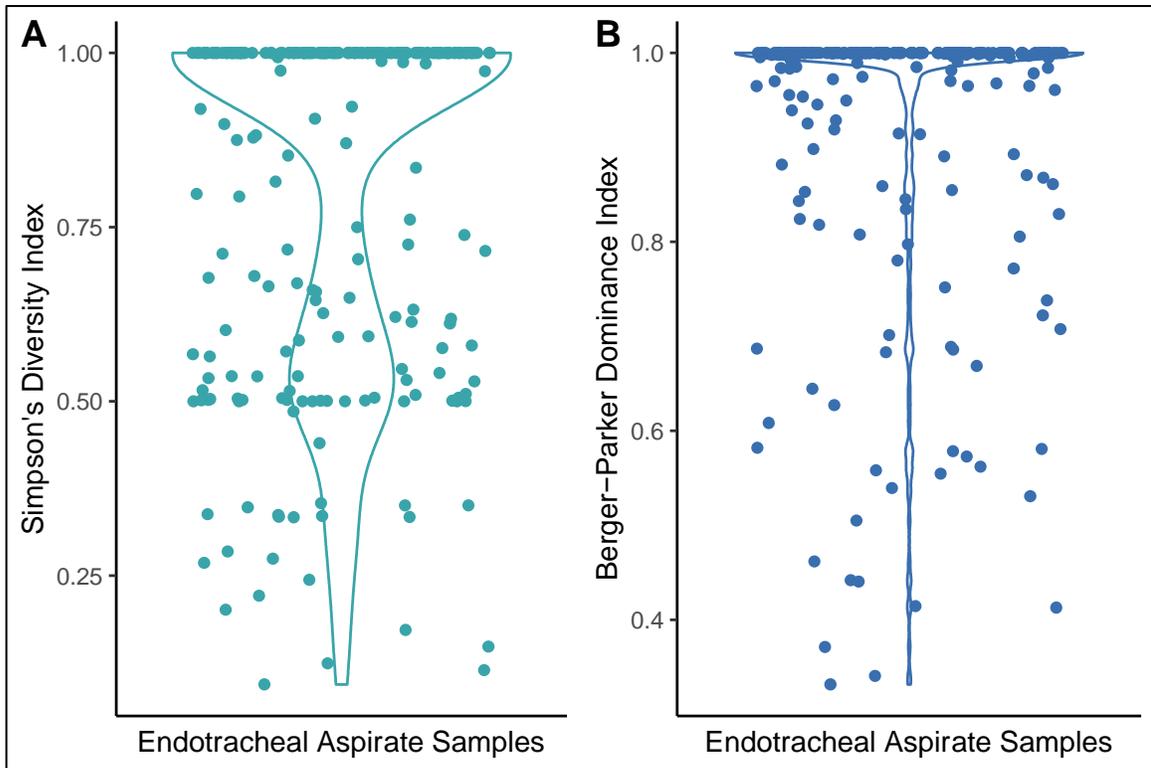


Figure 2.5: Mycobionne Evenness in ETA Samples

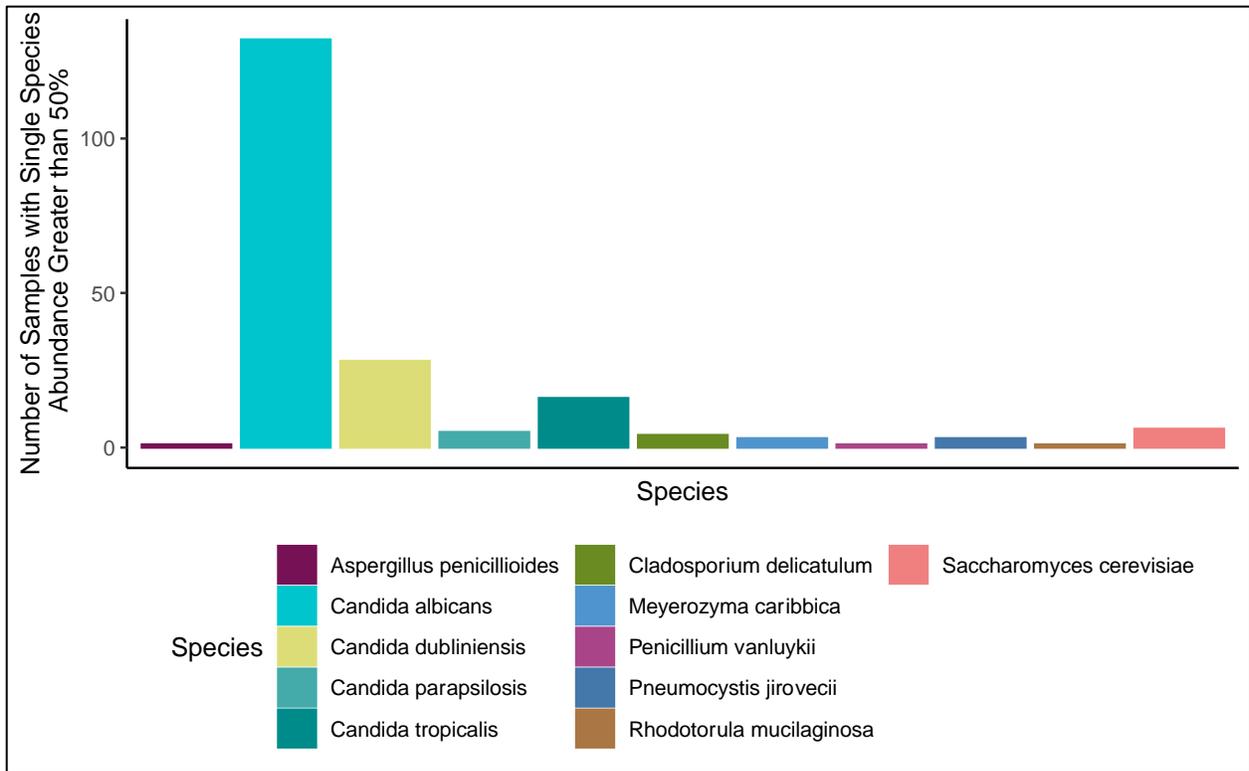


Figure 2.6: Samples with Single Species Abundance Greater than 50%

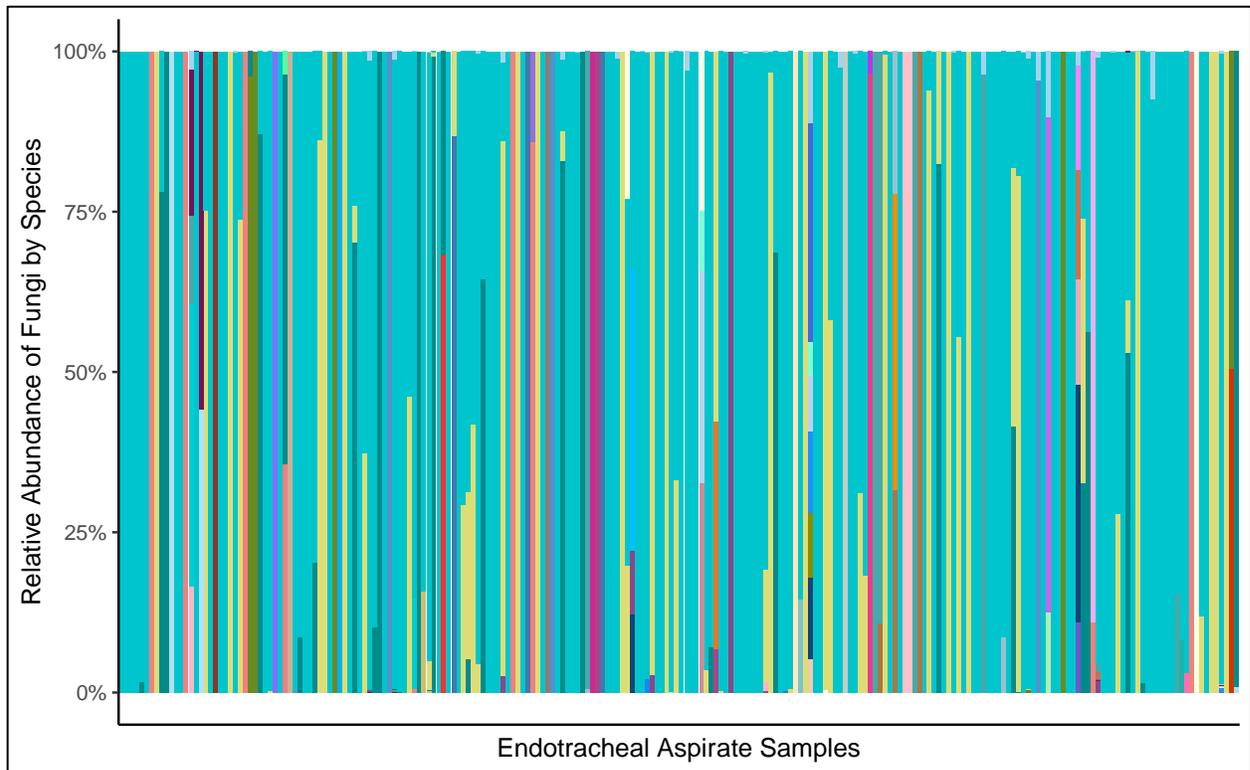


Figure 2.7: Relative Abundance of ETA Samples by Species

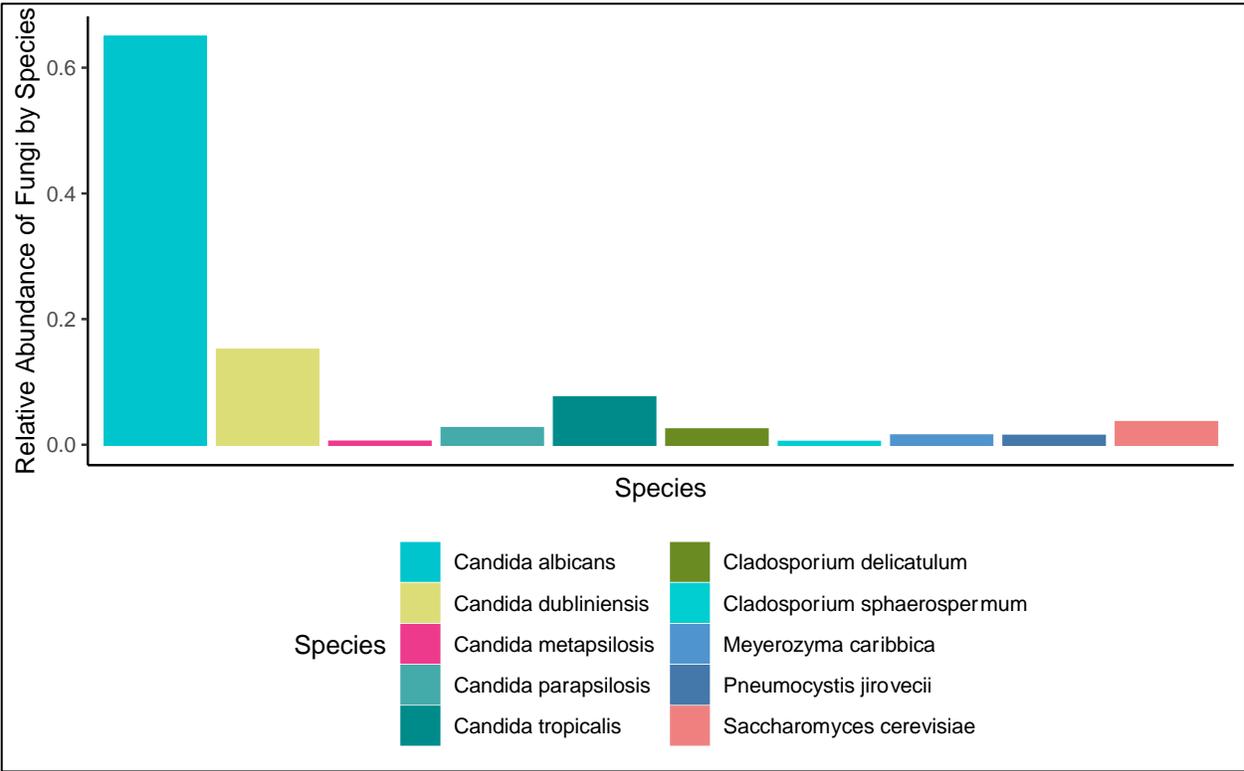


Figure 2.8: Relative Abundance by Species in ETA Samples

3.0 Manuscript 2: The Role of the Lung Mycobiome in Clinical Outcomes in Critical Illness and ARDS

3.1 Introduction

Background: In critically ill patients, variation in the host inflammatory responses has been associated with poor outcomes. However, the biological mechanisms underlying this heterogeneity have not yet been defined. We investigated whether variation in fungal communities (mycobiome) in the respiratory tract are associated with host inflammation and clinical outcomes.

Methods: We collected endotracheal aspirates (ETA) and plasma samples from 226 critically ill patients. We used extracted DNA from ETAs and performed fungal rRNA gene sequencing (internal transcribed spacer (ITS)) on the Illumina MiSeq platform to characterize the lower respiratory tract mycobiome. Statistical analyses included permutational multivariate analysis of variance (PERMANOVA), alpha (within-sample) diversity measures, and changes in taxonomic abundance. We classified patients into alpha diversity subgroups based on the Shannon Index with a Shannon index greater than 0 as high diversity and a Shannon Index of 0 as low diversity. We examined associations between these diversity metrics and clinical outcomes.

Results: Fungal communities from ETAs had very low alpha diversity with 51% of samples comprised of a single fungal species. Low alpha diversity was associated with a higher rate of ARDS diagnosis ($p=0.03$) and increased severity of disease as measured by a modified Sequential Organ Failure Score (SOFA) ($p=0.0003$). Critically ill patients in the low alpha diversity subgroup had higher levels of biomarkers and cytokines associated with inflammation and immune system activation (plasma IL-10 ($p=0.02$), TNFR1 ($p=0.02$) and ST-2 ($p=0.04$)) as well as host-response

to infection (pentraxin-3 (p=0.03), procalcitonin (p=0.02)) compared to patients in the high alpha diversity subgroup. Assignment to the lower diversity subgroup was associated with fewer ventilator-free days (adjusted p=0.04) and a longer time-to-liberation from mechanical ventilation (adjusted, p=0.05) compared to the high diversity subgroup. Higher Shannon Index values (as a discrete value) were associated with increased ventilator-free days (p=0.05), and increased survival (p=0.04).

Conclusion: Our hypothesis-generating findings suggest that the fungal communities of the lung may play a role in accentuated host inflammation and adverse clinical outcomes in critical illness.

3.2 Background

While there has been substantial progress in identifying risk factors for and contributors to the pathogenesis of the Acute Respiratory Distress Syndrome (ARDS), broadly efficacious treatments and pharmacological therapies for ARDS have yet to be discovered. The difficulties associated with identifying a treatment for ARDS are due in part to the biological heterogeneity present in ARDS.^{9,294} Recent research in ARDS has focused on developing an understanding of the biological heterogeneity and has revealed distinct subgroups of patients with or at risk for ARDS identified by a differential innate immune response and associated with differences in organ dysfunction, response to treatment, and clinical outcomes.^{16,22–24,154,295} The biological basis of these differential immune responses is not yet understood.

In the last ten years, technological improvements in culture-independent microbiology have allowed for the discovery that the lungs, which were previously thought to be sterile, play host to a unique and dynamic community of microbes, including bacteria, fungi, and viruses.^{66,70}

The vast majority of research into the lung microbiome has focused on bacteria. It has been demonstrated that bacteria are present in the lung in states of health, are altered in respiratory disease due in part to both anatomical and pathophysiologic changes caused by the disease process, are associated with variation in innate immunity, and are potential contributors to patient outcomes in both acute and chronic lung diseases.^{69,126,239,240,242–244,296} The bacterial component of lung microbiota of critically ill patients differs significantly from what has been observed in healthy subjects, and these alterations are correlated with an increase in both systemic and alveolar inflammation. Recent studies have hypothesized that in critically ill patients alveolar inflammation and injury may be perpetuated by perturbations of the bacterial communities in the lung and have successfully demonstrated that the diversity of bacterial communities in the lung are correlated with worse clinical outcomes in critical illness.^{25,237}

In contrast, very little research has focused on the role of the fungal component of the lung microbiota, or lung mycobiome. Despite the low relative abundance (0.1% of sequencing reads) of fungal DNA compared to bacterial DNA, the mycobiome is a fundamental part of the human microbiome.^{245,297} Lung mycobiota have been identified in health and are altered in both acute and chronic respiratory disease including chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), bronchiectasis, asthma, lung transplant, and fungal infection.^{39,70,81,121,246–248}

Similar to bacteria, fungi contain pathogen-associated molecular patterns (PAMP), which are recognizable by pattern recognition receptors that activate macrophages, B and T cells, thereby triggering inflammation. However, there have been few studies utilizing culture-independent techniques to examine the mycobiome in critical illness and the interaction of fungal PAMPs from the mycobiome with innate immune functions and subsequent inflammation in critical illness.^{251,298} In critical illness and acute respiratory failure, endotracheal intubation exposes the

lungs to the microbes from the upper respiratory tract and oral cavity while damaged alveoli flooded with nutrient-rich edema provide a rich environment for aberrant microbial reproduction and growth.^{7,25,132,237}

This study is motivated by the hypothesis that alteration of the lung mycobiota of patients with critical illness is associated with a systemic inflammatory response and adverse clinical outcomes. To determine if lung mycobiota at the time of intubation predict clinical outcomes in critically ill patients, we characterized key features of the lower respiratory tract mycobiome (diversity, community composition) in a cohort of critically ill mechanically ventilated patients and examined the association of features of the lung mycobiome with host inflammatory response and clinical outcomes in the ICU.

3.3 Methods

We have utilized the Acute Lung Injury Registry and Biospecimen Repository (ALIR), an ongoing prospective observational cohort of critically ill patients at the UPMC Presbyterian Hospital, Pittsburgh, Pennsylvania for this analysis. We enrolled a convenience sample of adult patients who were intubated and mechanically ventilated in the Medical or Cardiac Intensive Care Units (ICU) at UPMC from April 2015– September 2019. Participants were enrolled within 48 hours of being intubated. Exclusion criteria included: i) patients with tracheostomy, due to frequent airway colonization with pathogens, ii) expected survival <48hrs, and v) inability to obtain informed consent from a legally authorized representative. We collected ETA samples at the time of enrollment and utilized the electronic medical record to obtain clinical data. This study was

approved by the University of Pittsburgh Institutional Review Board (protocol PRO10110387), and written informed consent was provided by all participants or their surrogates.

3.3.1 Clinical Sample Collection

In a manner similar to the ETAs obtained for microbiologic culture studies, distal tracheal secretions were suctioned through a closed endotracheal tube suctioning system. We instilled 5cc of sterile saline through the tubing system and then repeated suctioning in cases that suctioning did not return an adequate (> 5 ml) amount of ETAs. We also retained the saline not used for sample collection (~ 5 cc) as a negative experimental control to identify contamination during sample collection. All samples were collected directly in sputum collection traps, labeled, and frozen to -80°C as soon as possible until sample processing.

At study enrollment, we also collected blood samples (10 cc) (through central venous or arterial access or phlebotomy) in sodium citrate anticoagulated. We performed same-day centrifugation and plasma collection in the research laboratory and stored samples at -80°C .

3.3.2 Clinical Microbiology

We utilized clinical microbiologic results obtained within 48hrs of research biospecimens in order for the clinical microbiologic results to be of the same infectious process being studied by the next-generation sequencing. All clinical cultures were obtained at the discretion of the treating physicians and were not obtained specifically for research purposes. All specimen cultures were performed by the clinical microbiology laboratory at UPMC. Samples were used to inoculate agar plates: trypticase soy agar with 5% sheep blood, chocolate agar (for fastidious organisms),

Columbia Naladixic Acid Agar (*CAN Agar for gram-positives*), and MacConkey (for gram-negatives). Samples are incubated for up to 24hrs and the following day, isolated colonies are identified with matrix-assisted laser desorption/ionization with time-of-flight mass spectroscopy (MALDI-TOF MS) (Bruker Biotyper) and susceptibility testing is performed with the MicroScan WalkAway platform (Beckman Coulter).

Respiratory samples (sputum, ETA, or bronchoalveolar lavage (BAL)) where pathogenic bacteria or fungi were isolated by the clinical laboratory were considered to be culture positive. When “normal respiratory flora” or no organism growth was reported for respiratory samples by the clinical laboratory, we considered samples to be culture negative. We considered blood cultures as positive when the clinical laboratory reported the growth of organisms that were not deemed skin contaminants by the treating physicians.

3.3.3 Mycobiome Experiments

We utilized the Powersoil (MoBio) extraction kit to isolate microbial DNA directly from ETA samples, as previously described.²⁵⁷ ETA samples are highly viscous and required pretreatment with Dithiothreitol (0.1% DTT in phosphate-buffered saline) in 1:1 dilution in order to dissolve the mucus to allow for the use of DNA extraction columns.

We amplified the microbial DNA by PCR using the method of Caporaso et al.²⁵⁸ and the Q5 HS High-Fidelity polymerase (NEB) regions 1 and 2 of the internal transcribed spacer (ITS) rRNA gene. Four microliters per reaction of each sample were amplified with a single barcode in triplicate 25 microliter reactions. To obtain an adequate number of amplicons, we employed a 2-step nested PCR protocol. The initial cycle conditions were 98°C for 30 seconds, 15 cycles of 98°C for 10 seconds, 55°C for 30 seconds, and 72°C for 90 seconds followed by 72°C for 2 minutes.

We utilized 5 microliters of the initial PCR reaction in the second cycle and were processed using the following conditions: 98°C for 30 seconds, 15 cycles of 98°C for 10 seconds, 55°C for 30 seconds, and 72°C for 1 minute followed by 72°C for 2 minutes. Amplified DNA was then purified using AMPure XP beads (Beckman) at a 0.7:1 ratio (beads:DNA). A pooled sample was created by combining 30 microliters of each sample and was further purified using the MinElute PCR purification kit. A second purification of the sample using AMPure XP beads and a final cleanup using the Purelink PCR Purification Kit (Life Technologies) were performed. The purified pool was quantitated in triplicate through high-resolution microfluidics-based automated electrophoresis using the 2100 Bioanalyzer instrument (Agilent).²⁹⁹ The sequencing pool was prepared according to instructions by Illumina with an additional incubation at 95°C for 2 minutes immediately following the initial dilution to 20 picomolar.²⁵⁷ The sequencing pool was then diluted to a final concentration of 7 pM + 15% PhiX control. We utilized the Illumina Miseq platform for sequencing of the amplicons. Reagent controls were included for DNA extraction and PCR amplification.

Sequencing reads were demultiplexed into individual sample/replicate fastq files. Reads were then processed, trimmed and quality filtered with a quality control pipeline utilizing DADA2.²⁵⁹ We merged paired sequences with forward and reverse reads passing the quality filtering and trimming steps. Chimeras were removed. The Unite database was used to classify reads into amplicon sequence variants (ASVs) using the naïve Bayesian classifier method.^{260,261} Results of ASV annotation were converted into per-sample taxonomic profiles in a taxa table. The taxa table was then filtered for singletons and low abundance taxa (relative abundance, <0.005%). Samples that generated fewer than 50 reads in the range of negative control samples were filtered from the analysis.

3.3.4 Host-Response Experiments

We measured validated biomarkers of ARDS in plasma and ETA samples using a custom Luminex multi-analyte panel (R&D Systems, Minneapolis, MI, United States) for our host-response experiments. We included biomarkers targeting the innate immune response (IL-6, IL-8, IL-10, Tumor necrosis factor receptor 1 (TNFR1), suppression of tumorigenicity-2 [ST-2], fractalkine), *epithelial injury* (receptor of advanced glycation end-products [RAGE]), *endothelial injury* (Angiopoietin-2) and *host-response to infection* (procalcitonin and pentraxin-3) to assess host response in relationship to microbial profiles.^{16,22,145} (1→3)-β-D-Glucan was quantified using the Fungitell assay (Associates of Cape Cod, Inc.) to assess the presence of fungal cell wall products present in the serum and ETA samples.^{146,147} The fungal cell-wall constituent (1,3)-β-d-glucan (BDG) represents a pathogen-associated molecular pattern (PAMP) that can stimulate innate immunity and is used as a clinical biomarker of fungal infection. We defined a positive BDG as greater or equal to the conventional threshold of 60 pg/ml.³⁰⁰ As a biomarker of intestinal permeability, we measured levels of fatty acid binding protein-2 (FABP-2) with the Quantikine® Human FABP2/I-FABP Immunoassay (R&D Systems, Minneapolis, USA).

3.3.5 Clinical Characteristics

We collected prospective detailed data abstracted from the electronic medical record (EMR) for each patient. On the day of enrollment, demographic and medical history data were collected from the EMR. The physiologically worst values from vital signs, laboratory results, mechanical ventilation parameters, and chest radiography within 24hrs from enrollment were recorded. We measured the physiologically worst sequential organ failure assessment (SOFA)

scores; however, we did not include the neurologic component of SOFA score because all patients were intubated and receiving sedative medications.³⁰¹ Lung Injury Prediction Scores (LIPS) were calculated from baseline variables.³⁰²

We recorded whether patients had received antibiotics in the 30 days prior to ICU admission, and then modeled the systemic antibiotic exposure in the ICU (before microbiome sampling) with a published antibiotic exposure score that took into account dosing duration, the timing of administration, and specific antibiotic type.³⁰³ Retrospective classifications of the etiology and severity of acute respiratory failure (sepsis, ARDS, pneumonia or aspiration, and intubation for airway protection without risk factors for ARDS per established criteria) were performed by a consensus committee of clinical experts for all enrolled patients after review of all available data.^{3,27,304,305} Classifications were performed without knowledge of microbiome sequencing or host biomarker data. We followed patients prospectively for cumulative mortality and ventilator-free days at 30 days as well as for time-to-liberation from mechanical ventilation and survival up to 30 days from intubation. Prospective clinical outcomes included survival at 30 days, duration of ICU stay, acute kidney injury³⁰⁶ incident shock (defined as the need for vasopressors), and ventilator-free days (VFD).³⁰⁷

3.3.6 Statistical Analysis

3.3.6.1 Alpha Diversity Subgroups

We derived alpha diversity metrics (Shannon Index) and based on the observed distribution of the Shannon Index, we classified patients into alpha diversity-based subgroups with a Shannon index greater than 0 classified as high diversity and a Shannon Index of 0 classified as low

diversity. We examined descriptive statistics of demographic and clinical characteristics, and biomarker values were log-transformed for analysis.

We sought to identify associations with the Shannon Index (linear regression model) and alpha diversity subgroups (logistic regression model) and biomarkers. To assess the independence of the association between diversity and biomarkers, we adjusted for potential clinical confounders that were differently distributed between alpha diversity subgroups in models for Shannon Index and alpha diversity subgroups. For biomarker analyses, regression models were adjusted for history of immunosuppression, administration of antibiotics prior to ICU admission, LIPS score, SOFA score, PaO₂:FiO₂ ratio, shock, and acute kidney injury.

We examined for associations with the Shannon Index clinical outcomes (ventilator-free days, ICU length of stay, survival at 30 days, and time-to-liberation from mechanical ventilation) using regression models and proportional hazards models. We examined for associations between the alpha diversity subgroups and with clinical outcomes (survival at 30 days, time-to-liberation from mechanical ventilation, ventilator-free days, and ICU length of stay) using proportional hazard and regression models. For time-to-event analyses (survival at 30 days and time-to-liberation from mechanical ventilation), time zero was defined as the time of intubation. For survival at 30 days and time-to-liberation from mechanical ventilation, Cox proportional hazards and Fine-Grey proportional hazards (treating death as a competing risk) models, respectively, for the independent effects of the Shannon Index and alpha diversity subgroups were adjusted for potential clinical confounders that were differently distributed between subgroups. For time-to-event analyses, proportional hazards models were adjusted for history of immunosuppression, administration of antibiotics prior to ICU admission, LIPS score, SOFA score, PaO₂:FiO₂ ratio,

shock, and acute kidney injury. Regression models for Shannon Index and alpha diversity subgroup were adjusted for the same variables.

3.3.6.2 Predictor/Responder Analysis

Following alpha diversity subgroup-level analyses, we then pursued species-level analyses to delineate the contributions of individual species in outcome prediction. We statistically quantified and analyzed microbiota profiles using 3 distinct methods: distribution-based methods using the tail statistic and Shannon index, abundance-based methods using the additive-log ratio transformation, and distance-based methods using the Manhattan distance metric to identify inter-sample difference.

We divided clinical variables of interest into covariates and “grouped” variables. We designated clinical variables as covariates if they were less plausibly affected by the mycobiota. These included age, gender, and administration of antibiotics before sample collection. We considered variables to be “grouped” if they shared related measurements or related phenotypes and there was a possibility of correlation or collinearity between them and if it was more plausible that they affected or responded to the mycobiota. We included shock, extra-pulmonary sepsis, LIPS score, pneumonia, PaO₂:FiO₂ ratio, history of COPD, ARDS status, acute kidney injury, and clinical culture positive for fungus. We fit linear models and performed a predictor-responder analysis to determine whether grouped variables were stronger predictors of or responders to the mycobiota. Covariates were always included as predictors and were never included as potential responders to the mycobiota, where grouped variables were treated as either multivariate responders (Y) or multiple regression predictors (X) depending on the model being fit. Mycobiota features (taxonomic abundances or diversity metrics) were placed on the opposite side of the regression equation relative to the grouped variable to allow us to determine the grouped variables

association with the mycobiota. This results in two different models being fit: $Mycobiota = Covariates + Grouped Variables$; where the mycobiota are acting as the responders and $Grouped Variables = Covariates + Mycobiota$; where the mycobiota is the predictor. Depending on the method being used (abundance-based or distance-based), the quantitative representation of the mycobiota differed. After both the predictor and responder models have been fit, the resulting estimated coefficient p-values are compared to establish whether a variable was a stronger predictor of a fungal species or diversity metric or was a stronger respondent to a fungal species as a predictor of a variable.

3.3.6.3 Distance-based Approach

In our distance-based approach, we utilized three different methods to assess the inter-sample distances. We visualized distances using ordination performed with multidimensional scaling. Firstly, we performed Permutational Multivariate Analysis of Variance (PERMANOVA) tests of the Manhattan distances using the Adonis function from the vegan package in R to identify factors associated with distancing between samples.^{308–310} The second method we applied was the application of hierarchical cluster analysis with multinomial logistic regression (HCAMLR). Using the Manhattan distances, we computed hierarchical clusters using Ward's minimum variance method. From the initial root node, at each division lower in the hierarchical cluster tree (sequentially increasing the cluster size by 1) we fit log-linear models for the probability that an individual was in a particular cluster and the model iteration with the smallest p-value for that predictor was considered to be the optimal cluster cutoff for that variable. We also determined an overall optimal cluster stopping level for the entire hierarchical cluster tree using a distance-based pseudo-F statistic using the Calinski-Harabasz criteria. Finally, we sought to identify cluster influencers, or the pairwise comparisons between clusters that identify taxonomic differences that

explain the separation between clusters. We identified clusters using Ward's minimum variance method with the Manhattan distance or by using categorical variables to define clusters within the data. We used analysis of variance (ANOVA) models to quantify the amount of variation a particular species was contributing to the intra- and inter-cluster membership. The \log_{10} ratio of the R^2 for a model without a particular species compared to the model with that same particular species was used to determine which species are influential in separating the pairwise clusters; a more negative $R^2\log_{10}$ ratio indicates greater separation.

3.3.6.4 Abundance-based Approach

In order to undertake our abundance-based approach, we first performed additive logarithm ratio (ALR) transformations of relative abundance on the top 20 most abundant species in our samples. The ALR transformation is required to mitigate spurious correlations among species that occur due to the compositional nature of microbiome data and to ensure that each species' abundance was normally distributed and made more independent. When group variables were modeled as responses, the covariates and the mycobiota ALR values were the predictors and when the group variables were modeled as predictors, the covariates and the mycobiota ALR values acted as the responders. We used univariate predictor/responder analyses to compare the relative significance and direction of the associations between individual fungal species and each group variable individually.

3.3.6.5 Distribution-based Approach

In our distribution-based method, we calculated alpha diversity indices using the vegan package in R.^{309,310} The Tail index was calculated as previously described.³¹¹ Using the lm and boxcox function from the standard stats and MASS packages in R, we performed diversity

regression.^{309,312} When treating alpha diversity metrics as a response, the Box-Cox transformation was applied to each of the alpha diversity indices to adjust for the non-normal distribution of the residuals from the fitted linear models. Using the predictor/response linear models described above, we determined whether each alpha diversity index was a stronger predictor or responder of the grouped clinical variables.

3.4 Results

3.4.1 Cohort Description

ETA samples from 226 mechanically ventilated patients (median age 60.6 years, 54% male) were included in this analysis (Figure 2.1, Table 3.1). Of the 226 participants, 47 (21%) were diagnosed with ARDS, 135 (60%) were considered at-risk for developing ARDS and 195 (86%) were receiving systemic antibiotics at the time of sample collection in the ICU (Table 3.2). Additional clinical characteristics of the cohort are reported in Table 3.3 and Table 3.4. An additional 120 control samples were used in our experimental pipeline to assess potential contamination. ETA samples from ICU patients had higher numbers of ITS reads compared to experimental control samples ($p < 0.001$) (Figure 2.2).

3.4.2 Alpha Diversity

Overall, the alpha diversity in this cohort was low and ranged from a Shannon Index of 0 (representing effectively mono-fungal communities) to 2.06 (in the range of a more typical

microbiome). 116 samples had a Shannon Index of zero and classified as having low diversity, and 110 samples had a Shannon Index of greater than zero and were classified as having high diversity. Patients with a diagnosis of ARDS had lower diversity than patients without ARDS ($p=0.03$). Among those with ARDS, the Shannon Index ranged from zero to 0.92, and 30 had a Shannon Index of zero and were classified as having low diversity and 17 samples had a Shannon Index of greater than zero and were classified as having high diversity.

3.4.2.1 Alpha Diversity Subgroups

More patients in the low alpha diversity subgroup had exposure to systemic antibiotics before study enrollment (93.1% vs 79.1%, $p=0.03$) and immunosuppression (31.9% vs 17.3%, $p=0.02$) compared to patients in the higher alpha diversity subgroup (Table 3.2). Patients in the low alpha diversity subgroup had higher LIPS scores (median 5.50 vs 5.0, $p=0.003$), SOFA scores (median 7.0 vs 6.0, $p=0.01$) and lower PaO₂:FiO₂ ratios (median 164 vs 205, $p=0.02$) and experienced shock (48.3% vs 31.8%, $p=0.02$) and acute kidney injury (70.7% vs 53.6%, $p=0.01$) at higher proportions than patients in the higher alpha diversity subgroup (Table 3.3). We did not identify significant differences in the taxonomic composition between the low diversity and the high diversity samples based on the beta diversity metrics. Using an NMDS plot to visualize variation among individual samples, many samples were clustered closely together indicating little variation in the taxonomic composition while other samples were distributed widely over the space of the NMDS plots indicating greater variation in mycobiome composition. (Figure 3.3) Patients in the high diversity strata had a significantly higher relative abundance of *Candida tropicalis* ($p=0.001$) and *Candida dubliniensis* ($p=0.01$) in their lung mycobiomes as compared to patients in the low diversity strata (**Error! Reference source not found.**).

After adjusting for potential confounders, classification to the lower diversity subgroup was associated with an increased length of ICU stay ($p=0.01$) and decreased survival at both 30 ($p=0.03$) and 90 ($p=0.02$) days (Table 3.2, Table 3.5). Classification to the lower diversity subgroup was associated with fewer ventilator-free days ($p=0.04$) and a longer time-to-liberation from mechanical ventilation ($p=0.01$) (Table 3.2, Table 3.5).

After adjustment for confounders, we identified that patients in the low alpha diversity subgroup had elevated levels of IL-10 ($p=0.006$), TNFR-1 ($p=0.01$), pentraxin-3 ($p=0.02$), ST-2 ($p=0.02$) and procalcitonin ($p=0.02$). Plasma BDG levels were elevated in patients assigned to the low alpha diversity subgroup ($p=0.05$) and compared to patients in the high alpha diversity group, patients in the low alpha diversity subgroup had a 2.71 (95% CI: 1.22, 6.36) times greater odds of having a 1,3)- β -d-glucan level greater than 60 pg/ml ($p=0.02$) (Table 3.7).

3.4.3 Distance-based Analyses

3.4.3.1 PERMANOVA

Using PERMANOVA, we identified associations with group variables as shown in In our ETA samples, we found significant associations with bacteremia ($R^2=0.01$, $p=0.05$) and pneumonia ($R^2=0.02$, $p=0.05$) (Appendix Table 4).

3.4.3.2 HCAML

Results of the HCAML for each of the covariates and group variables were determined. Appendix Table 5 shows the associations at the Calinski-Harabasz optimal cutoff for each variable grouping. Overall, more than half of the models suggested 8 clusters as an optimal cutoff; however,

aspiration and antibiotic analyses both suggested 3 clusters, and COPD and shock analyses both suggested 4 clusters. The variables associated with clustering tended to be associated with infection (bacteremia, pneumonia, and fungal culture). Clustering into 8 groups does result in clusters that are very diverse in size and includes some clusters that include very few samples.

3.4.3.3 Cluster influencers

Cluster influencing species for cluster membership at an 8-cluster cutoff are displayed in Appendix Figure 5. Overall, each taxon had one or two species that unified each cluster. *Candida albicans* was the unifying species in Cluster 1, Cluster 2, and marginally in Cluster 3. *Candida dubliniensis* was the unifying species in Cluster 2 and Cluster 3. *Candida spp.*, *Saccharomyces cerevisiae*, and *Pneumocystis jirovecii* were the primary taxa influencing the division in clusters.

Clustering within covariates and grouped variables is described in Appendix Table 6. We identified clusters between patients who had been administered antibiotics and immunosuppression, as well as those with infection (bacteremia, pneumonia, sepsis, positive clinical culture) and severity of disease (shock, LIPS score, ARDS status, PaO₂:FiO₂ ratio, acute kidney injury).

3.4.4 Distribution-based Analyses

Evenness was negatively correlated with pneumonia as a predictor (coefficient= -0.25, p=0.04). As a predictor, tail was negatively correlated with shock (coefficient =-0.21 , p=0.03). Shannon index was negatively correlated with shock (coefficient =-0.26 , p=0.04) and immunosuppression (coefficient =-0.20 , p=0.05) as a predictor (Appendix Figure 6, Appendix Figure 7).

As respondents, tail (coefficient =2.52 , p<0.001), the Shannon index (coefficient =3.78 , p<0.001), the Simpson index (coefficient =4.54 , p<0.001) and evenness (coefficient =3.67 , p<0.001) were all correlated positively with antibiotic administration (Appendix Figure 8, Appendix Figure 9) Results of the predictor/responder analysis found that a decrease in diversity, as measured by the Shannon and Simpson index as well as the Tail statistic and Evenness, predicted ARDS status (Appendix Figure 10).

3.4.5 Abundance-based Analyses

3.4.5.1 Species Abundance as Responders

Significant (p<0.05) correlations between particular species abundance and covariate and group variables as predictors are visualized in Appendix Figure 12. When considering the covariate and group variables as predictors, we identified that antibiotic exposure was correlated with an increase in the abundance of both *Candida albicans* (coefficient: 2.20) and *Candida dubliniensis* (coefficient: 1.97). A significant increase in *Candida albicans* abundance was also predicted by a diagnosis of sepsis (coefficient: 3.37). A decreased abundance of *Candida dubliniensis* was predicted by bacteremia (coefficient: -2.86) and pneumonia (coefficient:-1.69). Pneumonia also predicted a decrease in the abundance of *Saccharomyces cerevisiae* (coefficient: -0.76) and *Verticillium dahliae* (coefficient: -0.52). A significant increase in the abundance of *Verticillium dahliae* (coefficient: 1.26) was predicted by ARDS and ARDS also predicted an increase in the abundance of *Pneumocystis jirovecii* (coefficient: 1.77), *Cladosporium delicatulum* (coefficient: 1.01), *Hyphodermella rosae* (coefficient: 1.21), and *Kabatiella lini* (coefficient: 1.14). A history of aspiration predicted an increase in the abundances of *Candida parapsilosis* (coefficient: 1.03), *Verticillium dahliae* (coefficient: 0.54), and *Mycoacia fuscoatra* (coefficient:

0.52). LIPS score predicted a decrease in the abundance of *Cladosporium delicatulum* (coefficient: -0.90).

3.4.5.2 Species Abundance as Predictors

We visualized significant ($p < 0.05$) correlations between the abundance of particular species and grouped variables as respondents in Appendix Figure 11. A decrease in the relative abundance of *Candida dubliniensis* predicted bacteremia (coefficient= -0.02) and acute kidney injury (coefficient=-0.02). Acute kidney injury was also predicted by a decrease in *Candida tropicalis* (coefficient=-0.03). Sepsis was predicted by an increase in the abundance of both *Aspergillus penicillioides* (coefficient=0.09) and *Valsa pini* (coefficient=0.15). An increase in the abundance of *Aspergillus penicillioides* also predicted both bacteremia (coefficient: 0.09) and shock (coefficient: 0.11). PaO₂:FiO₂ ratio was predicted by a decrease in the abundance of *Rigidoporus pouzarii* (coefficient: -0.15). We found that a clinical culture positive for fungus was correlated with an increase in both *Candida metapsilosis* (coefficient: 0.10) and *Verticillium dahliae* (coefficient: 0.14).

3.4.5.3 Predictor/Responder Analyses

Results of the predictor/responder analyses in abundance found that ARDS was a reliable predictor of an increase in the abundance of *Hyphodermella rosae*, *Kabatiella lini*, *Pneumocystis jirovecii*, *Cladosporium delicatulum*, and *Verticillium dahliae*. We also identified that the abundance of *Candida metapsilosis* and *Verticillium dahliae* were more likely to predict a clinical culture positive for fungus. Inverse associations (a decrease in abundance) were identified between *Cladosporium delicatulum* as a responder to LIPS score and *Rigidoporus pouzarii* as a predictor of PaO₂:FiO₂ ratio (Appendix Figure 3).

3.5 Discussion

In a large cohort of mechanically ventilated ICU patients, we demonstrated that variation in the lung mycobiome within 72 hours of intubation is associated with systemic inflammatory responses and adverse clinical outcomes. Using culture-independent ITS rRNA gene sequencing in non-invasive samples from critically ill patients we identified that alpha diversity, as measured by the Shannon Index, was predictive of ARDS, severity of illness, length of ICU stay, and time to liberation from mechanical ventilation. ARDS status was predictive of both alpha diversity in ETA samples as well as a reliable predictor of an increase in specific fungal species. Through our investigation of the mycobiome at different levels (clusters, diversity, and relative abundance) and by considering the mycobiome as both a predictor and response of clinical features, we can start to understand which clinical features may be influenced by or influencing the mycobiome in critical illness. We identified consistent cluster-influencing fungal species associated with antibiotic and immunosuppression use as well as infection and with clinical correlates of severity of disease. At an abundance level, we identified significant increases in *Candida spp.* predicted by the use of antibiotics which likely contributed to the very low diversity and the high number of mono-fungal mycobiomes we observed through sequencing.

While the role of fungal colonization of the lungs in ICU patients has been estimated to be nearly 60% using culture-dependent methods, there has been a paucity of research into the lung mycobiome in critical illness using next-generation sequencing techniques.¹⁰⁰ The very little investigation into the role of the lung mycobiome in critical illness that has been undertaken has focused primarily on the description of the fungi present and the correlation of next-generation sequencing results and clinical cultures.^{101,251}

To our knowledge, our study is the first to demonstrate that variation in the lung mycobiome is predictive of clinical outcomes in critically ill patients and patients with ARDS. This core finding is in line with studies of the lung mycobiome in cystic fibrosis, bronchiectasis, asthma, and chronic obstructive pulmonary disease (COPD) that have found associations with clinical outcomes and variation in the lung mycobiome.^{56,89,90,93,282,313} Our results correspond with studies examining the bacterial component of the lung microbiome in respiratory diseases including critical illness, idiopathic pulmonary fibrosis, bronchiectasis, COPD, and cystic fibrosis which have found associations with alterations in the bacterial communities of the respiratory tract and clinical outcomes.³¹⁴⁻³²³ Additionally, our findings are complementary to recent studies of the bacterial component of the lung microbiome in critical illness that have reported that variation in the bacterial communities of the lungs, including low alpha diversity, have significantly worse clinical outcomes and provides new insights into the role of the broader lung microbiota in critical illness.^{25,237} Epidemiologic evidence supports the role of fungi in critical illness outside the context of conventionally defined invasive fungal infection. In critical illness, colonization of the airways with *Candida* has been associated with antibiotic use, which puts selective pressure on bacteria and allows for the proliferation of fungi in unoccupied environmental niches, as well as immunosuppression and poorer clinical outcomes.²⁷⁹

Our findings underline the lung mycobiome as a previously unexplored but potentially important contributor to heterogeneity between patients in critical illness. Efforts to better understand the heterogeneity in ARDS and sepsis have focused on clinical variables and plasma biomarkers have identified that there are sub-phenotypes of critically ill patients with differential response to treatment and clinical outcomes.^{16,24,295,324} The identification of clinical sub-phenotypes has focused research efforts on the biological determinants of increased host

inflammation.³²⁵ Our approach identified significant associations between low diversity and an increase in biomarkers associated with host inflammation in critical illness and ARDS. Thus, we hypothesize that some of the observed variation in host response and clinical features in critical illness may be accounted for by mechanisms of fungal PAMPs from the lung mycobiome interacting with the innate immune cells. Our use of samples collected at a single time-point and does not allow us to establish the directionality of the effects of the interaction between the host and the mycobiota and as such there is a possibility that inflammatory milieu in the alveolar space may lead to a secondary proliferation of fungi. There is support for both biological and temporal plausibility for the role of fungi stimulation host-response biomarkers. ETA samples were collected within 72 hours of intubation in our study and as such, respiratory fungi likely represent the primary insult for lung injury. In our analyses, we identified that patients with low diversity of fungal communities in the lung were twice as likely to have elevated levels of plasma BDG, which as a constituent of the cell wall of many fungi, is a PAMP recognized by complement receptor 3 and dectin-1 on the surface of dendritic cells, neutrophils, macrophages, and epithelial cells.^{326–328} When simultaneously stimulated with dectin-1, Toll-like receptors increase cytokine production through NF- κ B dependent signaling.^{327,329} While necessary for recognition and clearance of pathogenic fungi, a perpetual and aberrantly stimulated innate response could lead to increased mucosal inflammation and injury. Conversely, PAMPs arising from heat-inactivated *Candida* have been shown to reduce the activity of macrophages and to augment cytokine production, subsequently impairing first-line defenses of mucosal immunity.³³⁰ Many of the biomarkers associated with increased inflammation (e.g. TNFR-1 and procalcitonin) reflect known pathways of immune cell activation from PAMPs and were associated with low diversity fungal communities in the lung in our study.^{24,331–333} In our study, we identified that patients with low

diversity of fungal communities in the lung were twice as likely to have elevated levels of plasma BDG.

Our study has limitations that prompt the need for validation and further study. This study was conducted in a single tertiary care ICU and as such, the generalizability of our results should be validated in a wider population. While our study is the largest study of next-generation sequencing of fungal DNA in a critically ill population to date, results from our analysis of the subgroup of patients with ARDS require cautious interpretation as the effective sample size for this subgroup is relatively small. Additionally, we utilized endotracheal aspirate samples from a single time-point and as such we are unable to draw inferences about changes over time in the mycobiome and clinical outcomes. While our results remained significant after adjusting for clinical confounders, we were not able to control for all potential exposures and there is a possibility of residual confounding impacting our results.

Our results are based on ITS rRNA gene sequencing which is limited in that not all fungal species are detectable using that gene target and our use of DNA-based analyses limits our ability to analyze the viability of fungi and virulence factors of specific fungi. Fungi in the airways can present PAMPs to innate immune cells which can propagate the inflammatory cascade regardless of viability and ongoing growth and fungal proliferation.³³² Although we did identify a distinct fungal signal in our ETA samples, the biomass of these samples is very low with some samples not producing any sequencing reads and many producing a low number of reads that overlap with experimental controls. We only used ETA samples rather than bronchial alveolar lavage (BAL) samples and are limited by our ability to assess regional variability of fungal communities in the lung or directly study the host-mycobiota interactions in the alveolar space. We utilized ETA out of a concern for practical and ethical concerns (minimal risk exposure to participants) and due to

supporting evidence from comparative studies of the reliability of non-invasive samples and clinical practice guidelines.^{334,335}

In summary, in this prospective observational cohort study of mechanically ventilated patients, mycobiome diversity in the lung of patients with critical illness and ARDS is a predictor of the severity of disease and adverse clinical outcomes. We demonstrated that biomarkers associated with inflammation, immune system activation, and host-response to infection were elevated in patients with low fungal diversity of the lung. We identified *Candida spp.* as a unifying species in clusters of patients with infection, antibiotic, and immunosuppression use and with clinical features associated with the severity of disease. These associations are hypothesis generating for a potential mechanism of biological heterogeneity in critical illness involving interactions between the mycobiome and fungal PAMPs and the innate immune system. While the lung mycobiome has been understudied, it is very likely that it is an important source of biological heterogeneity and clinical variation among critically ill patients and may represent a potential therapeutic target for the prevention and treatment of lung injury and ARDS. The role of the mycobiome and the broader microbiome in critical illness and ARDS is complex and likely bidirectional and continued investigation of the respiratory microbiome with culture-independent next-generation sequencing approaches will allow for further delineation of interactions between the host and the microbiota as well as between bacteria and fungi in the respiratory tract of mechanically ventilated patients.

3.6 Tables

Table 3.1: Demographic Characteristics and Medical History by Alpha Diversity Subgroup

Demographics	Low Diversity N= 116	High Diversity N= 110	P-Value
Age, median [Q1, Q3], yrs.	60.8 [45.4, 69.8]	60.4 [43.9, 67.8]	0.78
Males, N (%)	60 (51.7%)	61 (55.5%)	0.67
BMI, median	29.7 [25.2, 36.3]	28.5 [24.4, 34.0]	0.18
Medical History			
Diabetes, N (%)	33 (28.4%)	42 (38.2%)	0.16
COPD, N (%)	31 (26.7%)	26 (23.6%)	0.70
Immunosuppression, N (%)	37 (31.9%)	19 (17.3%)	0.02
Chronic cardiac failure, N (%)	13 (11.2%)	20 (18.2%)	0.20
Chronic kidney disease, N (%)	21 (18.1%)	27 (24.5%)	0.31
Pulmonary Fibrosis, N (%)	13 (11.2%)	5 (4.5%)	0.11

Table 3.2: Clinical Characteristics by Alpha Diversity Subgroup

Clinical Characteristics	Low Diversity N= 116	High Diversity N= 110	P-Value
Systemic Antibiotics during ICU admission prior to sampling, N (%)	108 (93.1%)	87 (79.1%)	0.03
Respiratory Culture Positive for Culture, N (%)	29 (25.0%)	22 (20.0%)	0.46
WBC, median [Q1, Q3], x 10 ⁹ per liter	11.7 [8.88, 16.0]	12.1 [9.23, 17.4]	0.99
ARDS Status			
ARDS, N (%)	30 (25.9%)	17 (15.5%)	0.08
At Risk For ARDS, N (%)	71 (61.2%)	64 (58.2%)	0.24
Risk factors for ARDS			
Pneumonia, N (%)	47 (40.5%)	37 (33.6%)	0.35
Sepsis, N (%)	20 (17.2%)	14 (12.7%)	0.45
Aspiration, N (%)	18 (15.5%)	16 (14.5%)	0.98
LIPS score, median [Q1, Q3]	5.50 [4.00, 7.13]	5.00 [3.00, 6.00]	0.003

Table 3.3: Severity of Illness by Alpha Diversity Subgroup

Severity of Illness	Low Diversity N= 116	High Diversity N= 110	P-Value
SOFA score, median [Q1, Q3]*	7.00 [5.00, 9.00]	6.00 [4.00, 8.00]	0.01

PaO ₂ :FIO ₂ ratio, median [Q1, Q3], mmHg	164 [115, 205]	205 [137, 235]	0.02
Plateau pressure, median [Q1, Q3], cm	20.0 [16.8, 25.0]	19.0 [16.0, 23.0]	0.18
Shock, N (%)	56 (48.3%)	35 (31.8%)	0.02
Acute Kidney Injury, N (%)	82 (70.7%)	59 (53.6%)	0.01

Table 3.4: Associations Between Clinical Outcomes and Alpha Diversity Subgroup

	Unadjusted Odds Ratio* (95% CI)	P-Value	Adjusted Odds Ratio* (95% CI)	P-Value
ARDS Diagnosis	1.89 (0.98, 3.72)	0.06	2.22 (1.06, 4.80)	0.04
Ventilator Free Days	0.97 (0.95, 0.99)	0.02	0.97 (0.95, 0.99)	0.04
ICU Length of Stay	1.50 (1.11, 2.04)	0.01	1.55 (1.22, 2.13)	0.01

*Odds ratios were calculated as low versus high alpha diversity subgroup

Table 3.5: Time To Event Analysis Between Clinical Outcomes and Alpha Diversity Subgroup

	Unadjusted Hazards Ratio* (95% CI)	P-Value	Adjusted Hazards Ratio* (95% CI)	P-Value
Time-to-Liberation from Mechanical Ventilation	1.59 (1.17, 2.14)	0.003	1.54 (1.13, 2.11)	0.01
Survival at 30 Days	0.55 (0.32, 0.94)	0.03	0.53 (0.31, 0.93)	0.03
Survival at 90 Days	0.55 (0.33, 0.92)	0.02	0.53 (0.31, 0.91)	0.02

*Hazards ratios were calculated as low versus high alpha diversity subgroup

Table 3.6: Distributions of Biomarkers by Alpha Diversity Subgroup

	Low Diversity N=116	High Diversity N=110	P-Value
Innate Immune Response			
IL-6, pg/ml	46.5 [12.0, 178]	38.1 [17.3, 108]	0.308
IL-8, pg/ml	16.6 [7.46, 32.7]	14.8 [7.20, 25.9]	0.28
IL-10, pg/ml	1.34 [1.00, 11.8]	1.00 [1.00, 3.14]	0.002
TNFR-1, pg/ml	6090 [3200, 12800]	3750 [1880, 9130]	0.007
ST-2, pg/ml	186000 [83500, 462000]	129000 [59500, 279000]	0.008
Fractalkine, pg/ml	1920 [826, 2880]	1310 [570, 2260]	0.03
Host Response to Infection			
Procalcitonin, pg/ml	744 [247, 3270]	343 [127, 1750]	0.005
Pentraxin-3, pg/ml	4250 [1250, 11200]	2290 [937, 5380]	0.003
Epithelial Injury			
RAGE, pg/ml	3280 [1770, 5670]	2370 [1420, 5000]	0.06
Endothelial Injury			
Angiopoietin-2, pg/ml	6660 [3570, 15300]	5600 [2550, 12100]	0.07
Fungal Translocation			
Plasma (1,3)- β -d-glucan, pg/ml	28.5 [16.0, 63.0]	25.0 [15.0, 40.5]	0.13
Plasma (1,3)- β -d-glucan greater than 60 pg/ml	25 (22.1%)	14 (12.4%)	0.05
ETA (1,3)- β -d-glucan, pg/ml	565 [124, 3220]	5700 [2180, 6800]	0.16
Intestinal Permeability			
Fatty Acid-Binding Protein, pg/ml	715 [392, 1230]	603 [406, 1180]	0.54

Table 3.7: Associations with Biomarkers and Alpha Diversity Subgroup

	Unadjusted		Adjusted	
	Odds Ratio (95% CI)	P-Value	Odds Ratio (95% CI)	P-Value
Innate Immune Response				
IL-6, pg/ml	1.08 (0.94, 1.25)	0.28	1.06 (0.91, 1.24)	0.45
IL-8, pg/ml	1.14 (0.93, 1.41)	0.22	1.11 (0.89, 1.40)	0.37
IL-10, pg/ml	1.32 (1.09, 1.63)	0.006	1.37 (1.10, 1.70)	0.006
TNFR-1, pg/ml	1.26 (1.08, 1.60)	0.05	1.23 (0.97, 1.58)	0.10
ST-2, pg/ml	1.36 (1.10, 1.70)	0.005	1.33 (1.06, 1.69)	0.02
Fractalkine, pg/ml	1.07 (0.91, 1.28)	0.40	1.06 (0.89, 1.27)	0.51
Host Response to Infection				
Procalcitonin, pg/ml	1.24 (1.05, 1.47)	0.01	1.22 (1.02, 1.45)	0.02
Pentraxin-3, pg/ml	1.23 (1.05, 1.45)	0.01	1.23 (1.04, 1.46)	0.02
Epithelial Injury				
RAGE, pg/ml	1.16 (0.88, 1.54)	0.30	1.10 (0.81, 1.48)	0.55
Endothelial Injury				
Angiopoietin-2, pg/ml	1.20 (0.94, 1.54)	0.15	1.21(0.93, 1.59)	0.16

Fungal Translocation				
Plasma (1,3)- β -d-glucan, pg/ml	1.38 (1.03, 1.89)	0.04	1.41 (1.02, 1.99)	0.05
Plasma (1,3)- β -d-glucan greater than 60 pg/ml	2.59 (1.25, 5.65)	0.01	2.71 (1.22, 6.36)	0.02
Intestinal Permeability				
Fatty Acid-Binding Protein, pg/ml	1.13 (0.84, 1.53)	0.43	1.19 (0.86, 1.51)	0.30

3.7 Figures

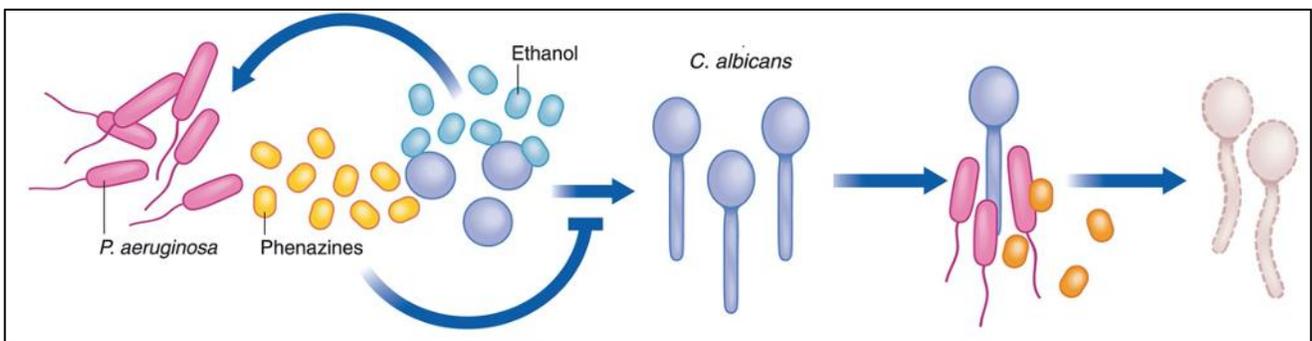


Figure 3.1: Example of Chemical and Physical Interactions Between *Pseudomonas aeruginosa* and *Candida albicans*

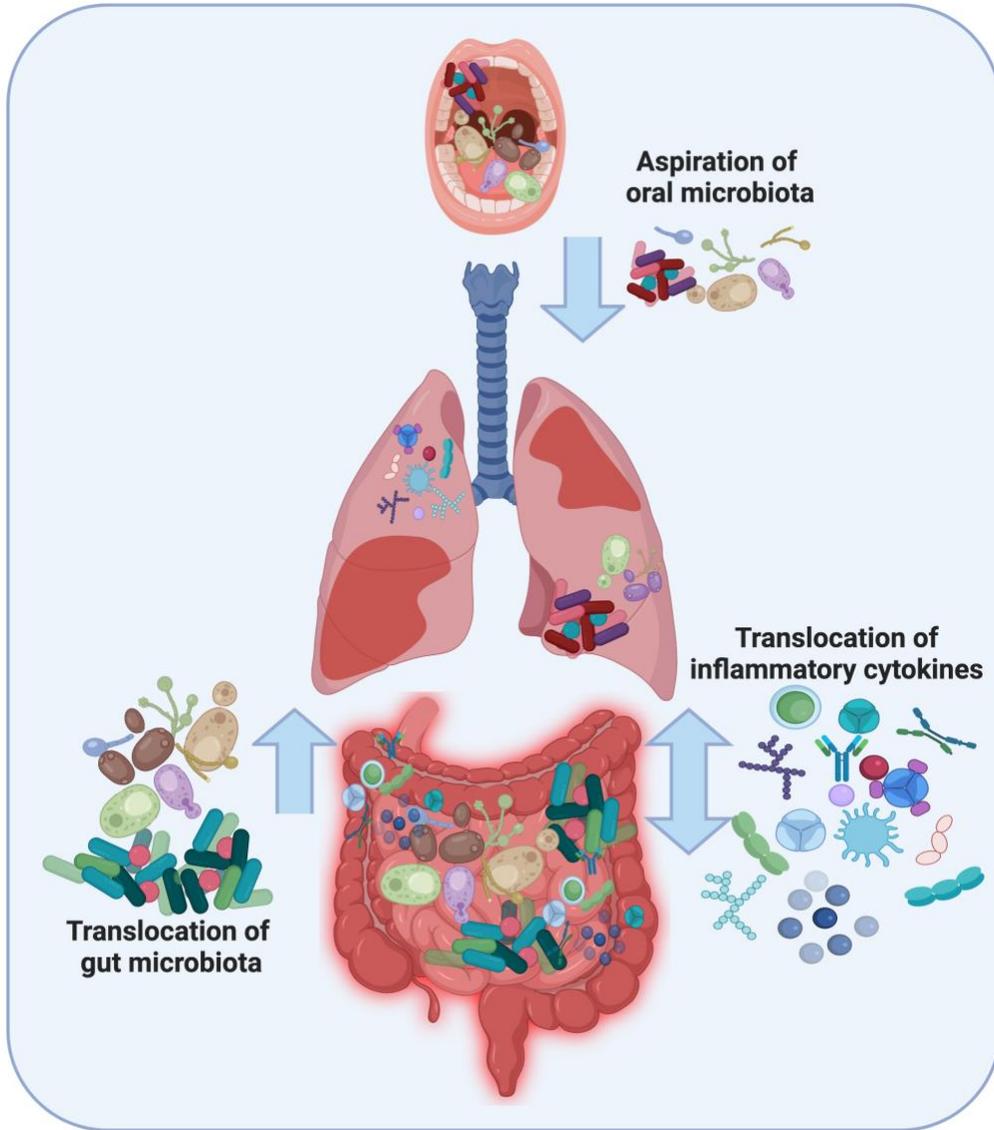


Figure 3.2: Visualization of The Gut-Lung Axis in Critical Illness

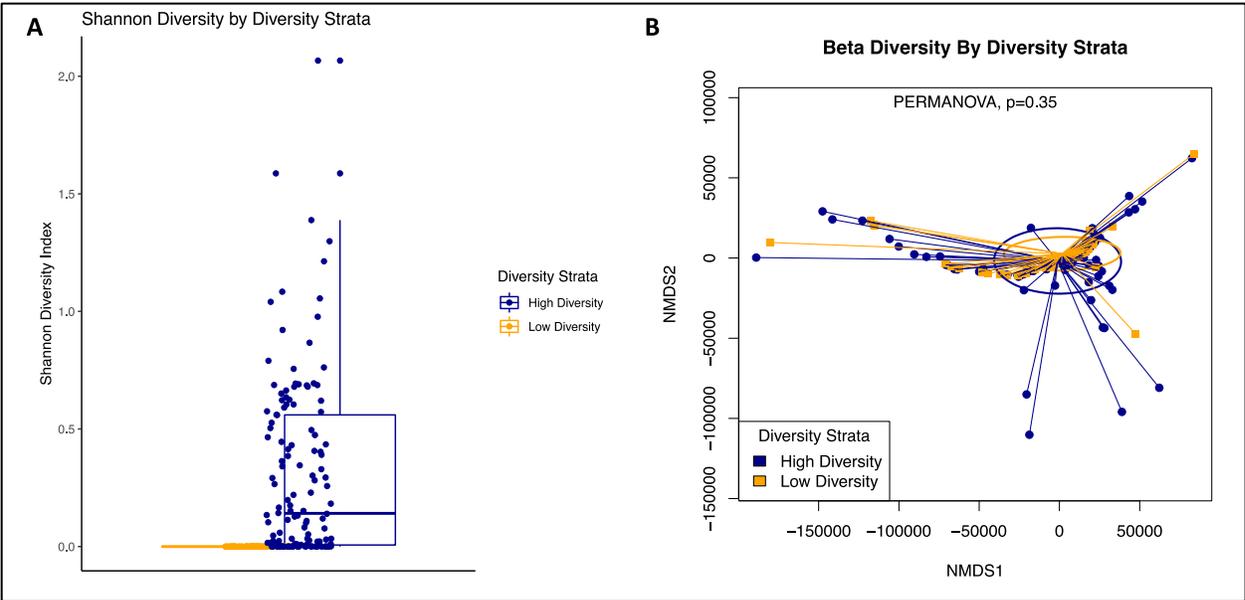


Figure 3.3: Differences in the Shannon Index and NMDS Ordination by Alpha Diversity Subgroup

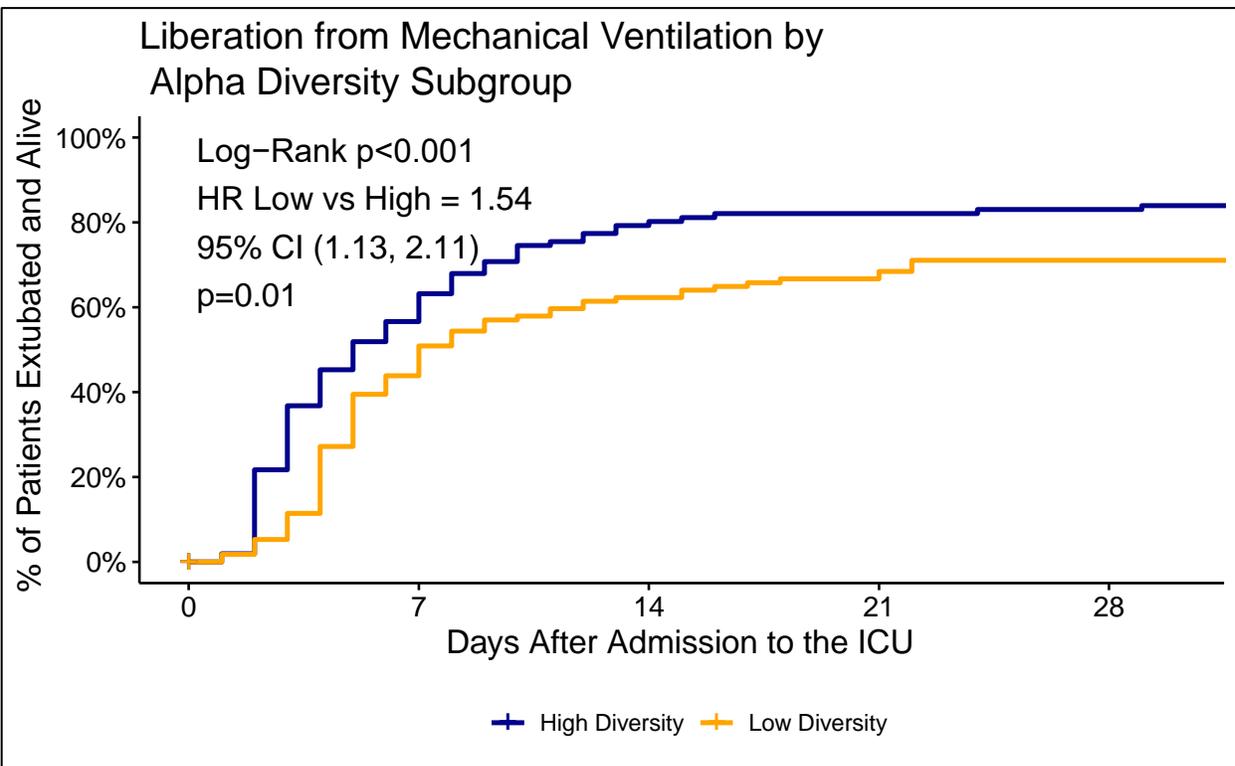


Figure 3.4: Kaplan-Meier Curves for Time to Liberation from Mechanical Ventilation by Alpha Diversity Subgroup

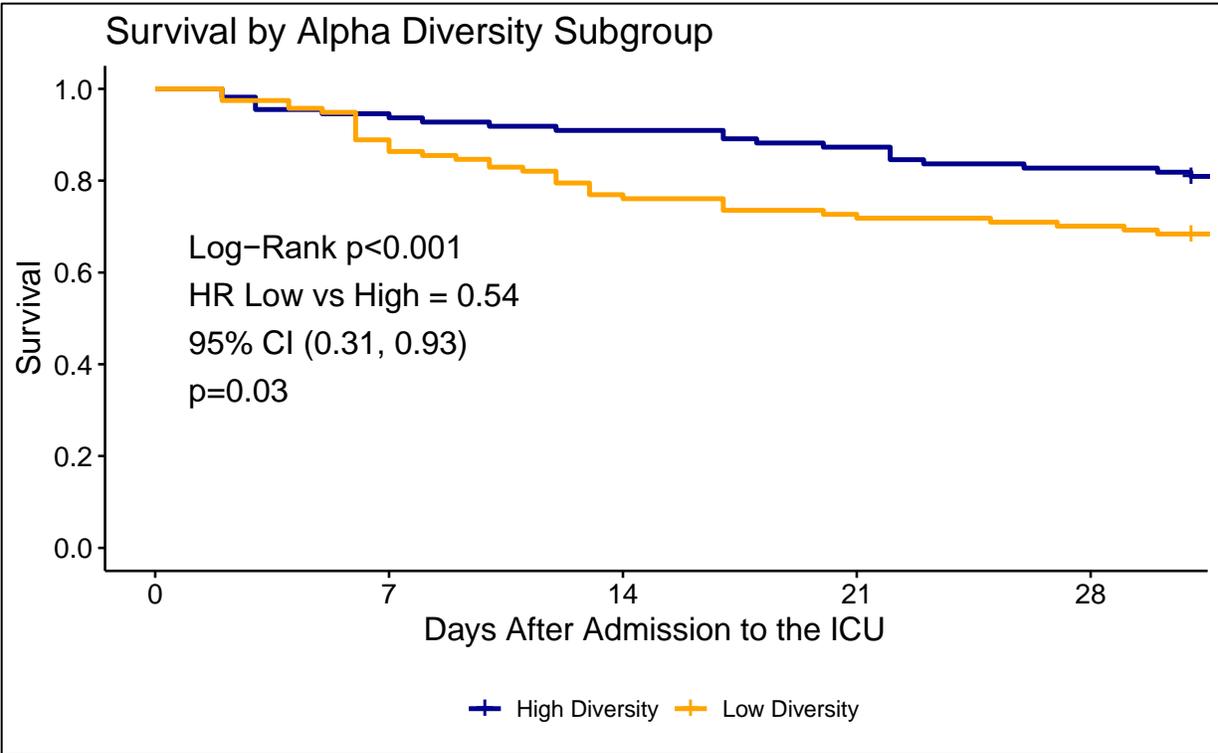


Figure 3.5: Kaplan-Meier Curves for Mortality at 30 Days by Alpha Diversity Subgroup

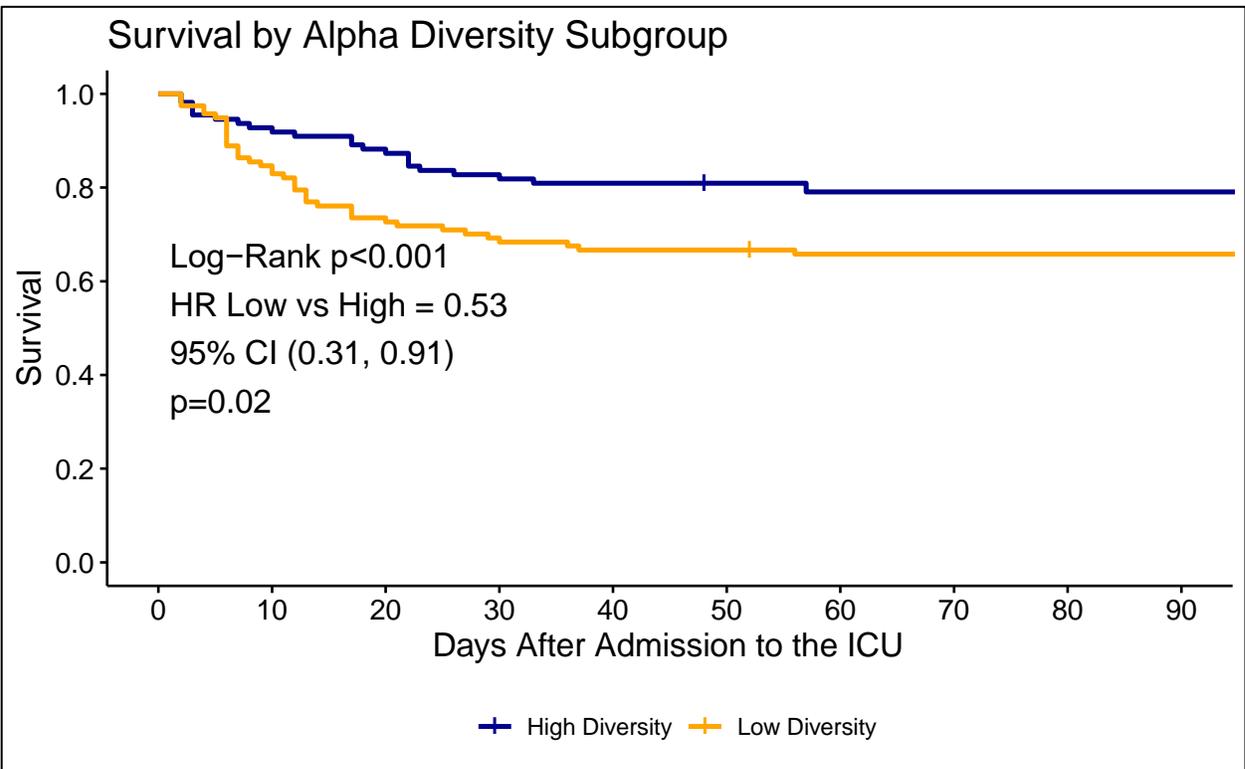


Figure 3.6: Kaplan-Meier Curves for Mortality at 90 Days by Alpha Diversity Subgroup

4.0 Manuscript 3: Interkingdom Interactions in Critical Illness

4.1 Introduction

Background: In critically ill patients, variation in the host inflammatory responses has been associated with poor outcomes. However, the biological mechanisms underlying this heterogeneity have not yet been defined. We investigated whether variation fungal communities (mycobiome) in the respiratory tract are associated with host inflammation and innate immune system activation and clinical outcomes.

Methods: We collected endotracheal aspirates (ETA) and plasma samples from 226 critically ill patients. We used extracted DNA from ETAs and performed fungal rRNA (ITS) and bacterial rRNA (16S) gene sequencing on the Illumina MiSeq platform to characterize the lower respiratory tract microbiome. We derived alpha diversity metrics to identify associations between bacterial and fungal alpha diversity and relative abundance of protective bacteria. We performed network analyses using probabilistic graphical models to identify associations between bacterial and fungal taxa, features of the microbiome, biomarkers clinical features and clinical outcomes.

Results: The number of total bacterial DNA reads produced by 16S sequencing was lower in patients assigned to the lower fungal alpha diversity subgroup (median [Q1, Q3]: 4368 [2821, 4861], $p=0.01$) compared to those assigned to the higher fungal alpha diversity subgroup (median [Q1, Q3]: 4759 [4337, 4759]). A higher fungal Shannon Diversity index was associated with an increased number of total 16S reads ($R=0.18$, $p=0.03$). The fungal Shannon diversity index was positively associated with higher proportions of protective bacteria ($R=0.2$, $p=0.01$) and patients assigned to the high fungal alpha diversity subgroup had higher relative abundance (median

[Q1,Q3]: 26% [4%, 58%] $p=0.05$) of protective oral-origin bacteria compared to those in the low fungal alpha diversity subgroup (median [Q1,Q3]: 19% [2%, 37%]). Gut-associated bacteria *Lachnospiraceae* were directly linked with *Candida tropicalis* and mortality at 30 and 90 days.

Conclusions: Our findings support previous work indicating an association between a lung microbiome enriched with gut-associated bacteria are hypothesis-generating for the possibility that microbiome-mycobiome-host interactions may play a role in adverse clinical outcomes in critical illness and ARDS.

4.2 Background

Recent research on the acute respiratory distress syndrome (ARDS) has focused on developing an understanding of the biological heterogeneity and has revealed distinct subgroups of patients with or at risk for ARDS identified by a differential innate immune response and associated with differences in organ dysfunction, response to treatment, and clinical outcomes. However, the biological basis of these differential immune responses is not yet understood and has limited the discovery of broadly efficacious therapies for ARDS.^{9,16,22–24,154,294,295} It has been recently proposed that in critically ill patients with ARDS alveolar inflammation and injury may be perpetuated by perturbations of the microbial communities in the lung and may account for some of the biological heterogeneity seen in ARDS.^{25,132,237,317,319,336} In critical illness, endotracheal intubation exposes the lungs to the microbes from the upper respiratory tract and oral cavity while damaged alveoli flooded with nutrient-rich edema provide a rich environment for aberrant microbial reproduction and growth.^{7,25,132,237} Several studies of critically ill patients have shown that alterations in the bacterial communities in the lung are correlated with an increase in both

systemic and alveolar inflammation as well as worse clinical outcomes in critical illness.^{25,126,237,319} However, very little research has been undertaken into the role of the fungal component of the lung microbiota, or lung mycobiome, and the interaction between lung mycobiota and the innate immune functions and subsequent inflammation in critical illness.^{39,70,251,298} Lung mycobiota have been identified in healthy individuals and in cystic fibrosis, bronchiectasis, asthma, and chronic obstructive pulmonary disease (COPD) alterations in the mycobiome have been associated with poor clinical outcomes.^{56,89,90,93,282,313}

Both bacteria and fungi contain pathogen-associated molecular patterns (PAMP) comprised of microbial nucleic acids and cell wall components such as the lipopolysaccharide of gram-negative bacteria and the 1,3-beta-D-glucan (BDG) for fungi. PAMPs are recognizable by pattern recognition receptors (PRRs) on immune cells that activate macrophages and B and T cells, thereby triggering immune activation and inflammation.^{250,329,333,337} Many of the biomarkers associated with detrimental clinical outcomes in critical illness (e.g. interleukins [IL]-6 and -8, soluble tumor necrosis factor receptor-1 [TNFR1], procalcitonin, and receptor for advanced glycation end-products [RAGE]) represent established pathways of immune cell stimulation from microbial PAMPs.^{24,331–333} Although present at significantly lower abundance than bacteria, fungi play an important role in the microbial community of the lung, and interactions between individual fungi and bacteria are well established and represent a potentially important factor in critical illness.^{53,56,249,338–343}

Bacteria and fungi can interact on multiple levels making polymicrobial interaction studies challenging. These interactions are complex and can take place when different microbiota occupy the same niches in the host and can result in differential effects that can be synergistic, antagonistic, or neutral. Fungal-specific factors have been shown to control bacterial behavior, virulence, and

survival, and conversely, bacterial-specific factors can influence fungal growth, physiology, and virulence. In this way, bacteria and fungi can provide mutual support for growth or exert more competitive effects upon each other, potentially resulting in the suppression of one microorganism and dominant growth of the other. Depending on specific conditions and changes in the microenvironment and specific stimuli from the host or other microorganisms, the interactions between bacteria and fungi can be mediated by different mechanisms that also operate as virulence factors including chemical exchanges of metabolites and toxins, biofilm formation, direct cell to cell interaction and quorum sensing.^{341,344–351}

Although data on the clinical relevance of bacterial–fungal interactions in the lung are limited, the relationship between *Candida spp.* and *Pseudomonas aeruginosa* is one of the most well studied fungal-bacterial relationships and provides an excellent example of the multi-faceted interaction between bacteria and fungi (Figure 3.1). The relationship between *Candida spp.* and *Pseudomonas aeruginosa* is primarily antagonistic; however, there is evidence that *Candida* and *Pseudomonas aeruginosa* work synergistically to increase virulence. *P. aeruginosa* secretes phenazines that are toxic to fungi as well as a quorum-sensing molecule, 3-oxo C12 homoserine lactone, in order to control the morphogenesis of *Candida spp.* by restricting the growth of hyphae.^{352–354} While phenazines and 3-oxo C12 homoserine lactone inhibit hyphal formation, they do not kill existing hyphae.^{352,355,356} *Pseudomonas aeruginosa* attaches to existing *Candida spp.* hyphae using carbohydrate components of the fungal cell wall, weakening the cell wall, and inducing death in the hyphae.³⁵⁷ This forms a fungal scaffold on which *Pseudomonas aeruginosa* forms a biofilm, thereby parasitizing *Candida spp.* .^{358–360}

This inhibitory effect of *Pseudomonas aeruginosa* on *Candida* biofilms has been demonstrated to impact multiple *Candida* species. *Candida tropicalis* and *Candida dubliniensis*

biofilms exposed to *Pseudomonas aeruginosa* had significantly decreased viability and were thinner compared to fungal biofilms not exposed to *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* lipopolysaccharide (LPS) has been shown to inhibit *Candida glabrata*, *Candida krusei*, and *Candida dubliniensis* biofilm formation and maturation, suggesting that bacterial LPS may have played a role in *Candida* biofilm development.^{361,362}

Conversely, a quorum-sensing molecule produced by *Candida spp.*, farnesol, inhibits swarming motility in *Pseudomonas aeruginosa*, which may lead to enhanced *Pseudomonas aeruginosa* biofilm formation on surfaces such as catheters or endotracheal tubing.^{363–366} *Candida spp.* and *Pseudomonas aeruginosa* were among the most common microorganisms identified in endotracheal tube biofilms and in tracheal secretions in patients with ventilator-associated pneumonia (VAP).³⁶⁷ This observation was followed by studies that demonstrated that colonization by *Candida spp.* increases host susceptibility to *Pseudomonas aeruginosa* infection and that risk of bacterial infection can be reduced via antifungal treatment.^{277,280,368–370} This increased susceptibility could be explained in part by Roux et al's work in rats which showed that colonization with *Candida albicans* increased levels of lung tumor necrosis factor alpha (TNF α) and interferon gamma (IFN- γ) and elicited a Th1-Th17 immune response that favored the development of bacterial pneumonia via the inhibition of bacterial phagocytosis by alveolar macrophages. The risk of bacterial pneumonia was reduced in colonized rats with antifungal treatment.³⁶⁸ Chen et al identified that ethanol produced by *Candida albicans* promotes *Pseudomonas aeruginosa* colonization of lung epithelial cells, and further work by Greenberg et al found that *Candida albicans* produced ethanol that could impede the clearance of *Pseudomonas aeruginosa* from the lungs by reducing macrophage recruitment in a rat model of infection.^{366,371}

In a zebrafish model of swimbladder infection, co-infection with *Candida albicans* and *Pseudomonas aeruginosa* was associated with the upregulation of the proinflammatory cytokine IL-6 and of IL-8, a potent chemoattractant of neutrophils and demonstrated a synergistic virulence associated with increased *Candida albicans* pathogenesis and inflammation.³⁷² However, in a murine model, infection with *Candida albicans* mediated a protective effect against lung tissue damage caused by *Pseudomonas aeruginosa* by triggering the production of IL-17 and IL-22 which stimulated the production of antimicrobial peptides by the host and contributed to the mobilization of phagocytic cells.³⁷³

In humans, the interaction between *Candida albicans* and *Pseudomonas aeruginosa* has been studied in the lungs of mechanically ventilated patients who are particularly susceptible to colonization by *Candida* and subsequent *Pseudomonas aeruginosa* VAP.²⁷⁷ In critically ill patients with VAP, mortality was significantly increased with culture-dependent identification of *Candida* colonization and *Candida* colonization was an independent predictor of mortality in patients with VAP.²⁸⁰ However, continued work by Delisle et al questioned whether colonization of the respiratory tract by *Candida* serves only as an indication of disease severity or contributes significantly to the worse clinical outcomes observed in patients with colonization of *Candida* in the setting of VAP.²⁷⁸ In the lungs of patients with CF, co-colonization with *Candida albicans* and *Pseudomonas aeruginosa* was associated with a significant decline in lung function as measured by FEV₁ compared to patients without colonization of *Candida albicans*.³⁷⁴ It is possible that *Candida* colonization could inhibit an antibacterial immune response by increasing levels of TNF α and IFN- γ in the lung and impairing alveolar macrophage function as demonstrated in animal models.⁵³

While the relationship between *Candida albicans* and *Pseudomonas aeruginosa* represents a well-studied example of inter-kingdom (bacterial-fungal-host) interaction, much less is known about other bacterial-fungal interactions or wider microbiome-mycobiome interactions with the host. Krause et al undertook one of the only culture-independent studies of both the bacteria and *Candida* in the lung of critically ill patients with pneumonia found that there was no specific bacterial microbiota profile based on the presence or absence of particular bacteria associated with the presence of *Candida spp.*; however, their investigation did not take into account associations between particular bacterial and fungal taxa or the role of overall community diversity in relation to potential bacterial-fungal interactions.²⁵¹ This next-generation sequencing (NGS) based description of bacteria and *Candida spp.* in critical illness represents an innovative perspective on the potential interactions of bacteria and fungi and encourages continued culture-independent research into the role of bacteria and fungi in the lungs of critically ill patients. It is not yet clear whether factors such as systemic antibacterial therapy, host immune status, or exposure to hospital-acquired pathogens simply predispose a patient to colonization by both bacteria and fungi or are important factors in the dynamic microbiome-mycobiome interaction in the lungs of critically ill patients.

As we develop an improved understanding of the pivotal role played by microbial communities in critical illness and ARDS, our appreciation for the potential role played by fungal communities in the modulation of the systemic immune system and inflammation in the lung grows. As our understanding of how factors such as bacteria and fungi separately and their interaction impact host-response increases, so will we improve our ability to predict the significance of changes in the microbiota on host status, opening up the microbiome as a potential target for therapeutics in critical illness and ARDS in the future. Potential therapeutic targets may

include antimicrobials, anti-inflammatory agents including steroids and drugs targeting specific cytokine production.

However, analyzing and converting microbiome data into meaningful biological insights remain very challenging as microbiota are complex in both compositional variability and in their interactions with each other and with their host. This complexity can be well represented and modeled using graphical network approaches that can facilitate microbiome analysis and enhance our understanding of intricate ecological and biological processes impacting health and disease. The idea that architectural features of networks seem to be universal in complex systems, including molecular interaction networks computer networks, microcircuits, and microbiomes is an essential feature of network theory.³⁷⁵ This commonality allows for the application of tools used primarily in non-biological systems where the goal is to characterize intricate relationships and reveal patterns in high dimensional data to be applied to host-microbiota and microbiota-microbiota interactions and ecological processes present in the human microbiome.³⁷⁶ Graphical network approaches applied to microbiome studies can be used to model co-occurrence of microbiota, identify relationships between microbiota essential for community stability and provide evidence for the influence of microbial and environmental interactions on pathogenesis and disease process in the host. Application of network approaches to the microbiome of the lung in critical illness have been limited to Kitsios et al's use of a probabilistic graphical model (PGM) to identify relationships between bacterial taxa, clinical features, and respiratory culture positivity.³¹⁸ Their use of a PGM extended their univariate taxonomic analyses and identified that respiratory culture positivity was positively associated with the pathogen abundance, *Enterobacteriaceae*, *Haemophilus*, *Escherichia*, and *Enterococcus* abundance, and was negatively associated with *Prevotella* abundance and hemoglobin levels. The ability to visualize the complex relationships of

large numbers of variables that interact with each other including clinical features, environmental factors, and microbiota co-occurrence and abundance in critical illness may lead to the discovery of direct links between microbiota and clinical outcomes in the host. This improvement in our understanding of the microbiological underpinnings of the disease process in ARDS and critical illness may lead to the identification of new therapeutic targets and treatment strategies.

As such, we sought to determine whether there is evidence of microbiome-host and microbiome-mycobiome interactions in the lung of critically ill mechanically ventilated patients and to identify clinical and microbiological features independently associated with clinical outcomes including length of ICU stay, duration of mechanical ventilation, and mortality. To comprehensively examine for direct associations between outcomes, baseline clinical variables, biomarkers of host response, and bacterial and fungal taxa we utilized the PGM framework in our cohort of critically ill mechanically ventilated patients.

4.3 Methods

For this investigation, we utilized the Acute Lung Injury Registry and Biospecimen Repository (ALIR). ALIR is an ongoing prospective observational cohort of critically ill patients admitted to the medical or Cardiac ICU at the University of Pittsburgh Medical Center (UPMC). From this cohort, we enrolled a convenience sample of adult patients who were being mechanically ventilated from April 2015 through September 2019. Participants were enrolled within 72 hours of intubation and exclusion criteria included i) patients with tracheostomy, due to frequent airway colonization with pathogens, ii) expected survival <48hrs, and v) inability to obtain informed consent from a legally authorized representative. At enrollment, we collected endotracheal aspirate

(ETA) and blood samples and obtained clinical data from the electronic medical record. This study was approved by the University of Pittsburgh Institutional Review Board (protocol PRO10110387), and written informed consent was provided by all participants or their surrogates.

4.3.1 Clinical Sample Collection

ETAs were collected by suctioning distal tracheal secretions through a closed endotracheal tube suctioning system in a manner similar to the ETA collection for clinical microbiologic culture. 5cc of sterile saline was instilled through the tubing system and suction was repeated in cases in which suctioning did not return >5ml of ETA. Samples were collected in sputum collection traps directly and were frozen to -80°C as soon as possible before storage until sample processing. We stored the saline not used for sample collection as a negative experimental control sample to allow for the identification of potential contamination during sample collection.

We also collected 10cc of blood (through central venous or arterial access or phlebotomy) in sodium citrate anticoagulated at study enrollment. We performed same-day centrifugation and plasma collection in the research laboratory and stored samples at -80°C until sample processing.

4.3.2 Clinical Microbiology

In order for the clinical microbiologic results to be indicative of the same infectious process as being studied by the next-generation sequencing, we utilized clinical microbiologic results obtained within 72 hours of collection of research biospecimens. All clinical cultures were obtained at the discretion of the treating physicians and were not obtained specifically for research purposes. All specimen cultures were performed by the clinical microbiology laboratory at UPMC.

Samples were used to inoculate agar plates: trypticase soy agar with 5% sheep blood, chocolate agar (for fastidious organisms), Columbia Naladixic Acid Agar (CAN Agar for gram-positives), and MacConkey (for gram-negatives). Samples are incubated for up to 24hrs and the following day, isolated colonies are identified with matrix-assisted laser desorption/ionization with time-of-flight mass spectroscopy (MALDI-TOF MS) (Bruker Biotyper) and susceptibility testing is performed with the MicroScan WalkAway platform (Beckman Coulter).

Respiratory samples were considered to be culture positive where pathogenic bacteria or fungi were isolated in the sputum, ETA, or bronchoalveolar lavage (BAL) by the clinical laboratory. In samples where no organism growth or “normal respiratory flora” were reported by the clinical laboratory, we considered them to be culture negative. Blood cultures were considered to be positive if organisms not deemed to be skin contaminants by treating physicians were reported by the clinical laboratory.

4.3.3 Microbiome Experiments

4.3.3.1 DNA Extractions

We pretreated ETA samples with Dithiothreitol (0.1% DTT in phosphate buffered saline) in a 1:1 dilution to dissolve mucous and reduce the viscosity of samples for processing in DNA extraction columns. Microbial DNA was extracted ETA samples using the Powersoil (MoBio) extraction kit as per the manufacturer’s instructions as previously described.²⁵⁷ We utilized reagent controls from the DNA extraction process.

4.3.3.2 16S rRNA gene sequencing

We amplified extracted bacterial DNA by PCR using the method described by Caporaso et al and Q5 High-Fidelity polymerase (NEB) targeting the V4 hypervariable region of the 16S rRNA gene.²⁵⁸ We amplified four microliters per reaction of each sample with a single barcode in triplicate 25 microliter reactions. Cycle conditions were 98°C for 30s, then 33 cycles of 98°C for 10s, 57°C for 30s, 72°C for 30s, with a final extension step of 72°C for 2 min. We utilized reagent controls from the 16S rRNA gene amplification process. We combined triplicates and purified with the AMPure XP beads (Beckman) at a 0.8:1 ratio (beads:DNA) to remove primer-dimers. Eluted DNA was quantitated on a Qubit fluorimeter (Life Technologies). We created a pooled sample on ice by combining 20 ng of each purified band. For negative experimental controls and poorly performing samples, 20 microliters of each sample were added to the pooled sample. We further purified the sample pool using the MiniElute PCR purification kit (Qiagen), AMPure XP beads, and the PureLink PCR Purification kit (Life Technologies). The final pooled sample was quantitated in triplicate on the Qubit fluorimeter and prepared for sequencing using the instructions provided by Illumina with an additional incubation at 95°C for 2 minutes immediately following the initial dilution to 20 picomolar. The pool was then diluted to a final concentration of 7 pM + 15% PhiX control. Amplicons were sequenced on the Miseq platform.

4.3.3.3 ITS rRNA gene sequencing

Fungal DNA was amplified by PCR using the method of Caporaso et al. and using Q5 HS High-Fidelity polymerase (NEB) regions 1 and 2 of the internal transcribed spacer (ITS) rRNA gene.²⁵⁸ Four microliters per reaction of each sample were amplified with a single barcode in triplicate 25 microliter reactions. A 2-step nested PCR protocol was utilized to obtain an adequate number of amplicons. The conditions for the first step were 98°C for 30 seconds, 15 cycles of

98°C for 10 seconds, 55°C for 30 seconds, and 72°C for 90 seconds followed by 72°C for 2 minutes. We utilized 5 microliters of the initial PCR reaction in the second cycle and ran the second cycle as follows: 98°C for 30 seconds, 15 cycles of 98°C for 10 seconds, 55°C for 30 seconds, and 72°C for 1 minute followed by 72°C for 2 minutes. Reagent controls were included in ITS rRNA gene amplification. Triplicate samples were combined and then purified using AMPure XP beads at a 0.7:1 ratio (beads:DNA).

Samples were then pooled together by combining 30 microliters of each sample. Further purification was undertaken using the MinElute PCR purification kit, an additional round of clean up using AMPure XP beads, and a final purification using the Purelink PCR Purification kit. We then quantitated the purified pool in triplicate through a high-resolution microfluidics-based automated electrophoresis using the 2100 Bioanalyzer instrument (Aligent). The sequencing pool was prepared according to instructions by Illumina with an additional incubation at 95°C for 2 minutes immediately following the initial dilution to 20 picomolar. The sequencing pool was then diluted to a final concentration of 7 pM + 15% PhiX control. We utilized the Illumina Miseq platform for the sequencing of the amplicons.

4.3.3.4 Positive and Negative Experimental Control Samples

We considered three types of experimental negative control samples used to assess for possible contamination events from sample collection to DNA extraction and PCR amplification: endotracheal aspirate collection controls, DNA extraction, and PCR amplification negative controls. ETA controls consisted of left-over sterile saline not used for sample collection as a negative control to identify potential contamination of the ETA sample from microbial populations present in clinically sterile saline syringes. We included these ETA controls in DNA extraction, PCR amplification, and sequencing alongside clinical samples. DNA extraction negative controls

were comprised of sterile water in one DNA extraction column per batch of clinical samples undergoing DNA extraction. We added sterile water in the place of template DNA in our PCR reaction mix as negative PCR amplification controls. We also included PCR amplification positive controls to confirm effective amplification. We used the ZymoBIOMICS Microbial Community DNA Standard (Zymo Research, Irvine, CA), a mock microbial community consisting of genomic DNA of eight bacterial strains and 2 fungal strains as our PCR amplification positive control.

4.3.4 Analytics

4.3.4.1 16S Sequence Quality Control and Annotation

From the pooled sequencing run, sequences were demultiplexed into individual sample/replicate fastq files. Each fastq file was processed through the Center for Medicine and the Microbiome (CMM) custom modular read QC pipeline configured to perform the following steps: low complexity filtering, quality value trimming, Illumina sequencing adapter trimming, and 16S primer trimming. Paired sequences with forward and reverse reads passing the Quality Control filtering and trimming steps were then combined (end aligned and a consensus sequence computed) using the `make.contigs` function of `Mothur`.³⁷⁷ Sequences meeting screening requirements were then processed through the CMM's 16S clustering and annotation pipeline. For statistical modeling and analyses, we utilized taxonomic tables at the genus level, with taxonomic assignments performed using a naïve Bayes k-mer classifier in conjunction with the Ribosomal Database Project (RDP) 16S rRNA gene sequences.³⁷⁸ The taxa table was filtered for low abundance taxa (relative abundance <0.005%) and singletons. Clinical samples with low reads were excluded from analyses. We calculated the relative abundance per sample of protective (i.e. oral-origin bacteria, such as *Streptococcus*, *Prevotella*, and *Veillonella*) bacteria in each sample,

Shannon Diversity, Simpson Diversity, and clustered samples by bacterial taxonomy as previously described.²⁵

4.3.4.2 ITS Sequence Quality Control and Annotation

Sequencing reads were demultiplexed into individual sample/replicate fastq files. Each fastq file was processed through a custom quality control pipeline configured to perform the following steps: low complexity filtering, quality value trimming, Illumina sequencing adapter trimming, and ITS primer trimming utilizing cutadapt and the R packages Biostrings and ShortRead.^{378–380} Utilizing DADA2, we merged paired sequences with forward and reverse reads passing the quality filtering and trimming steps. Chimeras were removed. The Unite database was used to classify reads into amplicon sequence variants (ASVs) using the naïve Bayesian classifier method.^{260,261} Results of ASV annotation were converted into per-sample taxonomic profiles in a taxa table. The taxa table was then filtered for singletons and low abundance taxa (relative abundance, <0.005%). Samples that generated a low number of reads in the range of negative control samples were filtered from the analysis. Ecological analyses were performed with Phyloseq, vegan, microbiome, and other R packages.³⁸¹

4.3.5 Host-Response Experiments

We measured validated biomarkers of the innate immune response (IL-6, IL-8, IL-10, Tumor necrosis factor receptor 1 (TNFR1), suppression of tumorigenicity-2 [ST-2], fractalkine), epithelial injury(receptor of advanced glycation end-products [RAGE]), endothelial injury(Angiopoietin-2), and host response to infection (procalcitonin and pentraxin-3) in plasma using a custom Luminex multi-analyte panel (R&D Systems, Minneapolis, MI, United States) for

our host-response experiments.^{16,22,145} As a biomarker of intestinal permeability, we measured levels of fatty acid binding protein-2 (FABP-2) with the Quantikine® Human FABP2/I-FABP Immunoassay (R&D Systems, Minneapolis, USA). We quantified (1→3)-β-D-Glucan using the Fungitell assay (Associates of Cape Cod, Inc.) to assess the presence of fungal cell wall products present in the serum and ETA samples.^{146,147}

4.3.6 Clinical Characteristics

Prospective demographic, medical history, and clinical data were abstracted from the electronic medical record (EMR) for each patient at study enrollment. For vital signs, laboratory results, mechanical ventilation parameters, and chest radiography, the physiologically worst values from within 24 hours of enrollment were recorded. We calculated sequential organ failure assessment (SOFA) scores for each patient; however, we did not include the neurologic component of SOFA score because all patients were intubated and receiving sedative medications.³⁰¹ Lung Injury Prediction Scores (LIPS) were calculated from baseline variables.³⁰² We recorded whether patients had received antibiotics in the 30 days prior to ICU admission and if patients were receiving antibiotics at the time of study enrollment and sample collection. We modeled the systemic antibiotic exposure in the ICU (before microbiome sampling) with a published antibiotic exposure score that took into account dosing duration, the timing of administration, and specific antibiotic type.³⁰³ A consensus committee of clinical experts performed retrospective classifications of the etiology and severity of each patient's acute respiratory failure (sepsis, ARDS, pneumonia or aspiration, and intubation for airway protection without risk factors for ARDS) using established criteria and all available clinical data.^{3,27,304,305} Classifications were performed without knowledge of microbiome sequencing or host biomarker data. Prospective

clinical outcomes included 30 and 90-day mortality, duration of ICU stay, acute kidney injury, incident shock (defined as the need for vasopressors), and ventilator-free days.^{306,307}

4.3.7 Statistical Analyses

We derived alpha diversity metrics (Shannon Index) and based on the observed distribution of the Shannon Index, we classified patients into alpha diversity-based subgroups with a Shannon index greater than 0 classified as high diversity and a Shannon Index of 0 classified as low diversity. We examined descriptive statistics of demographic and clinical characteristics, and biomarker values were log-transformed for analysis. We utilized Wilcoxon tests and Spearman's correlations to examine associations between bacterial microbiome features including bacterial load, Shannon Diversity Index, Simpson's Diversity index and percentage of protective bacteria and mycobiome features including fungal load, Shannon Diversity index, Shannon Diversity subgroup, and Simpson's Diversity Index as well as relative abundance, presence, and dominance of individual fungal taxa.

4.3.8 Network Analyses

To both investigate relationships between bacterial and fungal taxa in critical illness and as further validation for the significant associations of clinical features and outcomes with mycobiome alpha diversity, we performed network analysis with PGMs. PGMs estimate and graphically represent the relationships between a large number of variables with complex interactions with each other and allow for the discovery of direct links between variables based on their conditional dependencies. We used the CausalMGM (Causal Mixed Graphical Model) R

package which utilizes an algorithm that accurately identifies the underlying graphical model structure over both continuous and categorical data types.^{382–384} The ability to include mixed data types allowed us to graphically represent clinical features, measures of mycobiome alpha diversity, clinical outcomes, and individual taxa data. In order to visualize networks, we used Cytoscape (3.8.2).³⁸⁵

We generated networks including variables representing the abundance of bacterial and fungal taxa identified in 16S and ITS sequencing, clinical features, and measures of mycobiome alpha diversity separately for each clinical outcome. In an attempt to reduce the number of variables included in the network, we also generated networks that included features of the microbiome including the percentage of bacteria considered to be protective and bacterial Shannon diversity index, fungal taxa, mycobiome features, clinical features and outcomes but excluded individual bacterial taxa.

From those networks we identified variables with were independently associated (first neighbors) with the outcome variable. PGMs theory posits that first neighbors of a variable represent the best predictors of that variable, making PGMs a useful tool for feature selection in predictive modeling.²⁴

4.4 Results

The number of total bacterial DNA reads produced by 16S sequencing was lower in patients assigned to the lower fungal alpha diversity subgroup (median [Q1, Q3]: 4368 [2821, 4861], $p=0.01$) compared to those assigned to the higher fungal alpha diversity subgroup (median [Q1, Q3]: 4759 [4337, 4759]). A higher fungal Shannon Diversity index was associated with an

increased number of total 16S reads ($R=0.18$, $p=0.03$). Alpha diversity of the fungal communities as measured by the Simpson's Diversity Index was also associated with a higher number of 16S reads ($R=-0.19$, $p=0.02$). There was not a significant relationship between the number of reads identified by ITS sequencing and those identified by 16S sequencing ($R=0.005$, $p=0.95$). Bacterial alpha diversity as measured by both the Simpson's Diversity Index ($R=-0.001$, $p=0.99$) and Shannon diversity index ($R=0.005$, $p=0.95$) were not associated with fungal alpha diversity as measured by the Shannon Diversity Index.

In previous work by our group, we demonstrated that there were 3 distinct clusters of samples marked by pathogen abundance and low bacterial alpha diversity.²⁵ Cluster 2 was considered to have both low alpha diversity and to be enriched with bacterial taxa known to be pathogenic. Clusters 1 and 3 had higher abundance of taxa considered to be normal respiratory flora or of oral origin, although cluster 1 had lower alpha diversity compared to Cluster 3. Patients with ETA microbiomes aligned with Cluster 2 had significantly worse clinical outcomes including worse 30 day survival rates and longer time to liberation.²⁵ We found no association between these bacterial taxa-based clusters and fungal alpha diversity (Shannon Diversity Index, $p=0.49$; Simpson Diversity Index, $p=0.97$) or the number of total fungal DNA (ITS) reads ($p=0.62$). There was no association between bacterial clusters and the abundance of fungal taxa commonly dominating ETA samples (*Candida albicans*, $p=0.53$; *Candida dubliniensis*, $p=0.71$). We did identify an association between the relative abundance of protective oral-origin and bacterial taxa considered to be normal respiratory flora and fungal alpha diversity. The fungal Shannon diversity index was positively associated with higher proportions of protective bacteria ($R=0.2$, $p=0.01$) and patients assigned to the high fungal alpha diversity subgroup had higher relative abundance

(median [Q1,Q3]: 26% [4%, 58%] $p=0.05$) of protective oral-origin bacteria compared to those in the low fungal alpha diversity subgroup (median [Q1,Q3]: 19% [2%, 37%]).

We examined associations between microbiome characteristics and the relative abundance, presence, and dominance of the most abundant fungal species identified in our samples: *Candida albicans* and *Candida dubliniensis*. Compared to samples with single taxa dominance of *Candida albicans*, samples in which *Candida albicans* was not the only fungal species present had a higher number of bacterial DNA 16S reads ($p=0.05$) and a higher relative abundance of protective oral origin bacteria ($p=0.03$). The relative abundance of *Candida albicans* was negatively associated with the relative abundance of protective oral-origin bacteria ($R=-0.16$, $p=0.05$). In samples where *Candida dubliniensis* was present, the relative abundance of protective bacteria was higher ($p=0.004$) compared to those where *Candida dubliniensis* was not identified and the relative abundance of *Candida dubliniensis* was associated positively with the relative abundance of protective bacteria ($R=0.21$, $p=0.008$).

When considering clinical outcomes in our PGM analyses, we constructed models that included both fungal taxa and bacterial taxa as well as models that excluded bacterial taxa but considered the microbiome through the inclusion of bacterial Shannon diversity index, relative abundance of protective bacteria, and bacterial cluster assignment. With our sample size ($N=226$), the graphical models including both bacterial and fungal taxa with 270 nodes are likely too large to be stable and motivated the construction of the smaller models (57 nodes) excluding bacterial taxa.

We identified 5 bacterial taxa directly associated with mortality at 30 days including *Corynebacteriaceae*, *Desulfobulbus*, *Firmicutes*, *Gammaproteobacteria*, and the bacteria from the *Lachnospiraceae NK4A136* group (Figure 4.3). In our reduced PGM with clinical features,

biomarkers, fungal taxa, and microbiome and mycobiome features, we identified the abundance of *Candida tropicalis* and *S. cerevisiae* and FABP-2 and antibiotic administration as direct links of 30-day mortality (Figure 4.4). The abundance of *Cardiobacterium*, *Corynebacteriaceae*, *Firmicutes*, bacteria from the *Lachnospiraceae NK4A136* group, and *Solobacterium* were directly linked with mortality at 90 days in our PGM (Appendix Figure 32). In the reduced model, we found that mortality at 90 days was directly linked with fungal Shannon diversity index, *S. cerevisiae* abundance, shock, and immunosuppressive use (Appendix Figure 33).

Time to liberation from mechanical ventilation was linked with the abundance of *Gallicola*, *Hungatella*, *Listeria*, *Phyllobacterium*, and *Sutterella* (Appendix Figure 30). Direct links with time to liberation from mechanical ventilation in our condensed probabilistic graphical model excluding bacteria included the clinical cultures positive for fungi, pneumonia, and COPD. The number of ventilator-free days was directly linked with *Coprococcus*, *Listeria*, *Negativicoccus*, *Phyllobacterium*, and *Sutterella* in our model incorporating bacterial taxa and was directly linked with shock and ARDS in our reduced model without bacteria (Appendix Figure 31). ICU length of stay was directly linked with the abundance of bacterial species *Gallicola*, *Hungatella*, *Listeria*, *Phyllobacterium*, and *Sutterella* (Appendix Figure 26). ARDS, pneumonia, a clinical culture positive for fungi, and antibiotic use were directly linked with ICU length of stay in our reduced model (Appendix Figure 27).

4.5 Discussion

While there is substantial *in vitro* research into the relationship between specific bacteria and fungi in single-species environments demonstrating the potential for both synergistic and

antagonistic relationships, very little investigation into the interaction of the lung bacterial microbiome and mycobiome with each other and the role of the host-response in these interactions has been undertaken. The focus of interkingdom interaction research in the lung has focused primarily on cystic fibrosis where relevant microbial crosstalk has been observed both *in vivo* and *in vitro* and is proposed to occur through several different pathways including physical interaction, production of antimicrobial metabolites, quorum sensing molecules, modulation of the host immune response and nutrient exchange through metabolite production.^{73,249,313,386–388} In a large cohort of mechanically-ventilated patients with critical illness and acute respiratory failure, we have utilized network analyses and probabilistic graphical models to identify bacterial taxa, fungal taxa, and microbiome features associated with clinical outcomes. Using culture-independent ITS rRNA and 16S rRNA gene sequencing in ETA non-invasive samples from critically ill patients we demonstrated an association between the number of bacterial DNA (16S) reads and both the fungal Shannon diversity index and Simpson diversity index and showed that the proportion of protective oral-origin bacteria was positively associated with increased fungal Shannon diversity. We identified that in patients dominated by *Candida albicans*, the number of bacterial reads was decreased, and the relative abundance of protective oral origin bacteria was lower in patients who are not dominated by *Candida albicans*. Our findings are complementary to recent studies of the lung microbiome in critical illness that have shown that alterations in the lung microbiota composition, alpha diversity, and burden are associated with alterations in the host-response associated with inflammation and immune system activation, increased mortality at 30 days, longer time to liberation from mechanical ventilation and ventilator-free days and may provide new insights into the role of the broader lung microbiota, including both bacteria and fungi, in critical illness.^{25,237} However, to our knowledge, this is the first study to examine the relationship

between the microbiome, the mycobiome, and the critically ill host and the implications of this relationship on clinical outcomes.

Given the substantial heterogeneity in critical illness and ARDS, identification of attributes that are connected to patient outcomes could be useful for developing prediction models and may offer mechanistic insights and identify risk factors and relationships between microbial communities and host-response which could be useful to develop more personalized approaches to prevention and treatment of ARDS in critical illness. We applied the CausalMGM algorithm to clinical variables, biomarkers, bacterial and fungal taxa, and microbiome features to identify variables that are directly linked to clinical outcomes including mortality at 30 and 90 days, time to liberation from mechanical ventilation, ventilator-free days, and ICU length of stay. These network analyses offer a unique perspective on the relationship on the microbiome-mycobiome-host interaction and provide both validation of recent research into the bacterial communities in ARDS and novel links with outcomes in critical illness.

We identified a direct link between mortality at both 30 and 90 days and gut-associated bacteria including Gammaproteobacteria, *Firmicutes*, and *Lachnospiraceae*. In a cohort of 91 patients with critically ill patients, Dickson et al found that the composition of bacterial communities was driven by the presence of bacteria from the *Lachnospiraceae* family in lower respiratory tract samples and was associated with worse ICU outcomes and strongly predictive of fewer ventilator-free days.²³⁷ Although our model adds validity to Dickson et al's finding that gut-associated bacteria in the lung microbiome is a risk factor for worse outcomes in critical illness, the role of both gut and lung microbiota in the pathogenesis of lung injury in critical illness and ARDS is not yet clear. Previous work by Dickson et al demonstrated that in ARDS the lung microbiome may be enriched with gut-associated bacteria and associated with increased severity

of both systemic and alveolar inflammation.¹²⁶ Panzer et al found that in a cohort of 74 mechanically ventilated trauma patients, ARDS onset was associated with the presence of gut-associated bacteria in ETA samples.³¹⁶ A limitation of Panzer et al and Dickson et al's investigations is difficulty in determining whether the gut-associated bacteria driving negative outcomes in critically ill patients are derived from the lower gastrointestinal tract or through aspiration of the oral-pharyngeal microbiota. Kitsios et al incorporated oral microbiome samples acquired concurrently with ETA samples in a larger cohort of 301 critically ill patients and derived three heterogeneous bacterial clusters based on alpha diversity and taxonomic composition. Patients with low alpha diversity, low abundance of protective oral bacteria, and high pathogen (e.g. *Staphylococcus* and *Pseudomonadaceae*) relative abundance were associated with a hyperinflammatory sub-phenotype, longer time to liberation from mechanical ventilation, and higher mortality at 30 days as compared to patients with higher alpha diversity and more typical oral bacterial communities.²⁵

Candida tropicalis is a direct link to *Lachnospiraceae* in our model. While the interaction between *Candida tropicalis* and *Lachnospiraceae* remains unclear, García et al hypothesized that metabolites of typical gut microbiota may be essential to maintain the virulence of *Candida albicans* and undertook an *in vitro* investigation to determine the impact metabolites of *Lachnospiraceae* on *Candida albicans* ability to form invasive hyphae. In the setting of metabolites from *Lachnospiraceae*, *Candida albicans* had significantly suppressed yeast-to-hyphae transition genes, reduced hyphal differentiation, and growth resulting in an attenuation of damage to epithelial cells.³⁸⁹ In a murine model, invasive candidiasis was associated with a decrease in gut-associated bacteria, including *Lachnospiraceae*, and a corresponding decrease in IL-17A production thought to increase susceptibility to *Candida* infection.³⁹⁰ It is not known if

this relationship is similar between gut-associated bacteria and NAC species; however, overgrowth of NAC has been associated with perturbations in the gut microbiome.³⁹¹ In our reduced model, we discovered a direct link between FABP-2, a validated biomarker of intestinal barrier integrity, and mortality at 30 days suggesting that there could be intestinal permeability leading to translocation of gut microbes (Figure 3.2).²⁰⁶ Our findings encourage continued investigation into the role of the microbiome in respiratory disease and the impact of remote (gut) and proximal (oral) microbiota on the microbiome-mycobiome-host interactions in the lung.

Recent research efforts have focused on better characterizing the heterogeneity in ARDS, sepsis, and critical illness by identifying sub-phenotypes of critically ill patients with different responses to potential treatments and with different outcomes.^{16,22–24,295} These sub-phenotypes have been differentiated through clinical variables and plasma biomarkers indicative of host inflammation and have encouraged research into the biological determinants of increased host inflammation and immune system activation.^{16,24,154,155,192,392,393} As such, we have hypothesized that some portion of the clinical heterogeneity observed in ARDS and critical illness may be accounted for by the consequences of the interaction between the bacteria and fungi comprising the microbiome and mycobiome respectively on the host and through the interaction of both the microbiome and mycobiome with the host's innate immune cells. Our findings underscore the interaction of the lung microbiome and mycobiome as a potentially important contributor to the heterogeneity seen between patients in critical illness.

Our study does have limitations that require further study and validation. This analysis utilized endotracheal aspirate samples from a single time-point and as such we are unable to draw inferences about changes over time in the microbiome, mycobiome, and their interactions as well as clinical outcomes. It may be valuable to construct PGMs based on samples acquired at a later

point in the ICU stay to determine if there are structural differences to the graphical models representing samples taken within 72 hours of intubation. Additionally, this study was conducted in a single tertiary care ICU and should be validated in a wider population to determine the generalizability of our results. To the best of our knowledge, our study represents the first culture-independent study of the role microbiome-mycobiome in critical illness and ARDS; however, PGM analysis requires that data be complete and without missing values, decreasing our sample size for PGM analysis. Models that included all bacterial taxa included more variables than samples and as such this analysis should be interpreted with caution but remain valuable for hypothesis generation.

Our results are based on both 16S and ITS rRNA gene sequencing which is limited in the ability to detect all fungal species using ITS 1 and 2 as a gene target. Additionally, using a culture-independent DNA based analysis limits our ability to analyze the viability of microbes and to determine virulence factors of specific microbiota. However, both bacteria and fungi can present PAMPs to the innate immune cells in the lung which can initiate the inflammatory cascade regardless of the viability of the microbes or ongoing growth.^{250,332} Our samples are from endotracheal aspiration rather than from bronchial alveolar lavage (BAL) samples and limit our ability to determine regional variability of the microbiota in the lung or to study the interactions occurring directly in the alveolar space. We chose to use ETA samples rather than BAL out of a concern for both practice and ethical issues (minimal risk exposure to patients) and due to clinical practice guidelines as well as significant supporting evidence supporting the use of ETA as a reliable comparison to BAL samples.^{334,335}

In summation, in this prospective, observational cohort study of mechanically ventilated patients, we demonstrated an association with the number of bacterial DNA reads and fungal alpha

diversity and between the relative abundance of oral origin bacteria with fungal alpha diversity and identified direct links between gut-associated bacteria in the lung with fungal communities on mortality at 30 and 90 days. These associations are hypothesis-generating for a potential mechanism of biological heterogeneity in critical illness involving interactions between the microbiome-mycobiome and microbiome-mycobiome-host immune system. While the lung broader lung microbiome, including fungi, has been understudied, it is likely that it is an important source of biological variation and clinical heterogeneity among critically ill patients and patients with ARDS and could represent a potential target for the development of preventive therapeutics or treatments for lung injury in critical illness and ARDS. There has been a significant push to identify subphenotypes of ARDS that we could incorporate into future clinical trials with the hope we could utilize predictive enrichment strategies in clinical trial design and enrollment, resulting in improved clinical outcomes. However, the biological mechanisms underlying the differential inflammatory states observed in the subphenotypes are not yet well understood. The interactions between the microbiome with the host have been proposed as a potential source of heterogeneity in the pathogenesis of the inflammatory response seen in ARDS. In the same cohort of patients utilized in this analysis, Kitsios et al found that low alpha diversity and enrichment of bacteria known to be pathogenic in the lung were independently associated with a hyperinflammatory subphenotype and with elevated biomarkers and worse clinical outcomes.²⁵ The analysis undertaken here is an extension of this important work and has considered the respiratory mycobiome and the interaction with both the microbiome and the host as a potential mechanistic source of heterogeneity in critical illness. If these mechanistic differences are proven, the subphenotype becomes an endotype with a biological basis. If a biologically plausible treatment can be successfully targeted to an endotypic mechanism, the endotype becomes a treatable trait.³⁹⁴

Through the continued refinement ARDS endotypes, it is hopeful that we will be able to improve public health through the identification of pharmaceutical treatments and other therapies that will improve outcomes in ARDS through a more precision-medicine like approach. Further investigation of the broader respiratory microbiome with culture-independent next-generation sequencing approaches as well as *in vitro* models of microbiota-microbiota interaction will allow for the continued development of our understanding of the role of the respiratory tract microbiome in critical illness and ARDS.

4.6 Figures

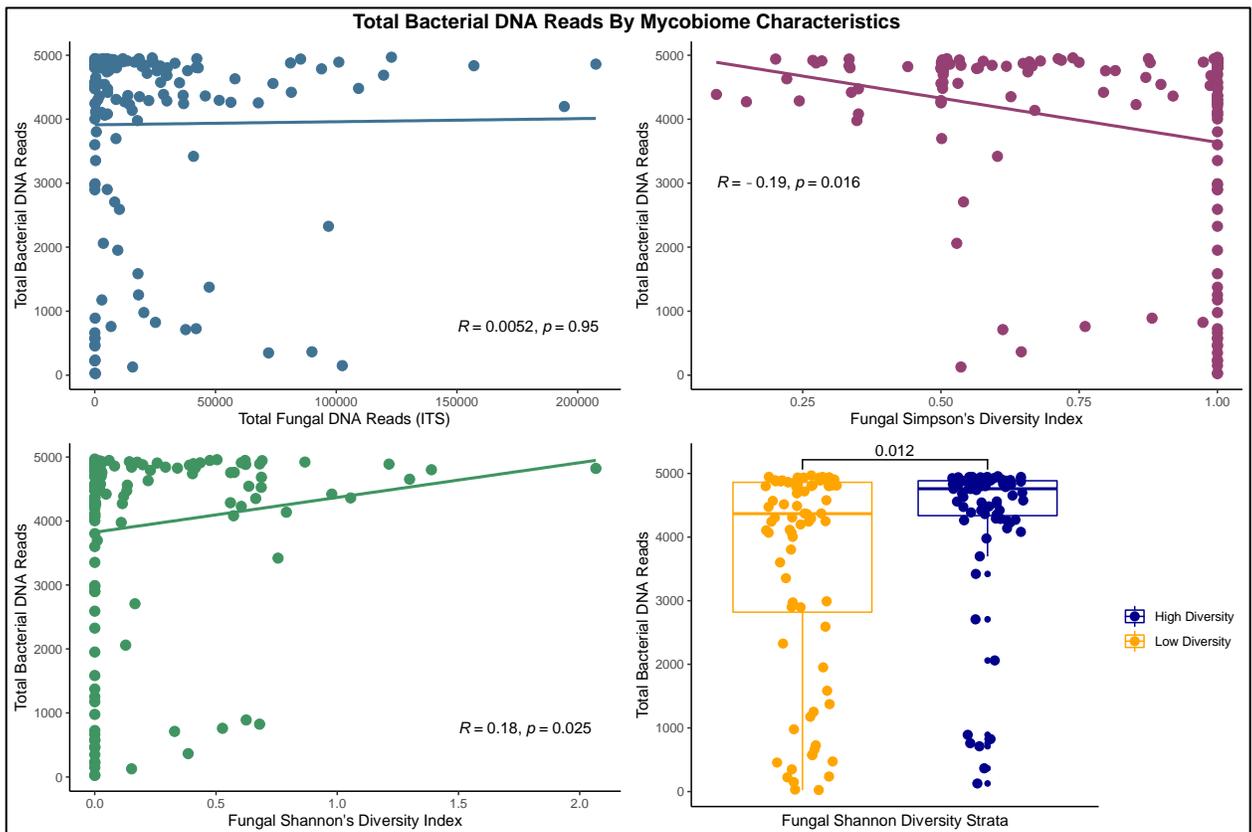


Figure 4.1: Bacterial Burden by Mycobiome Characteristics

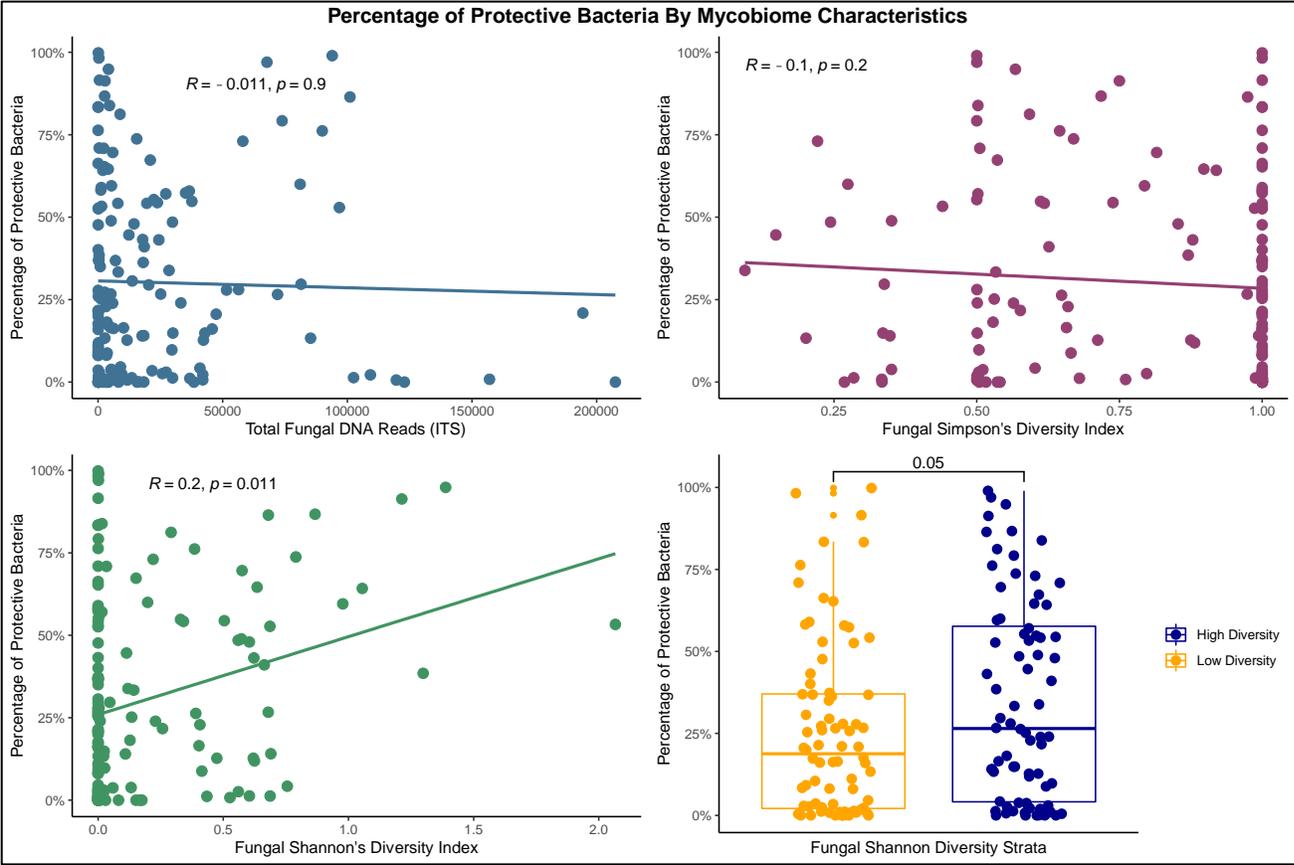


Figure 4.2: Percentage of Protective Bacteria by Mycobiome Characteristics

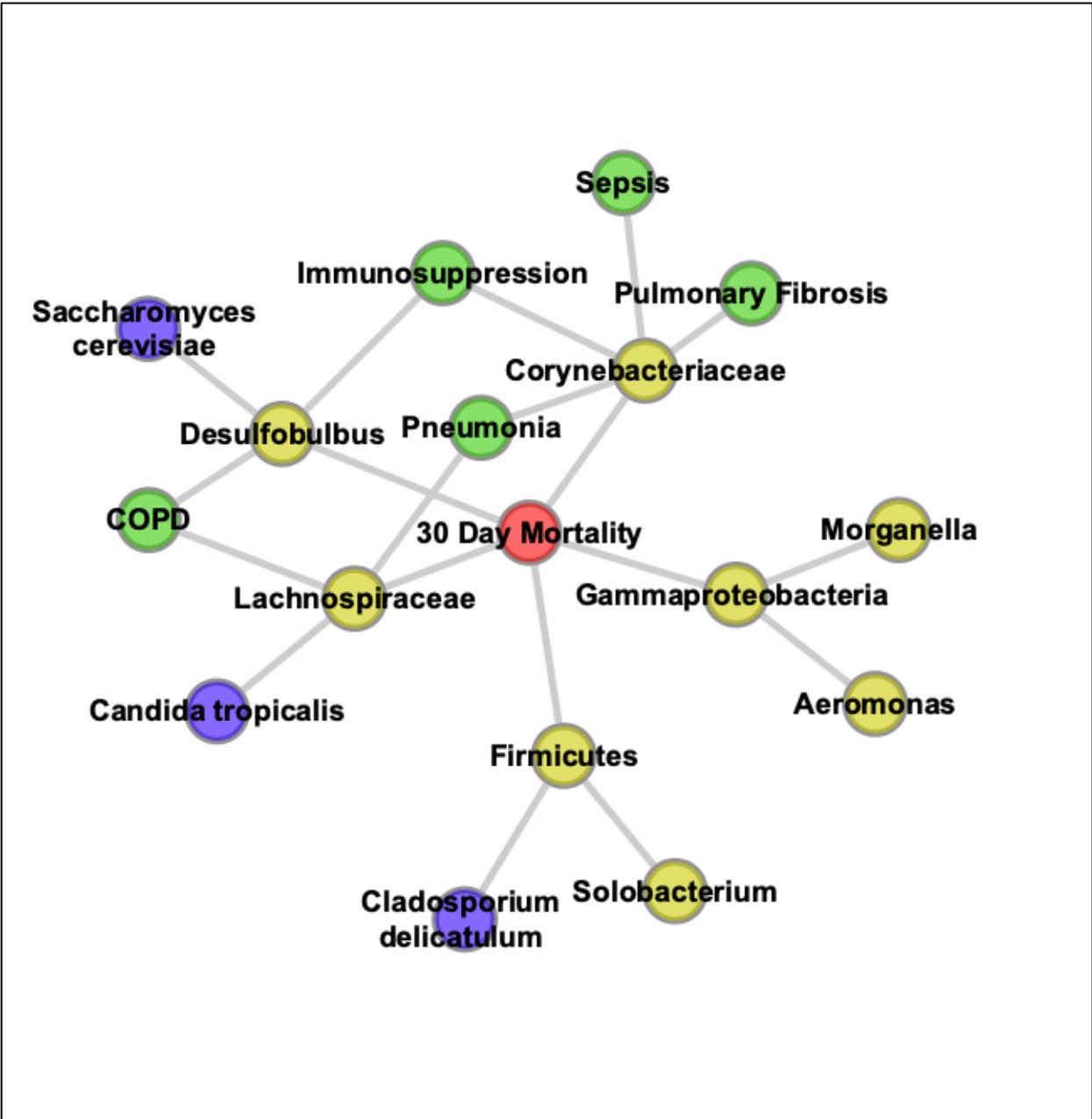


Figure 4.3: Probabilistic Graphical Model Network Analysis Demonstrating First and Second Neighbors of Mortality at 30 Days Including Bacterial Taxa

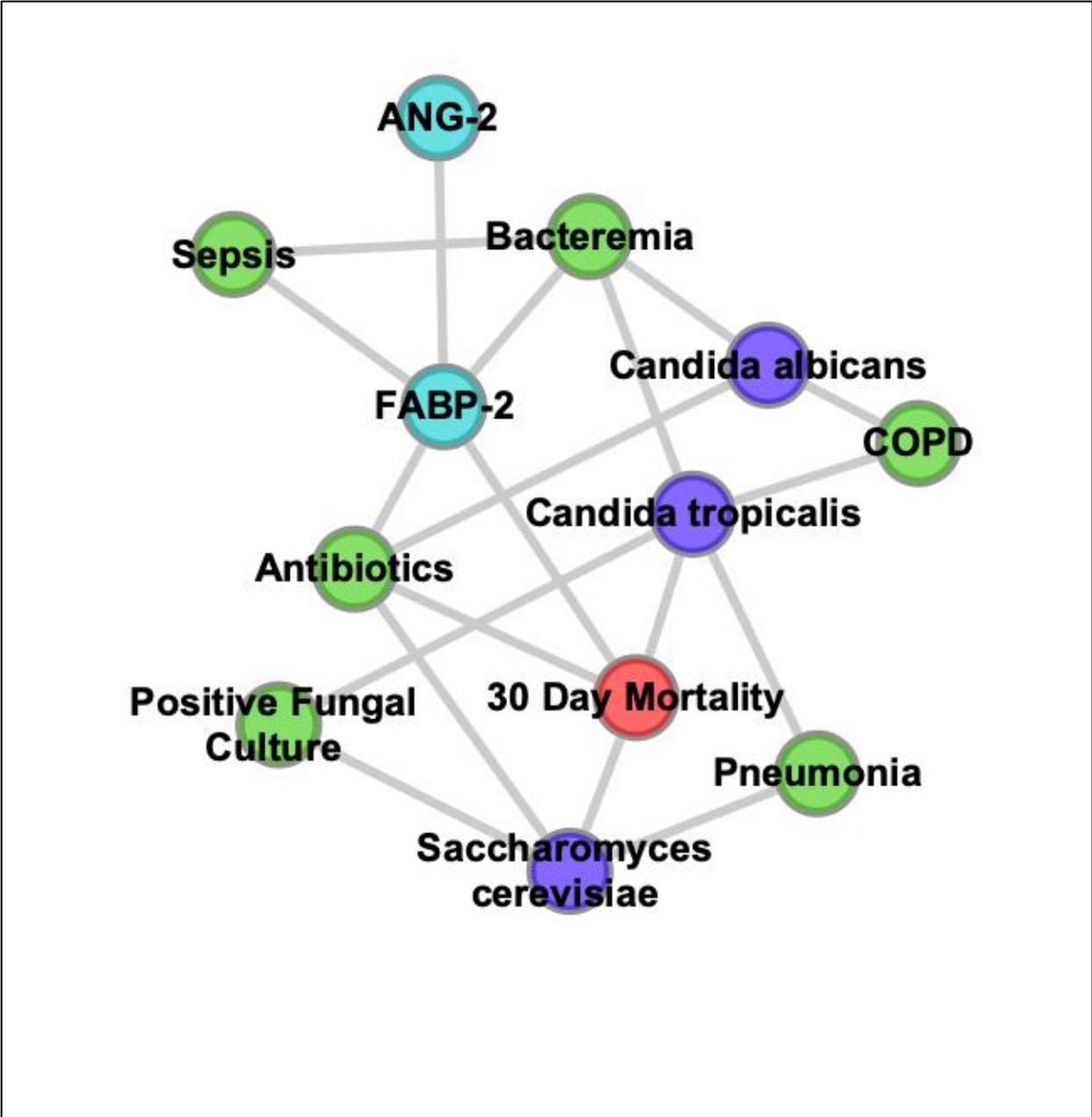


Figure 4.4: Probabilistic Graphical Model Network Analysis Demonstrating First and Second Neighbors of Mortality at 30 Days

5.0 Overall Conclusions and Public Health Significance

5.1 Dissertation Results Summary

This dissertation characterized the lung mycobiome, demonstrated a relationship between mycobiome diversity and biomarkers of host-response and identified associations with mycobiome diversity and composition and clinical outcomes in critically ill patients. It also applied a network analysis approach to identify potential direct links between bacteria and fungi occupying the same niche in the lung and clinical features and outcomes and found hypothesis-generating connections between fungi in the lung and bacteria implicated in worse outcomes in ARDS. Taken together, these manuscripts provide new information about an understudied community of micro-organisms in the lung and allow for a more comprehensive understanding of the role of the microbiota in the lung in critical illness and ARDS.

In Manuscript 1 we demonstrated that we were able to identify a distinct fungal signal in the ETA samples compared to experimental control samples. We found that the lung mycobiome of critically ill patients was very low with more than half of ETA samples containing reads representing a single fungal species. While we identified nearly 100 different fungal species across our samples including known fungal pathogens, we found *Candida* to be the most abundant genus by far.

Manuscript 2 further expanded on the associations of the mycobiome with clinical features and outcomes in critical illness. We constructed alpha diversity subgroups delineating mono-fungal communities from more diverse mycobiomes. We identified that patients classified to the low alpha diversity subgroup had higher levels of biomarkers of host response associated with

innate immune response (IL-10, TNFR-1 and ST-2) and response to infection (pentraxin-3 and procalcitonin) and observed evidence of potential fungal translocation (BDG) in the low alpha diversity subgroup. Using predictor/responder analysis we found that a decrease in diversity was predictive of both shock and ARDS. Patients with low alpha diversity had worse clinical outcomes with increased severity of disease, longer time-to-liberation from mechanical ventilation and a fewer ventilator free days.

We undertook species-level analyses to delineate the contributions of individual species in outcome prediction and identified clusters of samples unified by primarily by a single fungal species, in line with the low diversity and mono-fungal community structure we identified in Manuscript 1. We identified consistent cluster-influencing fungal species associated with antibiotic and immunosuppression use. Additionally, we found that antibiotic use predicted alpha diversity and was correlated with an elevated relative abundance of *Candida albicans*. We observed that a decrease in alpha diversity was predictive of immunosuppression use. These findings are consistent with previous evidence showing that airway colonization with *Candida* in critical illness is associated with antibiotic and immunosuppression use as well as worse clinical outcomes.²⁷⁹

The relationship between antibiotics, fungal diversity and poor clinical outcomes beg the question of what role bacteria are playing in this relationship and Manuscript 3 begins to explore the relationship of the microbiome (bacteria) and the mycobiome with clinical outcomes in our critically ill population. Utilizing 16S rRNA sequencing, we showed that patients in the low fungal alpha diversity subgroup had significantly lower number of bacterial sequencing reads than those in the higher fungal alpha diversity subgroup and that there was a significant positive correlation between the fungal Shannon diversity index and bacterial burden as measured by 16S reads. We

did not identify a relationship between fungal burden (by ITS reads) and bacterial burden, offering additional evidence that the composition of the fungal community may be more important in the bacterial-fungal interkingdom interaction than fungal burden. We identified that the relative abundance of protective oral-origin and bacterial taxa considered to be normal respiratory flora was positively correlated with fungal alpha diversity and that patients in the high fungal alpha diversity subgroup had significantly higher relative abundances of protective bacteria in their lungs. The relative abundance of *Candida albicans* was correlated negatively with protective bacterial abundance and in samples dominated by *Candida albicans* the relative abundance of protective bacteria in the lungs was significantly lower compared to samples not dominated by *Candida albicans*. These findings are consistent with previous evidence showing that airway colonization with *Candida* in critical illness is associated with antibiotic and immunosuppression use as well as worse clinical outcomes and recent work showing that higher bacterial alpha diversity and elevated relative abundance of protective oral-origin bacteria are associated with more ventilator-free days and greater survival in critical illness and ARDS.^{25,279}

We utilized network analyses and probabilistic graphical models to identify direct linkages between the abundance of individual bacteria and fungi with clinical features, biomarkers of host response and clinical outcomes. We identified a direct link between gut-associated bacteria, a biomarker of intestinal epithelial damage, FABP-2 and *Candida tropicalis* with mortality in critically ill patients. Recent studies have demonstrated an association between the relative abundance of gut-associated bacteria with mortality in ARDS but have not demonstrated a mechanism for how gut-associated bacteria enters the lung.^{126,237} Hypotheses for this bacterial migration have included both micro-aspiration and translocation from the intestine due to increased gut permeability. Identification of a link between a validated marker of gut permeability,

gut-associated bacteria and mortality provide additional support for a pathway supporting translocation of bacteria from the gut to the lung and encourage continued research into this line of inquiry.

Recent studies of the lung microbiome in critical illness that have shown that alterations in the lung microbiota composition, decreases alpha diversity, and increases in burden are associated with elevations in biomarkers of host-response, longer time to liberation from mechanical ventilation, fewer ventilator-free days and increased mortality at 30 days and are complementary to our findings in both Manuscript 2 and 3.^{25,237} Our results provide new insights into the role of the broader lung microbiota, including both bacteria and fungi, in critical illness. Use of network analyses allow for a unique perspective on the relationship on the microbiome-mycobiome-host interaction and provide both validation of recent research into the bacterial communities in ARDS and novel links with outcomes in critical illness.

5.2 Dissertation Public Health Significance

About 3 million patients per year are diagnosed with ARDS worldwide and 35-50% of those do not survive. Patients who do survive have a significantly impaired quality of life due to severe morbidity and disability.⁴ In the US, there are estimated 191,600 cases of critical illness requiring mechanical ventilation and 141,500 cases of ARDS. This patient population has an extremely high mortality rate and in the U.S. annually it is estimated that there are 74,500 deaths due to critical illness requiring mechanical ventilation and 59,000 ARDS-related deaths.²⁹

Patients with ARDS often have extended hospitalizations and require a significant amount of healthcare resources. Accounting for over 10% of intensive care unit admissions, patients with

ARDS require an estimated 2.7 million hospital days and over 1.6 million ICU days. Critically ill patients comprise an additional 23-30% of ICU admissions and account for another 3.6 million hospital days and 2.2 million ICU days.

These estimates were made before the COVID-19 pandemic and do not reflect the dramatic increase in ARDS related ICU admissions since March 2020. Rates of ARDS in COVID-19 vary regionally, but as of August 2020, an estimated one-third (33%) of patients hospitalized with COVID-19 develop ARDS and an additional 16% require invasive mechanical ventilation.³⁹⁵ As such, we expect rates of ARDS and critical illness requiring mechanical ventilation to remain elevated until the pandemic is resolved.

ARDS-related health care costs are approximately 4 times higher compared to those in non-ARDS critical illness during the initial hospitalization (\$117,137 vs. \$25,199 per person) and in the initial year following discharge (\$82,749 vs. \$22,670). Approximately one third (34%) of the estimated 100,000 patients who survive their acute illness are able to be discharged directly home, emphasizing the significant morbidity and long term health impacts of ARDS and critical illness including cognitive abnormalities, physical weakness, anxiety and depression, post-traumatic stress disorder and pulmonary disease.^{27,29-32} This profound morbidity not only severely impacts patient quality of life, but comes with ongoing direct health care costs estimated to be approximately \$50,000 for years 2-5 after the initial hospital discharge.³⁹⁶

Supportive care remains the only therapeutic option for patients with ARDS.^{6,7} In animal models, successful treatment of ARDS has been demonstrated; however, no effective therapies have been identified in humans. Our understanding of the biology and pathophysiology of ARDS are well developed and likely is not the driving factor behind our inability to identify a broadly efficacious treatment for ARDS. Substantial progress has been made in identifying pathogenic

contributors to the syndrome and improvements in the management of sedation, ventilatory support, sedation use and preventative therapies have contributed to improving the survival of ARDS.^{397–401} Given the substantial morbidity and mortality associated with ARDS, the need to identify successful treatments for ARDS is indisputable; however, there have been many well designed clinical trials of seemingly promising treatments that have not been able to identify a pharmacotherapy for ARDS.^{4,398,402–406}

There has been a significant push to identify phenotypes of ARDS on the basis of clinical, physiologic, biologic and microbiologic criteria that we could incorporate into future clinical trials with the hope that by identifying subsets of patients that may be more responsive to particular treatment strategies we could utilize predictive enrichment strategies in clinical trial design and enrollment, resulting in improved clinical outcomes. By focusing on specific phenotypes of ARDS we would be able to further homogenize patients being enrolled in clinical trials and allow for the development of a more precision medicine-based strategy in ARDS .

Current efforts in focused on identifying differences in patients with ARDS to determine subphenotypes within the syndrome have consistently identified two distinct group of patients: hyper- and hypoinflammatory subgroups.¹³ An increase in inflammation and an aberrant immune response have long been considered a hallmark of ARDS and biomarkers associated with host response have been consistently associated with lung damage and poor outcomes in patients with ARDS.^{17–21} Biomarkers of inflammation have been elevated in patients with differential responses to treatment with statins, fluid management and positive end-expiratory pressure levels.^{14–16,22,23}

With the intention of applying these subphenotypes in a clinical setting, a several clinical models for prediction of subphenotype assignment have been proposed.^{14–16,24} However, the biological mechanism underlying these differential inflammatory states is not yet well understood.

The interaction of the bacterial members of the microbiome with the host have been proposed as a potential source of heterogeneity in the pathogenesis of the inflammatory response seen in ARDS.^{25,114,126,237,244,319,407,408} In the same cohort of patients utilized in this dissertation, Kitsios et al found that low alpha diversity and enrichment of bacteria known to be pathogenic in endotracheal aspirate samples was independently associated with a hyperinflammatory subphenotype and was associated with elevated biomarkers and worse clinical outcomes.²⁵ Our findings highlight the respiratory microbiome as a previously understudied but potentially important contributor to patient-level heterogeneity in critical illness. Subphenotyping efforts for ARDS focused on modeling clinical variables and plasma biomarkers have identified subsets of patients with differential treatment-responses and outcomes.^{13,24,324}

Continued efforts in the subphenotyping of ARDS would allow for the enrichment of clinical trials in order to identify treatments that would improve outcomes for specific subsets of patients based on their clinical endotype. Prognostic enrichment involves enriching trial enrollment for patients with a high probability of an actionable outcome of interest, such as mortality, ventilator-free days or days alive and free of organ dysfunction (vasopressors, mechanical ventilation, dialysis). Prognostic enrichment aims to increase the frequency of the outcome of interest, which may increase the power to detect a beneficial treatment effect for a given sample size. In ARDS, efforts at prognostic enrichment have primarily focused on physiologic variables. Prognostic biomarkers may also be used for enrichment. Bedside measurement of biomarker levels to identify a hyperinflammatory phenotype could potentially identify ARDS patients with higher mortality. Various ICU risk scores have been tested unsuccessfully because they are not specific for ARDS and other clinical syndromes and because patients at the highest risk may not benefit from therapy. Predictive enrichment involves the

enrollment of patients who are more likely to respond to a given treatment based on the mechanism of benefit and thus is more specific than prognostic enrichment. Predictive enrichment has transformed cancer treatment trials, wherein analysis of genetic mutations in an individual's tumor is used to predictively enrich for enrollment in trials that mechanistically target these mutations. Enrichment strategies have both advantages and disadvantages. The major theoretical advantage of both prognostic and predictive enrichment is to increase the signal-to-noise ratio, reducing sample size and increasing the likelihood of detecting a therapeutic benefit. Predictive enrichment also may lead to a larger effect size. By excluding patients less likely to benefit from a specific treatment, predictive enrichment may also improve the benefit-to-risk ratio of a trial since patients who are unlikely to benefit from a therapy are still at risk of its adverse effects. The post-hoc application of the hypo and hyper inflammatory subphenotypes to the HARP-2 trial of simvastatin in ARDS by Dr. Carolyn Calfee and her group represents an example of predictive enrichment.^{14,409} The hyper-inflammatory phenotype of ARDS was associated with reduced mortality with simvastatin treatment in retrospective analysis of trial data, an effect that was not seen in the hypo-inflammatory phenotype nor in the trial as a whole. A future trial of simvastatin that enriches for the hyperinflammatory subphenotype might be more likely to show a treatment benefit. Understanding the mechanisms behind the differential inflammatory response observed will allow us to target treatments more precisely to affect those mechanisms. This work adds to public health through developing a better understanding of the biological mechanisms underlying heterogeneity in clinical characteristics and outcomes in critical illness and ARDS. Through the continued refinement of predictive models for subphenotyping of ARDS, it is hopeful that we will be able to identify pharmaceutical treatments and other therapies that will improve outcomes in ARDS through a more precision-medicine like approach.

Our characterization of the lung mycobiome and identification of the associations between the mycobiome and clinical outcomes in critical illness and ARDS offers a potential mechanistic pathway underlying the heterogeneity in ARDS and may represent an exciting line of inquiry in ARDS subphenotyping. This work adds to public health through developing a better understanding of the biological mechanisms underlying heterogeneity in clinical characteristics and outcomes in critical illness and ARDS. Through the continued refinement of predictive models for subphenotyping of ARDS, it is hopeful that we will be able to identify pharmaceutical treatments and other therapies that will improve outcomes in ARDS through a more precision-medicine like approach.

5.3 Future Directions

This dissertation was conducted using endotracheal aspirates from a single time-point, and thus cannot draw inferences about longitudinal evolution of microbial communities and host outcomes. As a part of the study protocol these samples were collected under additional samples have been collected at regular intervals following enrollment and provide an opportunity to extend this analysis to include longitudinal changes in both the mycobiome, microbiome, microbiome-mycobiome interactions and the resultant impact on the host. To our knowledge, this dissertation represents the first study of the lung mycobiome in critical illness and ARDS and represents a substantial increase in our understanding of the role of fungi in the host response in ARDS. However, the logical next steps for this study would be to consider the role of change over time in these relationships and to determine if changes in the broader lung microbiome contribute to disease progression over time.

While subphenotyping efforts remain a very promising advance in ARDS research, the applicability of biomarker testing and microbiome profiling to clinical testing remain inaccessible. Most of the biomarker tests are not available in a timely manner from hospital clinical labs and as such are not useful to clinicians. Similarly, microbiome analysis has primarily involved a lengthy process of sample preparation, sequencing and analytical pipeline processing. In order to provide clinical applicability to our inclusion of the microbiome in future predictive models, we must build real-time pipelines for microbiome analysis. Technological advancements such as the portable Oxford Nanopore Sequencer MinION has been shown to be a reliable and powerful tool for potential point-of-care metagenomic sequencing of samples. In work from Kitsios et al, the Oxford Nanopore sequencing technology was demonstrated to show excellent concordance with 16S sequencing and technical feasibility and proof-of-concept for utilizing point-of-care metagenomics.⁴¹⁰

Certainly, this analysis of this cohort could be expanded including examining concordance between clinical cultures and next generation sequencing results, investigating our sequencing method further by comparing our results to metagenomic sequencing, including samples from other body sites and longitudinal samples in our analysis. However, this work represents one of the only, and the largest, investigation of the mycobiome in critical illness. To expand our ability to consider the microbiome and mycobiome as part of an endotype of ARDS that could become potentially actionable, investigation of host–microbe interactions will be necessary to allow us to investigate the nature of the functional role microbes play in host response and ARDS pathogenesis. The focus of future mechanistic microbiome studies should be directed towards understanding interactions between the host and microbes, using not only metagenomics, but also metabolomics and metatranscriptomics, which can be integrated using network-based approaches.

To delineate if the inflammatory signal is first generated by the host or provoked by the microbiota, cellular studies should come into play. Isolation of bacterial and fungal strains of interest from the respiratory tract and consequent co-culture with immune cells, may be useful to pick apart cellular mechanisms involved in a specific host–microbe interaction. These *in vitro* studies of co-culture of bacterial and fungal species of interest with lung macrophages will provide important evidence for determining the mechanistic pathways in which crosstalk between the lung microbiota and airway innate immune cells occurs. In tandem with bacterial 16S rRNA sequencing from BAL samples from patients experiencing lung transplant rejection, Bernasconi et al have utilized a model of bacterial-fungal co-culture with lung macrophages to assess expression of genes known for their involvement in innate immune cell function and for levels of expression of biomarkers of inflammation.^{411,412} A similar mechanistic design could be employed in critical illness and ARDS to allow for a greater understanding of the crosstalk between specific bacteria and fungi and the impact on macrophage activation and expression of inflammatory markers that could be extrapolated to provide a deeper understanding of the interactions between the microbiome-mycobiome and host in ARDS and critical illness.

5.4 Figures

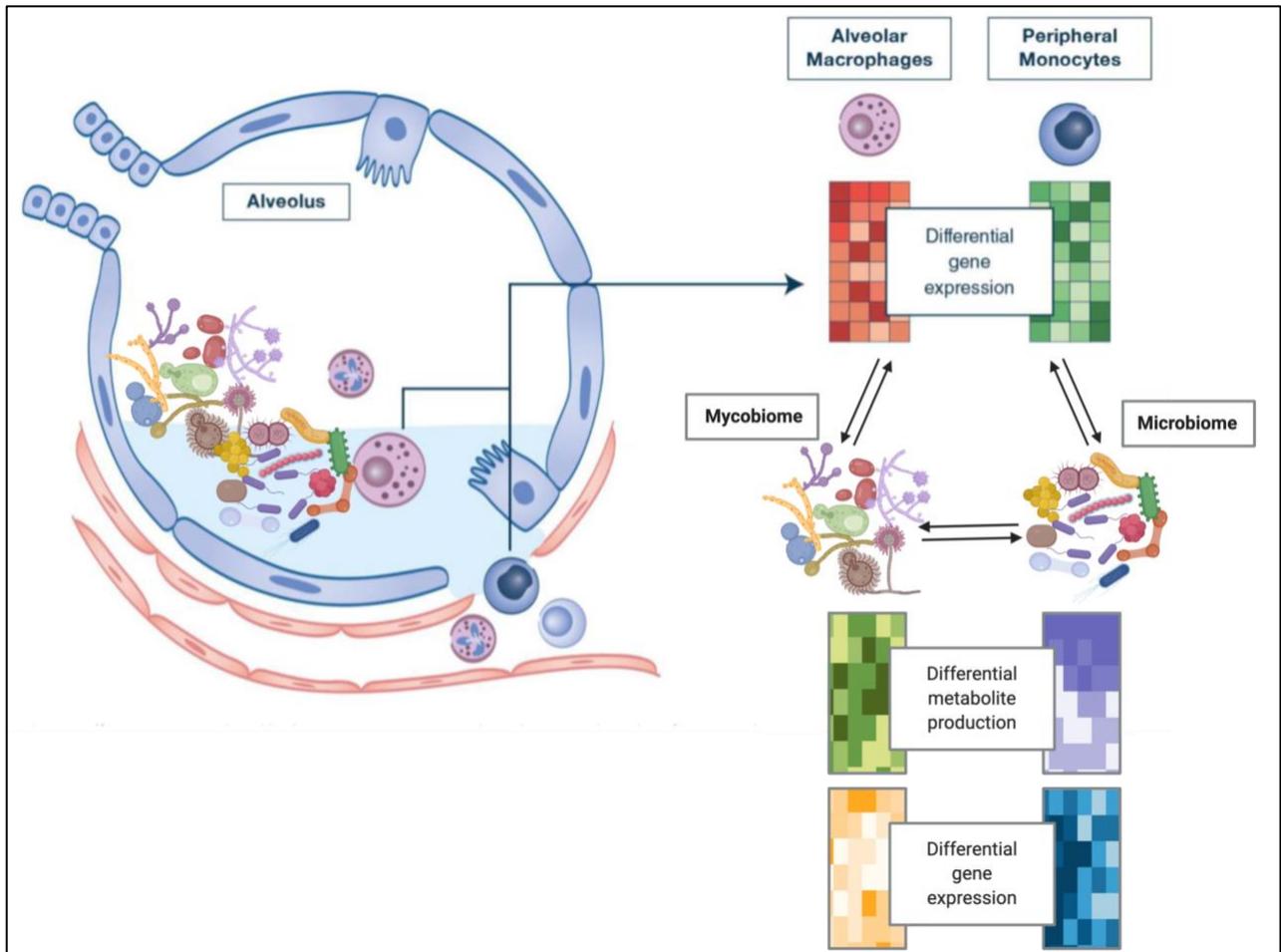


Figure 5.1: Visualization of Future Mechanistic Studies of Host-Microbiome Interactions in Critical Illness

Appendix A.1 : Manuscript 2

Appendix A.2 Tables

Appendix Table 1: Associations Between Clinical Outcomes and Shannon Index

	Unadjusted Odds Ratio (95% CI)	P-Value	Adjusted Odds Ratio (95% CI)	P-Value
ARDS Diagnosis	4.11 (1.19, 20.71)	0.05	6.94 (1.59, 43.12)	0.02
Ventilator Free Days	0.97 (0.95, 0.99)	0.02	0.97 (.95, 0.99)	0.04
ICU Length of Stay	1.50 (1.11, 2.04)	0.01	1.55 (1.22, 2.13)	0.01

Appendix Table 2: Time To Event Analysis Between Clinical Outcomes and Shannon Index

	Unadjusted Hazards Ratio (95% CI)	P-Value	Adjusted Hazards Ratio (95% CI)	P-Value
Time-to-Liberation from Mechanical Ventilation	2.02 (1.36, 3.00)	<0.001	1.88 (1.24, 2.86)	0.003
Survival at 30 Days	0.20 (0.05, 0.72)	0.01	0.28 (0.08, 0.93)	0.05
Survival at 90 Days	0.17 (0.04, 0.61)	0.007	0.23 (0.06, 0.84)	0.03

Appendix Table 3: Associations with Biomarkers and Shannon Index

Innate Immune Response	Unadjusted		Adjusted	
	Odds Ratio (95% CI)	P-Value	Odds Ratio (95% CI)	P-Value
IL-6, pg/ml	0.99 (0.97, 1.02)	0.72	0.99 (0.98, 1.02)	0.85
IL-8, pg/ml	0.96 (0.93, 0.99)	0.02	0.97 (0.94, 1.00)	0.09
IL-10, pg/ml	0.96 (0.93, 0.99)	0.004	0.96 (0.93, 0.99)	0.006
TNFR-1, pg/ml	0.96 (0.92, 0.99)	0.03	0.97 (0.94, 1.07)	0.11
ST-2, pg/ml	0.94 (0.91, 0.98)	<0.001	0.95 (0.92, 0.98)	0.005
Fractalkine, pg/ml	0.98 (0.95, 1.03)	0.09	0.98 (0.96, 1.01)	0.20
Host Response to Infection				
Procalcitonin, pg/ml	0.97 (0.94, 0.99)	0.009	0.97 (0.95, 0.99)	0.04
Pentraxin-3, pg/ml	0.96 (0.93, 0.98)	<0.001	0.96 (0.94, 0.99)	0.003
Epithelial Injury				
RAGE, pg/ml	0.98 (0.94, 1.03)	0.44	1.00 (0.96, 1.05)	0.99
Endothelial Injury				
Angiopoietin-2, pg/ml	0.96 (0.92, 0.98)	0.04	0.97 (0.93, 1.01)	0.14
Fungal Translocation				
Plasma (1,3)- β -d-glucan, pg/ml	0.94 (0.90, 0.99)	0.02	0.95 (0.91, 0.99)	0.03
Plasma (1,3)- β -d-glucan greater than 60 pg/ml	0.90 (0.80, 1.02)	0.08	0.91 (0.81, 1.02)	0.10
Intestinal Permeability				
Fatty Acid-Binding Protein, pg/ml	1.01 (0.96, 1.05)	0.74	1.01 (0.97, 1.05)	0.66

Appendix Table 4: PERMANOVA Associations with Covariates and Group Variables

Predictor	R ²	P-Value
Age	0.02	0.006
Gender	0.01	0.11
Antibiotic Use	0.01	0.16
Immunosuppression	0.003	0.73
COPD	0.005	0.56
Respiratory Culture Positive for Fungi	0.003	0.86
Pneumonia	0.02	0.05
Sepsis	0.006	0.37
Bacteremia	0.01	0.05
Aspiration	0.007	0.29
LIPS Score	0.002	0.96
PaO ₂ :FiO ₂ ratio	0.003	0.81
Shock	0.005	0.53
ARDS Risk Status	0.006	0.36
Acute Kidney Injury	0.005	0.53

Appendix Table 5: Results of the HCAML R for Variable Grouping

Group Variable	Optimal Cluster Cutoff (Calinski-Harabasz criteria)	Cluster Association and Direction
Aspiration	3	Cluster 2 (-)
Antibiotics	3	Cluster 3 (-)
COPD	4	Cluster 3 (+)
Shock	4	Cluster 1 (-) Cluster 4 (+)
Acute Kidney Injury	5	Cluster 4 (-) Cluster 5 (+)
Gender	7	Cluster 1 (-) Cluster 2 (+) Cluster 7 (+)
Age	8	Cluster 1 (+) Cluster 2 (-) Cluster 3 (-)
Bacteremia	8	Cluster 1 (+)
Pneumonia	8	Cluster 1 (+)
Fungal Culture	8	Cluster 2 (+)

Appendix Table 6: Clusters by Covariates and Group Variables Influencing Species

Group Variable	Group	Cluster Influencers (Unifiers)
Antibiotics (Yes/No)	Yes	<i>Candida albicans</i> , <i>Candida dubliniensis</i> , <i>Pneumocystis jirovecii</i> , <i>Kabatiella lini</i> , <i>Hyphodermella rosae</i> , <i>Candida metapsilosis</i>
Gender	Female	<i>Candida albicans</i> , <i>Candida tropicalis</i> , <i>Saccharomyces cerevisiae</i> , <i>Candida parapsilosis</i> , <i>Pneumocystis jirovecii</i> , <i>Hyphodermella rosae</i> , <i>Ramularia eucalypti</i> , <i>Naganishia adeliensis</i> , <i>Dentipellis fragilis</i> , <i>Trametes versicolor</i> , <i>Botryotrichum atrogriseum</i>
Shock (Yes/No)	Yes	<i>Saccharomyces cerevisiae</i> , <i>Candida parapsilosis</i> , <i>Pneumocystis jirovecii</i> , <i>Kabatiella lini</i> , <i>Rhodotorula</i> <i>mucilaginoso</i> , <i>Hyphodermella rosae</i> , <i>Meyerozyma</i> <i>caribbica</i> , <i>Valsa pini</i> , <i>Ramularia eucalypti</i> , <i>Mycoacia</i> <i>fuscoatra</i> , <i>Candida metapsilosis</i> , <i>Aspergillus</i>

		<i>penicillioides, Cladisporium sphaerospermum, Bannoa ogasawarensis, Hyphodontia microspora</i>
Bacteremia (Yes/No)	Yes	<i>Candida albicans, Saccharomyces cerevisiae, Cladosporium delicatulum, Rhodotorula mucilaginosa, Aspergillus penicillioides, Bannoa ogasawarensis, Phlebia chrysocreas, Resinicium pinicola, Oxyporus corticola, Ischnoderma resinosum</i>
COPD (Yes/No)	Yes	<i>Candida albicans, Candida tropicalis, Candida parapsilosis, Cladosporium delicatulum, Schizophyllum commune, Ramularia eucalypti, Aspergillus penicillioides, Cladosporium sphaerospermum, Phlebia chrysocreas, Resinicium pinicola, Oxyporus corticola, Hyphodontia microspora</i>
Sepsis (Yes/No)	Yes	<i>Candida albicans, Candida tropicalis, Saccharomyces cerevisiae, Cladisporium delicatulum, Rhodotorula mucilaginosa, Valsa pini, Aspergillus penicillioides, Bannoa ogasawarensis, Ischnoderma resinosum</i>
LIPS Score (Greater than/Less than 5)	Less than 5	<i>Candida tropicalis, Candida parapsilosis, Cladosporium delicatulum, Schizophyllum commune, Lycoperdon pyriforme, Rigidoporus pouzarii, Curvularia trifolii, Dentipellis fragilis, Oxyporus corticola, Botryotrichum atrogriseum</i>
ARDS Status (ARDS, At Risk, Not At Risk)	ARDS	<i>Candida albicans, Candida dubliniensis, Pneumocystis jirovecii, Kabatiella lini, Cladosporium delicatulum</i>
	Not At Risk	<i>Candida dubliniensis, Candida tropicalis, Saccharomyces cerevisiae, Candida parapsilosis, Schizophyllum commune, Lycoperdon pyriforme, Rigidoporus pouzarii, Phlebia chrysocreas, Curvularia trifolii, Dentipellis fragilis, Oxyporus corticola, Trametes versicolor, Botryotrichum atrogriseum, Hyphodontia microspora</i>
Immunosuppression (Yes/No)	Yes	<i>Candida albicans, Candida parapsilosis, Pneumocystis jirovecii, Cladosporium delicatulum, Hyphodermella rosae, Meyerozyma caribbica, Candida metapsilosis, Ischnoderma resinosum</i>
Pneumonia (Yes/No)	Yes	<i>Candida albicans, Candida tropicalis, Kabatiella lini, Cladosporium delicatulum, Hyphodermella rosae, Ramularia eucalypti, Mycoacia fuscoatra, Candida metapsilosis, Cladosporium sphaerospermum, Piptoporus betulinus, Trametes versicolor, microspore, Ischnoderma resinosum</i>
	No	<i>Candida dubliniensis, Pneumocystis jirovecii</i>
Pneumonia (Yes/No)	No	<i>Candida dubliniensis, Candida tropicalis, Saccharomyces cerevisiae, Hyphodermella rosae, Ramularia eucalypti, Rigidoporus pouzarii, Naganishia adeliensis, Plectosphaerella oratosquillae, Piptoporus betulinus, Phlebia</i>

		<i>chrysocreas, Curvularia trifolii, Resinicium pinicola, Oxyporus corticola, Trametes versicolor, Botryotrichum atrogriseum, Peniophora lycii</i>
Aspiration (Yes/No)	Yes	<i>Candida albicans, Candida tropicalis, Candida parapsilosis, Pneumocystis jirovecii, Verticillium dahliae, Mycoacia fuscoatra</i>
PaO2:FiO2 Ratio (Mild, Moderate, Severe)	Mild	<i>Candida tropicalis, Rhodotorula mucilaginosa, Rigidoporus pouzarii, Aspergillus penicillioides, Cladosporium sphaerospermum, Oxyporus corticola, Trametes versicolor, Hyphodontia microspora</i>
	Severe	<i>Kabatiella lini, Cladosporium delicatulum, Schizophyllum commune, Hyphodermella rosae, Meyerozyma caribbica, Lycoperdon pyriforme, Ramularia eucalypti, Mycoacia fuscoatra, Candida metapsilosis, Naganishia adeliensis, Phlebia chrysocreas, Resinicium pinicola, Oxyporus corticola, Botryotrichum atrogriseum</i>
Fungal Culture (Yes/No)	Yes	<i>Candida albicans, Candida dubliniensis, Candida parapsilosis, Pneumocystis jirovecii, Verticillium dahliae, Candida metapsilosis</i>
	No	<i>Candida tropicalis</i>

Appendix Table 7: Demographics and Medical History by ARDS Status

Demographics	ARDS N= 47	No ARDS N= 179	P-Value
Age, median [Q1, Q3], yrs.	45.5 [33.7, 63.5]	61.7 [50.7, 70.6]	<0.001
Males, N (%)	23 (48.9)	98 (54.7)	0.59
BMI, median	28.3 [23.8, 33.9]	29.2 [25.5, 36.0]	0.25
Medical History			
Diabetes, N (%)	11 (23.4)	64 (35.8)	0.15
COPD, N (%)	7 (14.9)	50 (27.9)	0.1
Immunosuppression, N (%)	17 (36.2)	39 (21.8)	0.07
Chronic cardiac failure, N (%)	4 (8.5)	29 (16.2)	0.27
Chronic kidney disease, N (%)	7 (14.9)	41 (22.9)	0.32
Pulmonary Fibrosis, N (%)	8 (17.0)	10 (5.6)	0.02

Appendix Table 8: Clinical Characteristics by ARDS Status

Clinical Characteristics	ARDS N= 47	No ARDS N= 179	P-Value
Systemic Antibiotics during ICU admission prior to sampling, N (%)	46 (97.9)	149 (83.2)	0.02
Respiratory Culture Positive for Culture, N (%)	22 (46.8%)	29 (16.2%)	<0.001

WBC, median [Q1, Q3], x 10 ⁹ per liter	12.5 [8.90, 18.1]	11.8 [9.10, 16.8]	0.80
Risk factors for ARDS			
Pneumonia, N (%)	34 (72.3)	50 (27.9)	<0.001
Sepsis, N (%)	7 (14.9)	27 (15.1)	1
Aspiration, N (%)	9 (19.1)	25 (14.0)	0.51
LIPS score, median [Q1, Q3]	6.50 [5.00, 7.75]	5.00 [3.50, 6.50]	<0.001

Appendix Table 9: Severity Of Illness by ARDS Status

Severity of Illness	ARDS N= 47	No ARDS N= 179	P-Value
SOFA score, median [Q1, Q3]*	7.00 [5.00, 9.00]	6.00 [4.00, 8.50]	0.05
PaO ₂ :FIO ₂ ratio, median [Q1, Q3], mmHg	134 [86.0, 182]	188 [133, 234]	<0.001
Plateau pressure, median [Q1, Q3], cm	23.0 [20.0, 30.0]	18.0 [15.8, 23.0]	<0.01
Shock, N (%)	22 (46.8)	69 (38.5)	0.39
Acute Kidney Injury, N (%)	34 (72.3)	107 (59.8)	0.15

Appendix Table 10: Clinical Outcomes by ARDS Status

Clinical Outcomes	ARDS N= 47	No ARDS N= 179	P-Value
Duration of mechanical ventilation, median [Q1, Q3], days	8.00 [5.50, 13.0]	5.00 [3.00, 8.00]	<0.001
ICU LOS, median [Q1, Q3], days	9.00 [8.00, 17.0]	7.00 [4.50, 12.0]	<0.001
VFD, median [Q1, Q3], days	15.0 [0, 21.5]	22.0 [0, 25.0]	<0.001
30 Day mortality, N (%)	14 (29.7)	44 (24.5)	<0.001
90 Day mortality, N (%)	16 (34.1)	47 (26.2)	<0.001

Appendix Table 11: Demographics and Medical History by Alpha Diversity Subgroup in ARDS

Demographics	Low Diversity N= 30	High Diversity N= 17	P-Value
Age, median [Q1, Q3], yrs.	49.4 [33.6, 61.3]	41.7 [34.7, 65.5]	0.96
Males, N (%)	15 (50%)	8 (47.1%)	1
BMI, median	28 [23.0, 34.5]	30 [24.2, 32.2]	0.92
Medical History			
Diabetes, N (%)	7 (23.3%)	4 (23.5%)	1
COPD, N (%)	4 (13.3%)	3 (17.6%)	1
Immunosuppression, N (%)	15 (50%)	2 (11.8%)	0.02
Chronic cardiac failure, N (%)	2 (6.7%)	2 (11.8%)	0.95

Chronic kidney disease, N (%)	5 (16.7%)	2 (11.8%)	0.98
Pulmonary Fibrosis, N (%)	7 (23.3%)	1 (5.9%)	0.26

Appendix Table 12: Clinical Characteristics by Alpha Diversity Subgroup in ARDS

Clinical Characteristics	Low Diversity N= 30	High Diversity N= 17	P-Value
Systemic Antibiotics during ICU admission prior to sampling, N (%)	17 (56.7%)	10 (58.8%)	0.77
Respiratory Culture Positive for Culture, N (%)	15 (50.0%)	7 (41.2%)	0.78
WBC, median [Q1, Q3], x 10 ⁹ per liter	11.1 [7.60, 17.2]	11.1 [7.60, 17.2]	0.32
Risk factors for ARDS			
Pneumonia, N (%)	20 (66.7%)	14 (82.4%)	0.42
Sepsis, N (%)	7 (23.3%)	0 (0%)	0.08
Aspiration, N (%)	7 (23.3%)	2 (11.8%)	0.56
LIPS score, median [Q1, Q3]	6.75 [6.00, 8.50]	5.50 [4.50, 5.50]	0.002

Appendix Table 13: Severity of Illness by Alpha Diversity Subgroup in ARDS

Severity of Illness	Low Diversity N= 30	High Diversity N= 17	P-Value
SOFA score, median [Q1, Q3]*	7.50 [6.0, 10.8]	6.00 [5.0, 7.0]	0.02
PaO ₂ :FIO ₂ ratio, median [Q1, Q3], mmHg	157 [86.3, 182]	130 [87, 167]	0.83
Plateau pressure, median [Q1, Q3], cm	26.0 [21.0, 30.3]	21.0 [18, 25]	0.06
Shock, N (%)	18 (60.0%)	4 (23.5%)	0.04
Acute Kidney Injury, N (%)	23 (76.7%)	11 (64.7%)	0.59

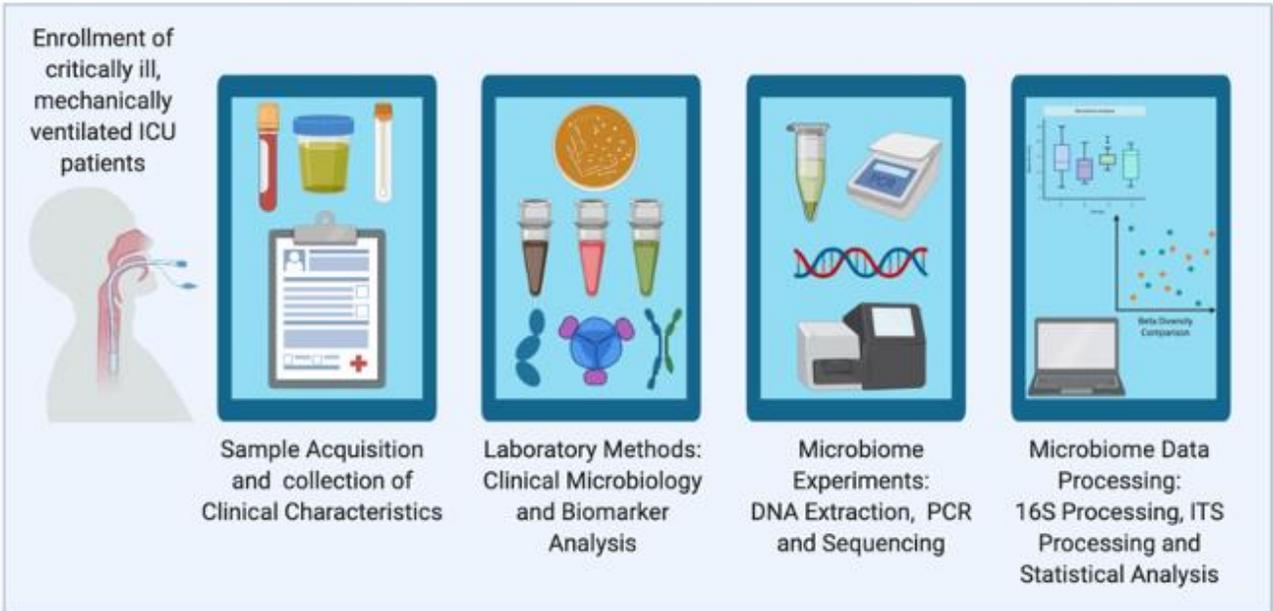
Appendix Table 14: Clinical Outcomes by Alpha Diversity Subgroup in ARDS

Clinical Outcomes	Low Diversity N= 30	High Diversity N= 17	P-Value
Duration of mechanical ventilation, median [Q1, Q3], days	7.5 [6.0, 13.0]	9.0 [5.0, 12.0]	0.86
ICU LOS, median [Q1, Q3], days	9.50 [8.0, 17.8]	9.0 [8.0, 17.0]	0.76
VFD, median [Q1, Q3], days	9.0 [0, 21.0]	11 [0, 24]	0.13
30 Day mortality, N (%)	11 (36.7%)	3 (17.6%)	0.30

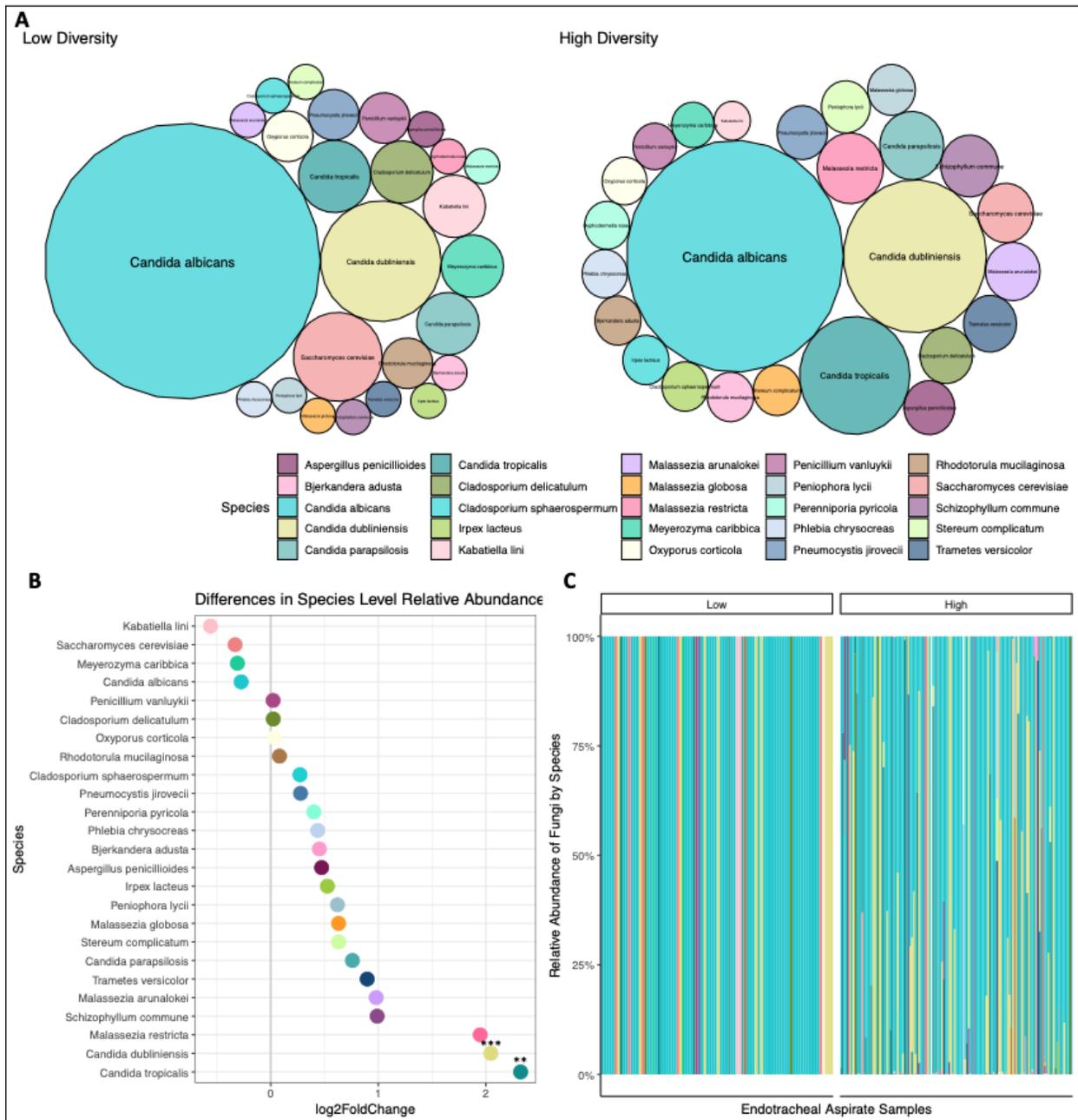
Appendix Table 15: Distribution of Biomarkers by Alpha Diversity Subgroup in ARDS

	Low Diversity N= 30	High Diversity N= 17	P-Value
Innate Immune Response			
IL-6, pg/ml	143 [29.1, 432]	58.6 [23.4, 358]	0.43
IL-8, pg/ml	19.1 [11.6, 47.5]	20.1 [9.08, 58.0]	0.85
IL-10, pg/ml	2.76 [1.00, 14.7]	1.00 [1.00, 5.02]	0.19
TNFR-1, pg/ml	7620 [3270, 10400]	3810 [2190, 15800]	0.46
ST-2, pg/ml	192000 [81900, 413000]	81000 [36100, 190000]	0.05
Fractalkine, pg/ml	2170 [1200, 3500]	1100 [584, 2020]	0.05
Host Response to Infection			
Procalcitonin, pg/ml	1490 [441, 4970]	606 [203, 817]	0.02
Pentraxin-3, pg/ml	4390 [2030, 11200]	3260 [2200, 6530]	0.59
Epithelial Injury			
RAGE, pg/ml	4110 [2200, 9410]	4480 [2710, 7870]	0.92
Endothelial Injury			
Angiopoietin-2, pg/ml	8110 [5020, 18300]	4860 [4370, 10100]	0.15
Fungal Translocation			
Plasma (1,3)- β -d-glucan, pg/ml	30.0 [13.0, 73.0]	25.0 [14.0, 41.0]	0.56
Plasma (1,3)- β -d-glucan greater than 60 pg/ml	9 (30.0%)	1 (5.9%)	0.21
Intestinal Permeability			
Fatty Acid-Binding Protein, pg/ml	521 [342, 859]	922 [576, 1230]	0.21

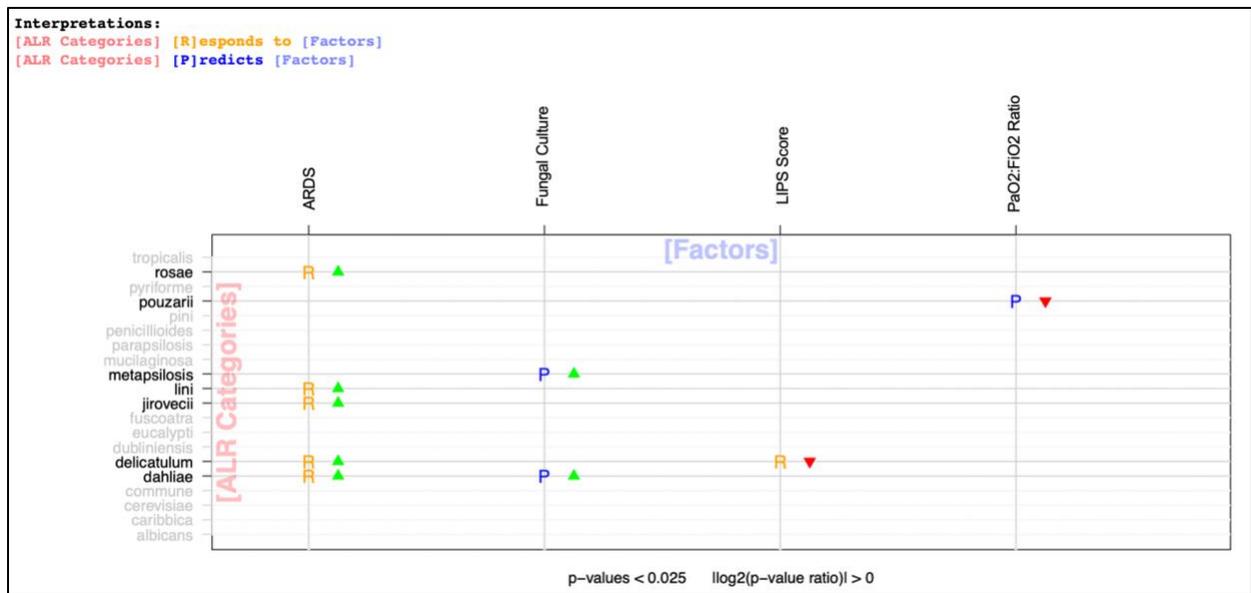
Appendix A.3 Figures



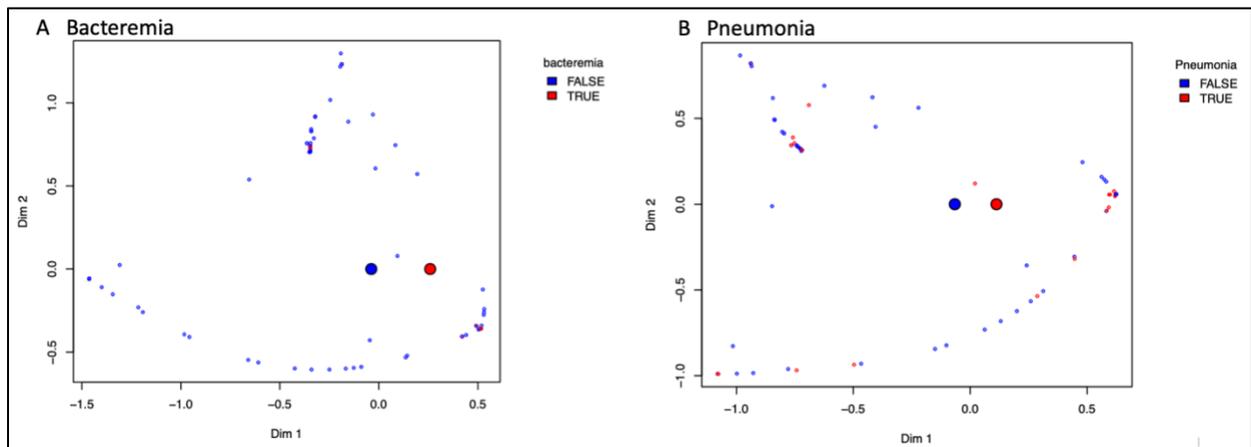
Appendix Figure 1: Visual Summary of the Study Protocol and Procedures



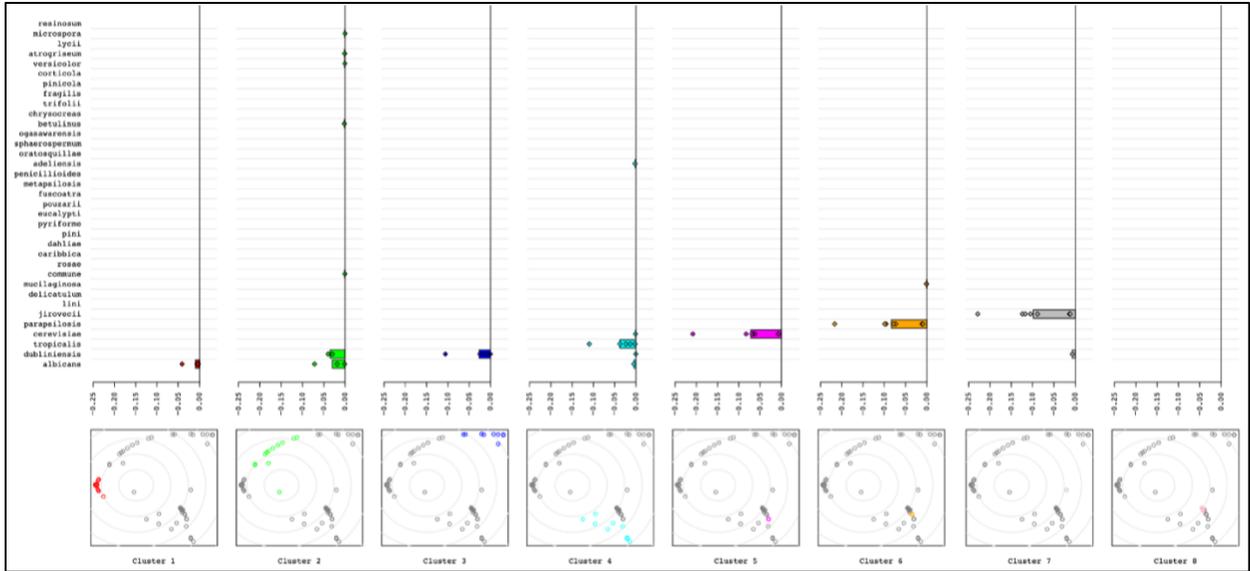
Appendix Figure 2: Taxonomic Differences by Alpha Diversity Subgroup



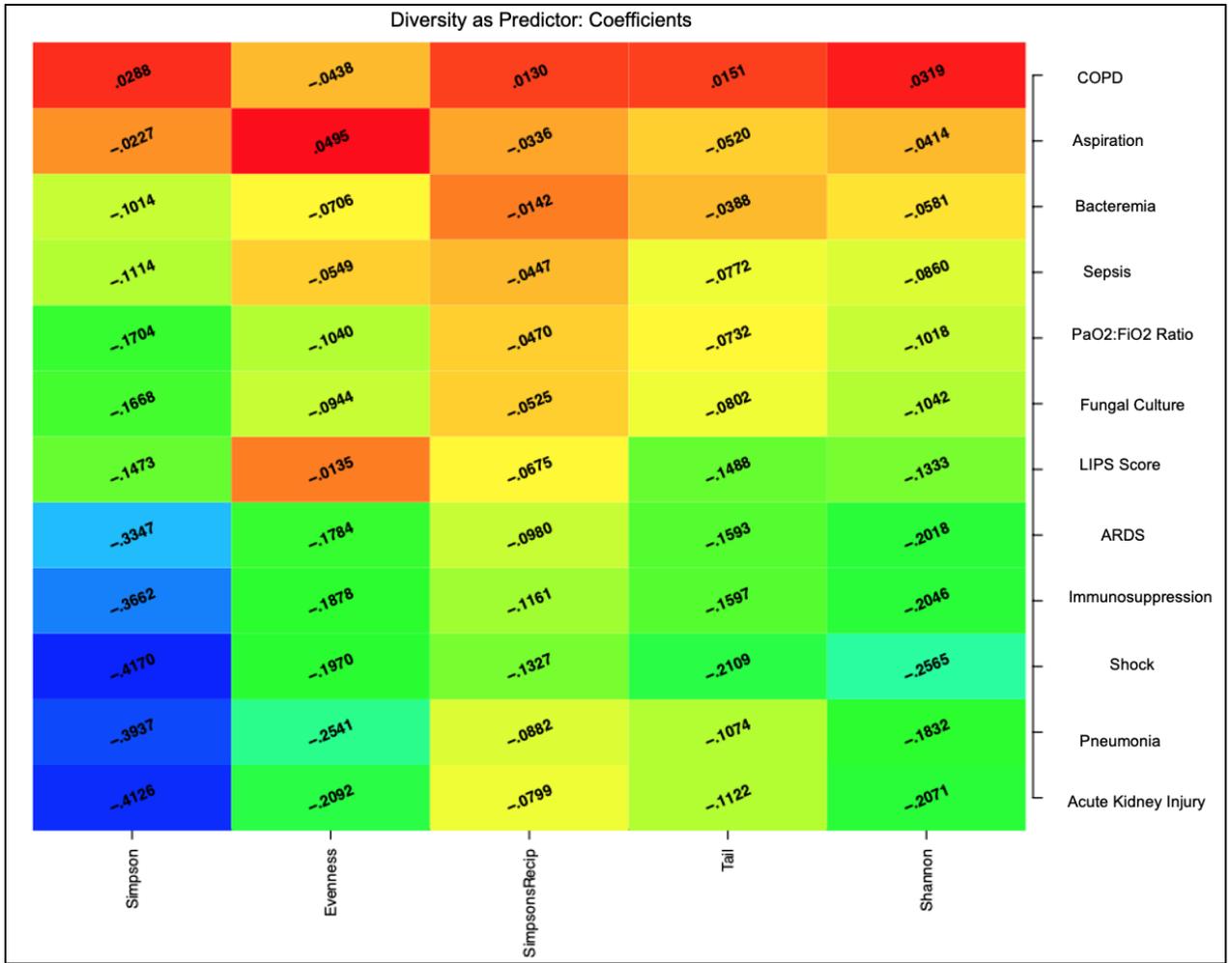
Appendix Figure 3: Taxa as Responders or Predictors with Group Variables



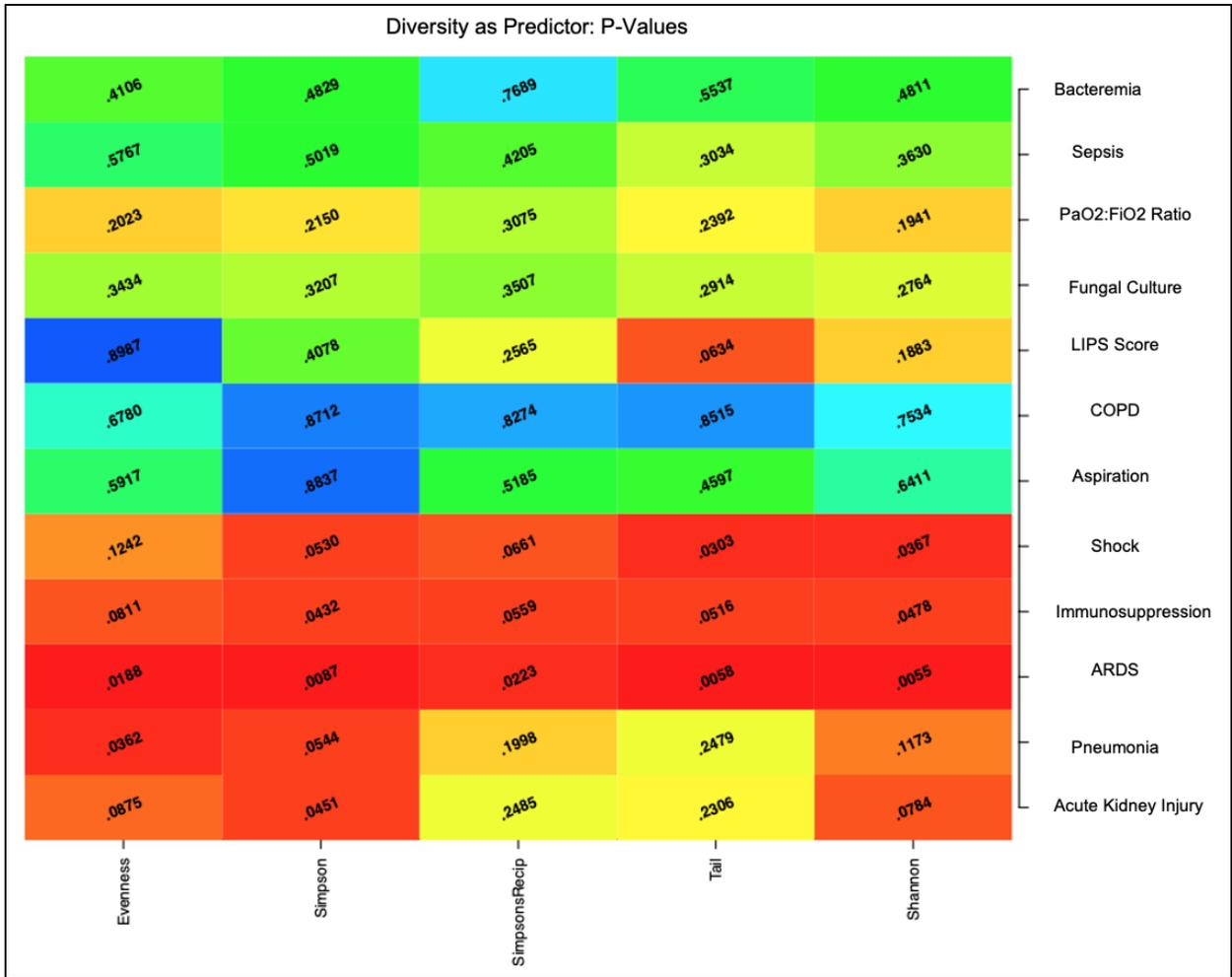
Appendix Figure 4: MDS Plot Demonstrating the Relative Similarity of Mycobiota Samples of Patients with and without Pneumonia and Bacteremia



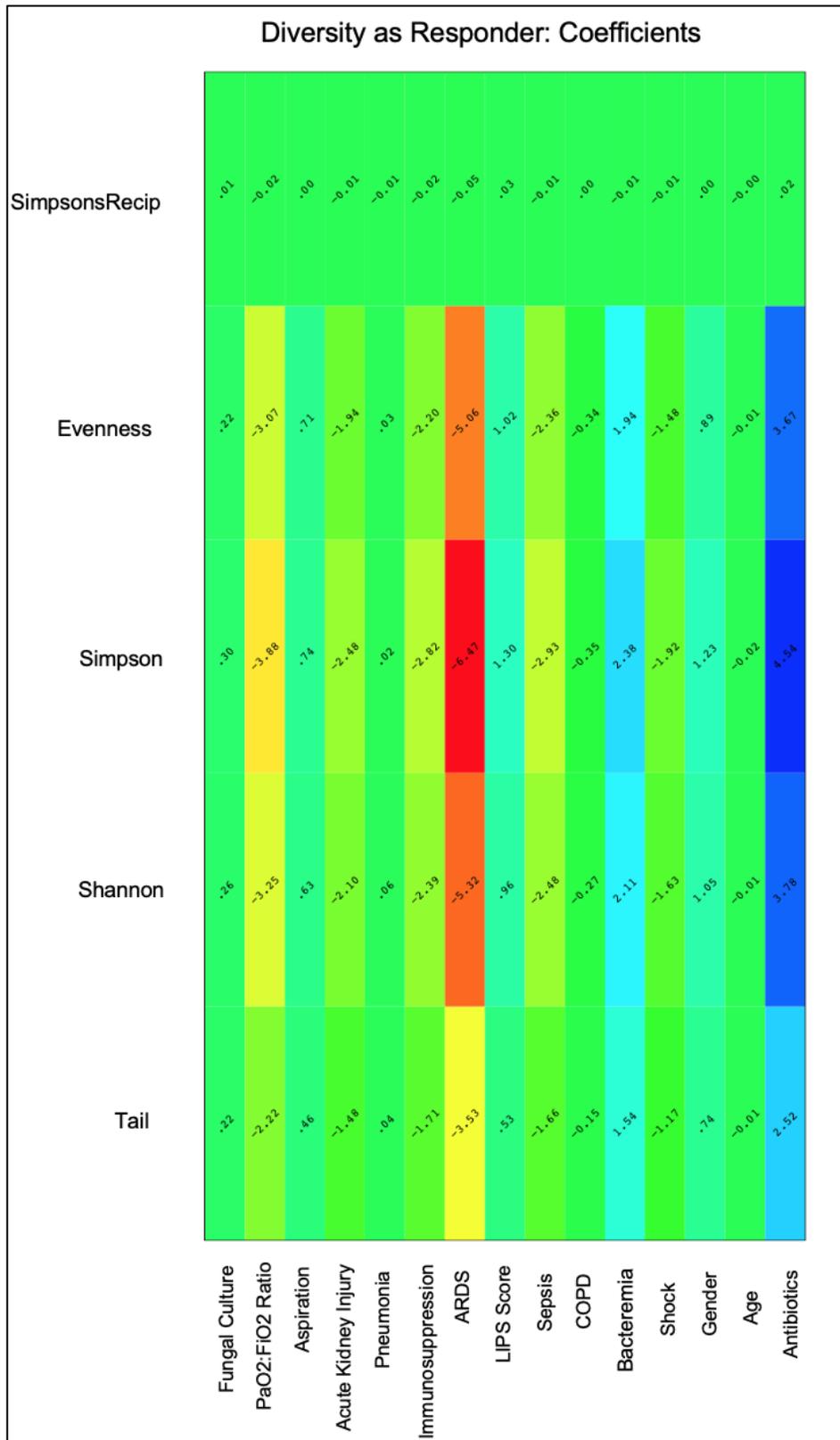
Appendix Figure 5: Species Unifying Clusters at an 8-Cluster Cutoff



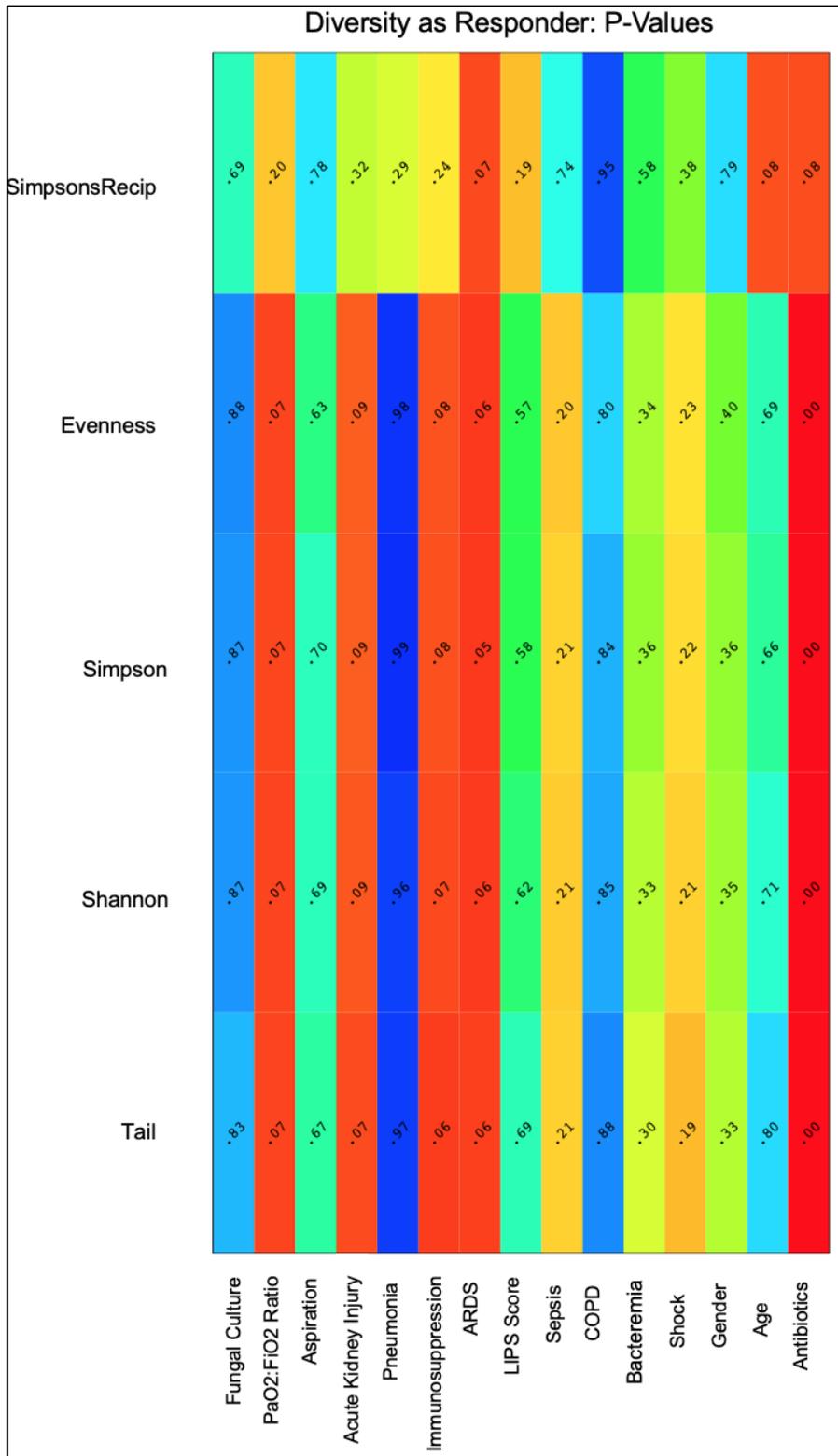
Appendix Figure 6: Diversity as a Predictor: Coefficients



Appendix Figure 7: Diversity as a Predictor: P-Values



Appendix Figure 8: Diversity as a Responder: Coefficients

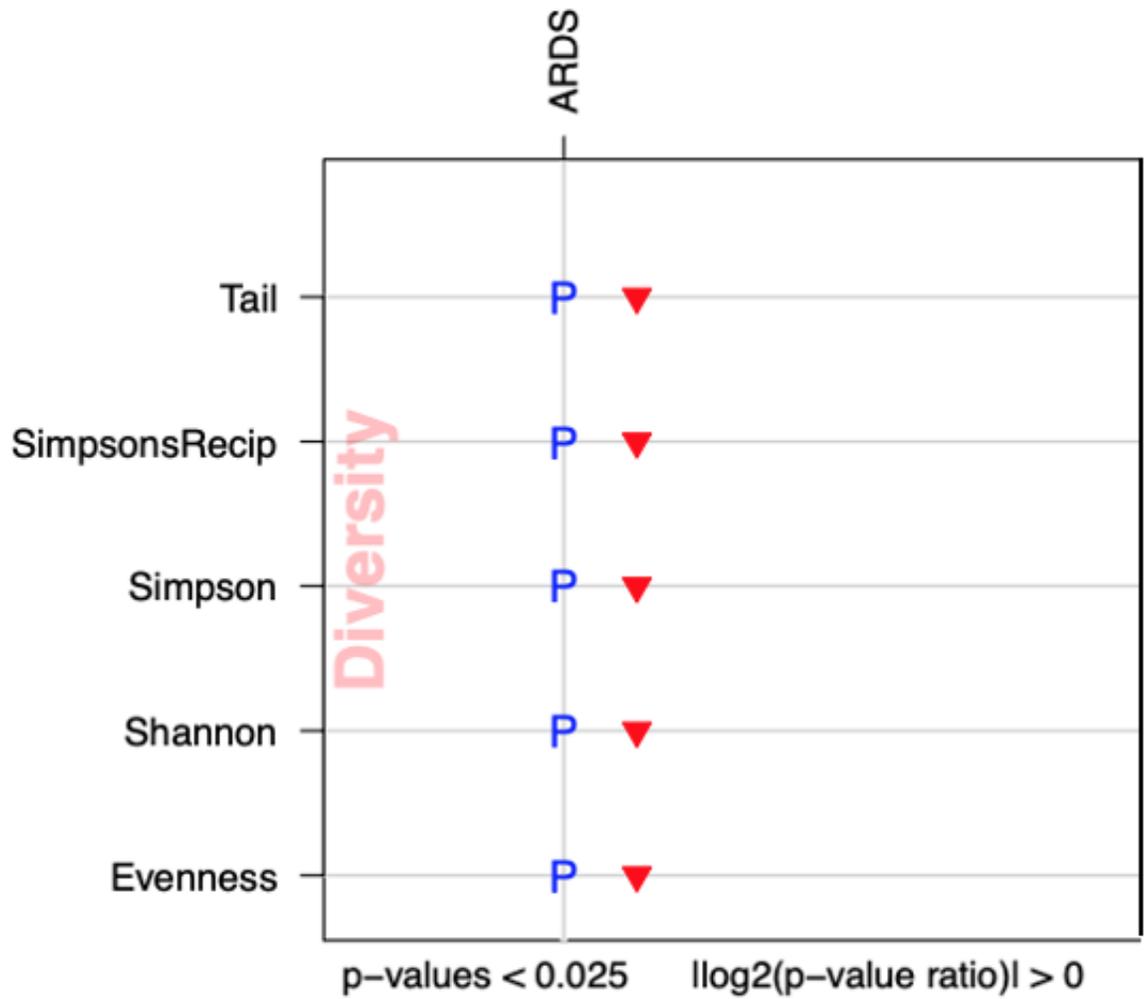


Appendix Figure 9: Diversity as a Responder: P-Values

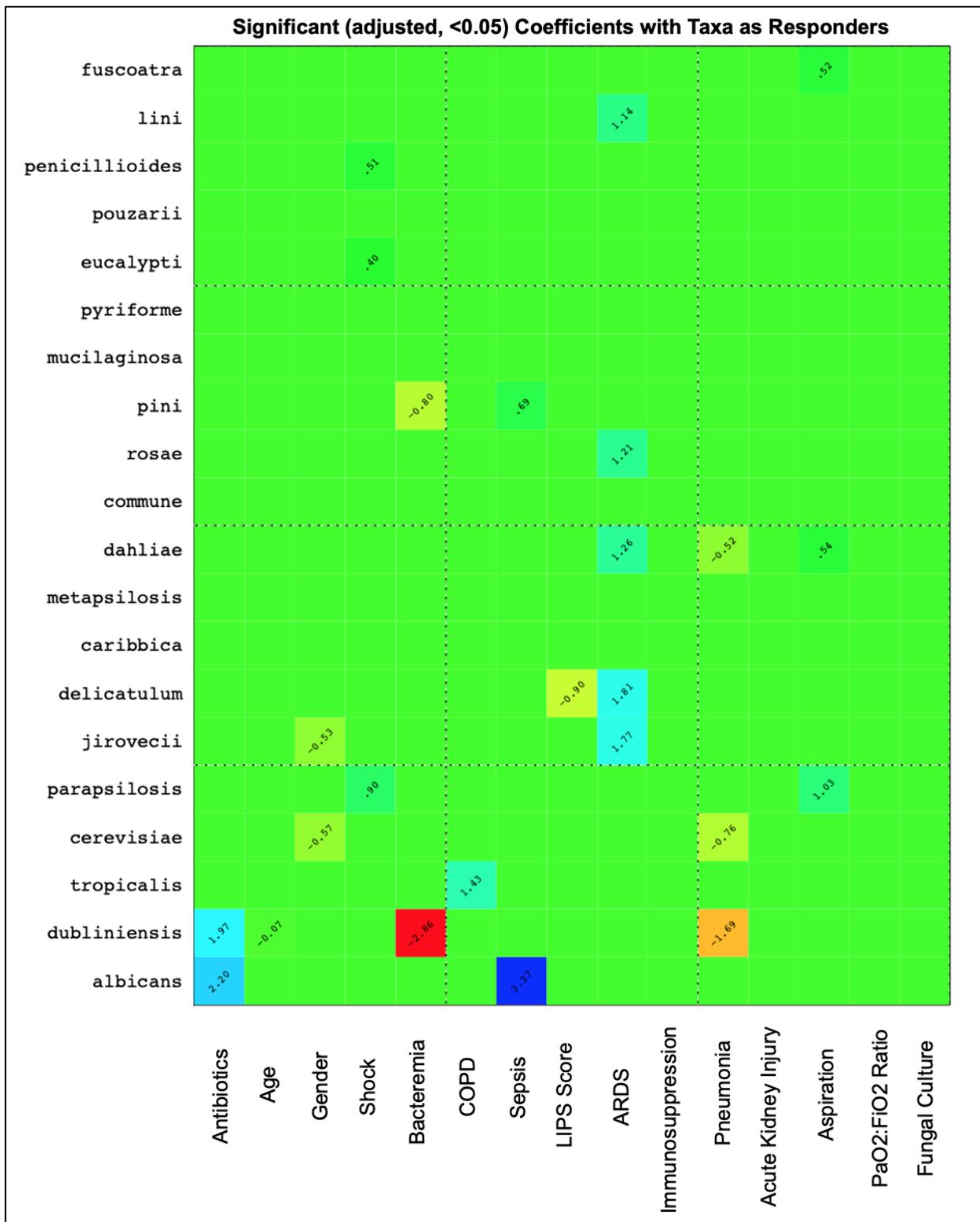
Interpretations:

Diversity [R]esponds to [Factors]

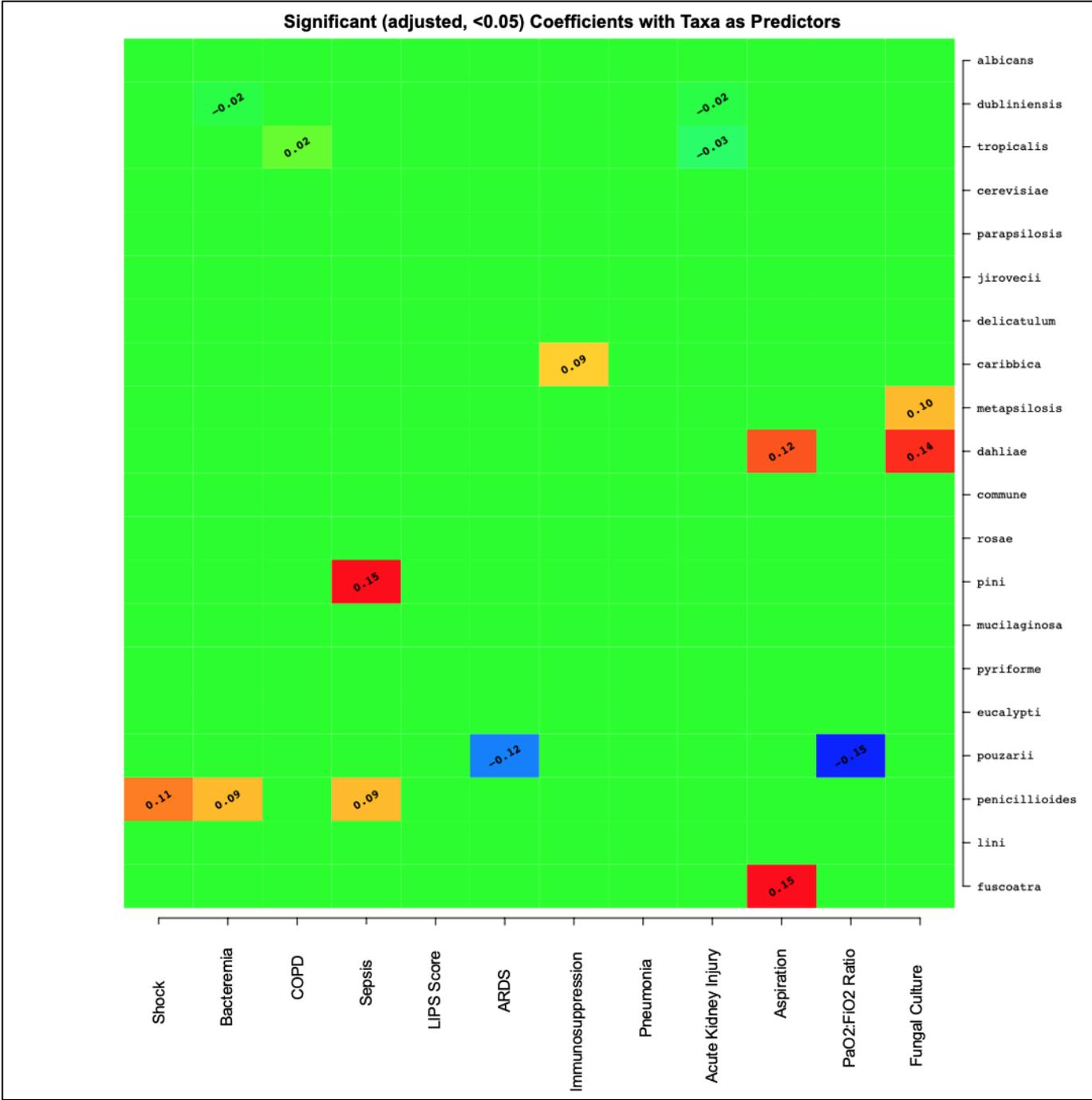
Diversity [P]redicts [Factors]



Appendix Figure 10: ARDS Predicts a Decrease in Alpha Diversity

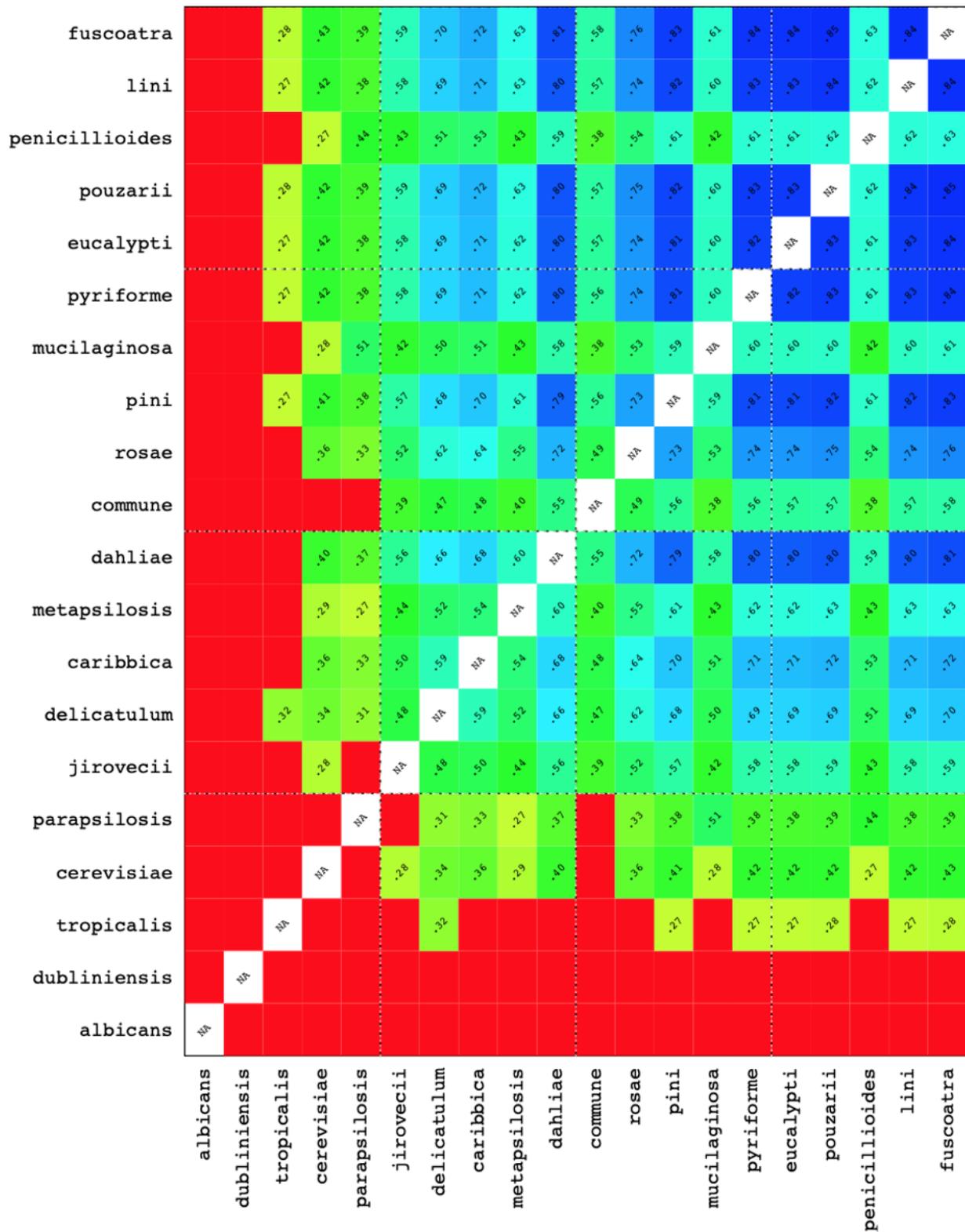


Appendix Figure 11: Significant Coefficients with Taxa as Responders



Appendix Figure 12: Significant Coefficients with Taxa as Predictors

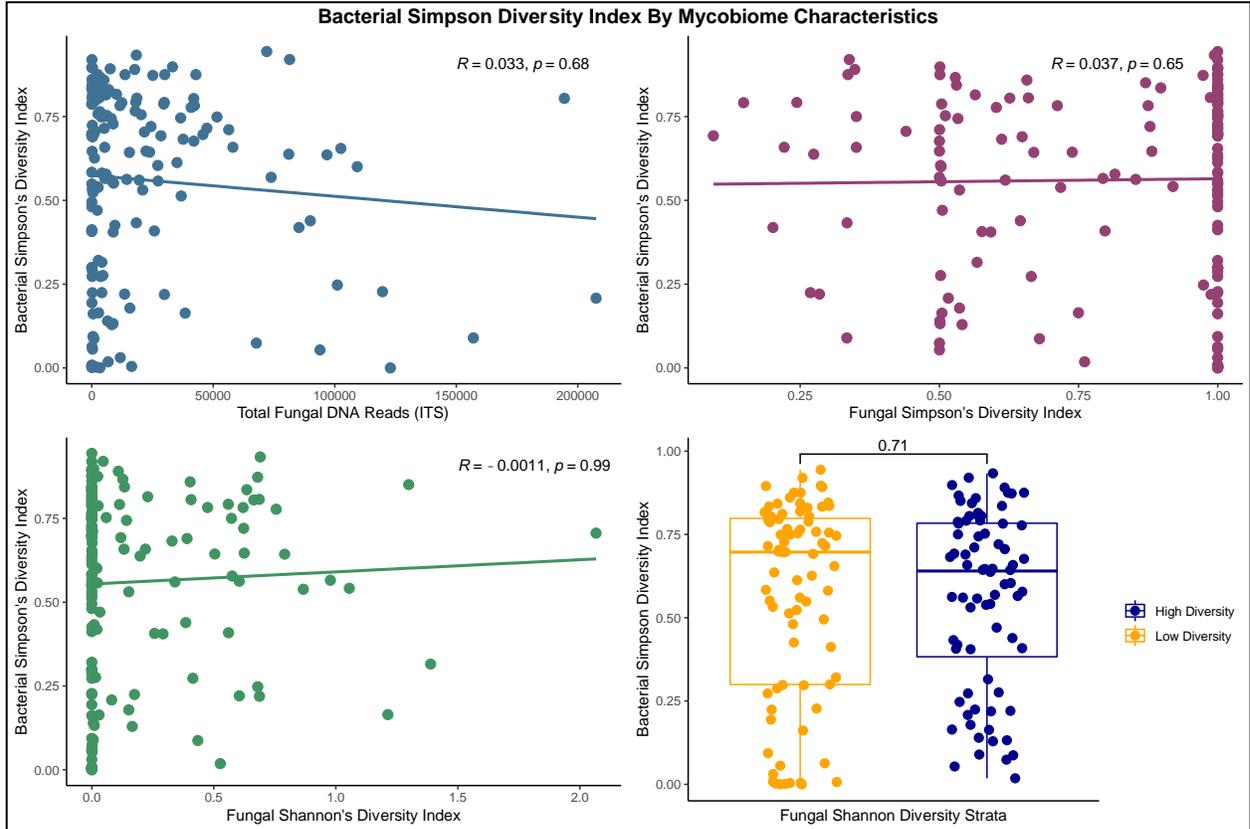
Significant (adjusted, <0.05) Correlations between Species Abundance



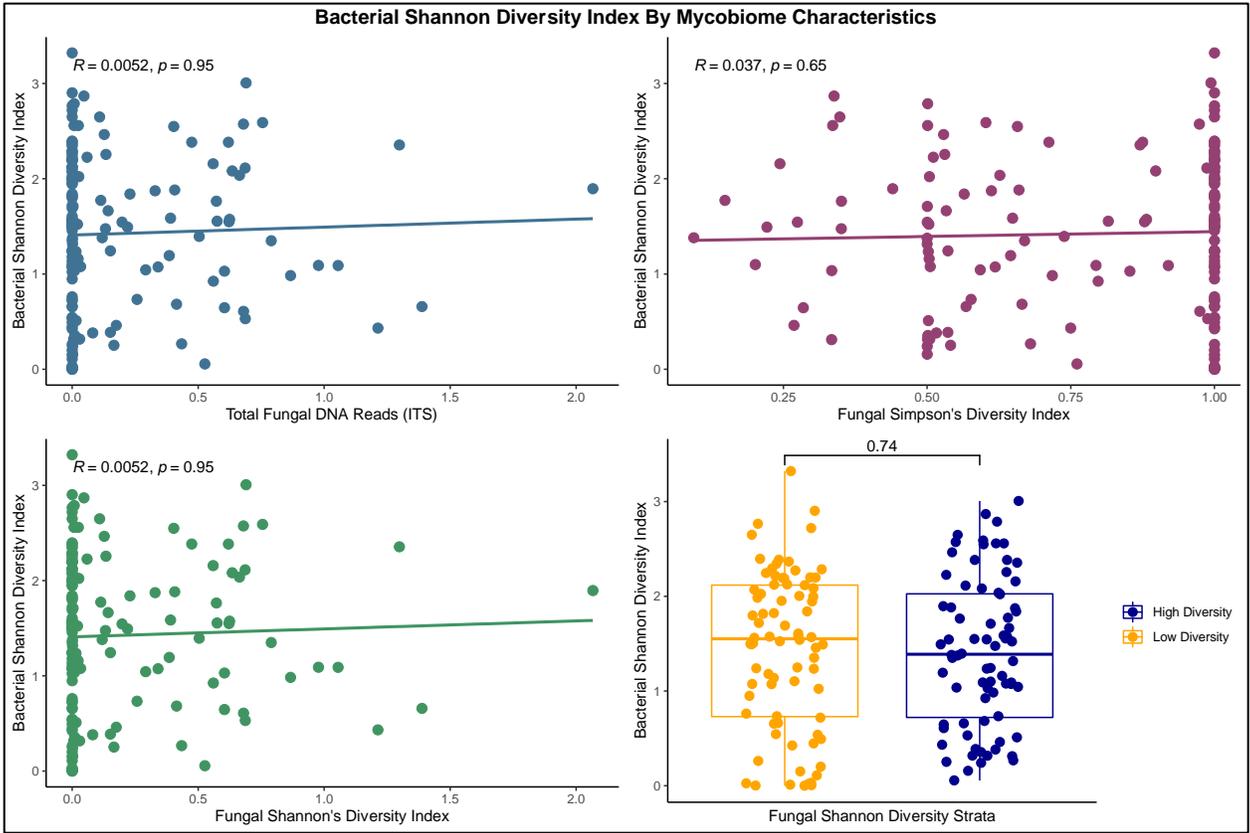
Appendix Figure 13: Significant Correlations Between Species Abundance

Appendix A.4 : Manuscript 3

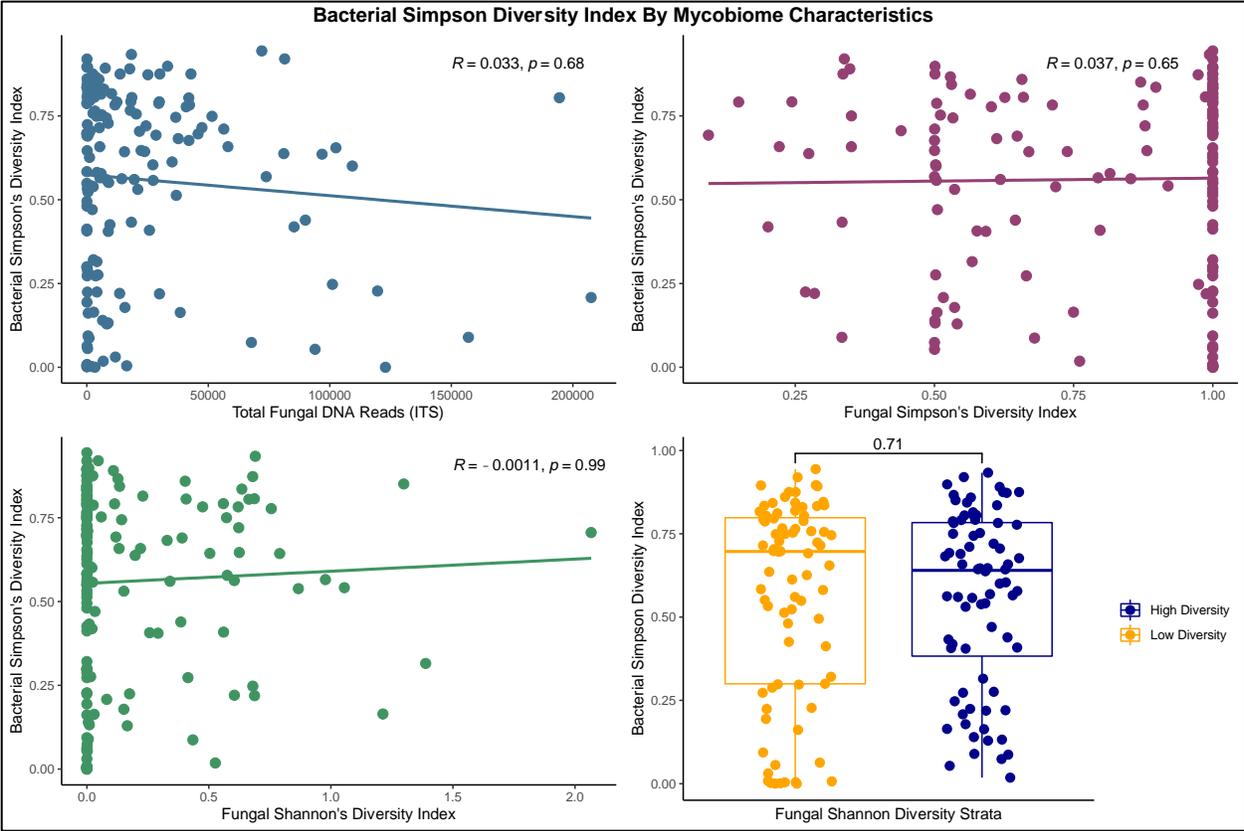
Appendix A.4.1 Figures



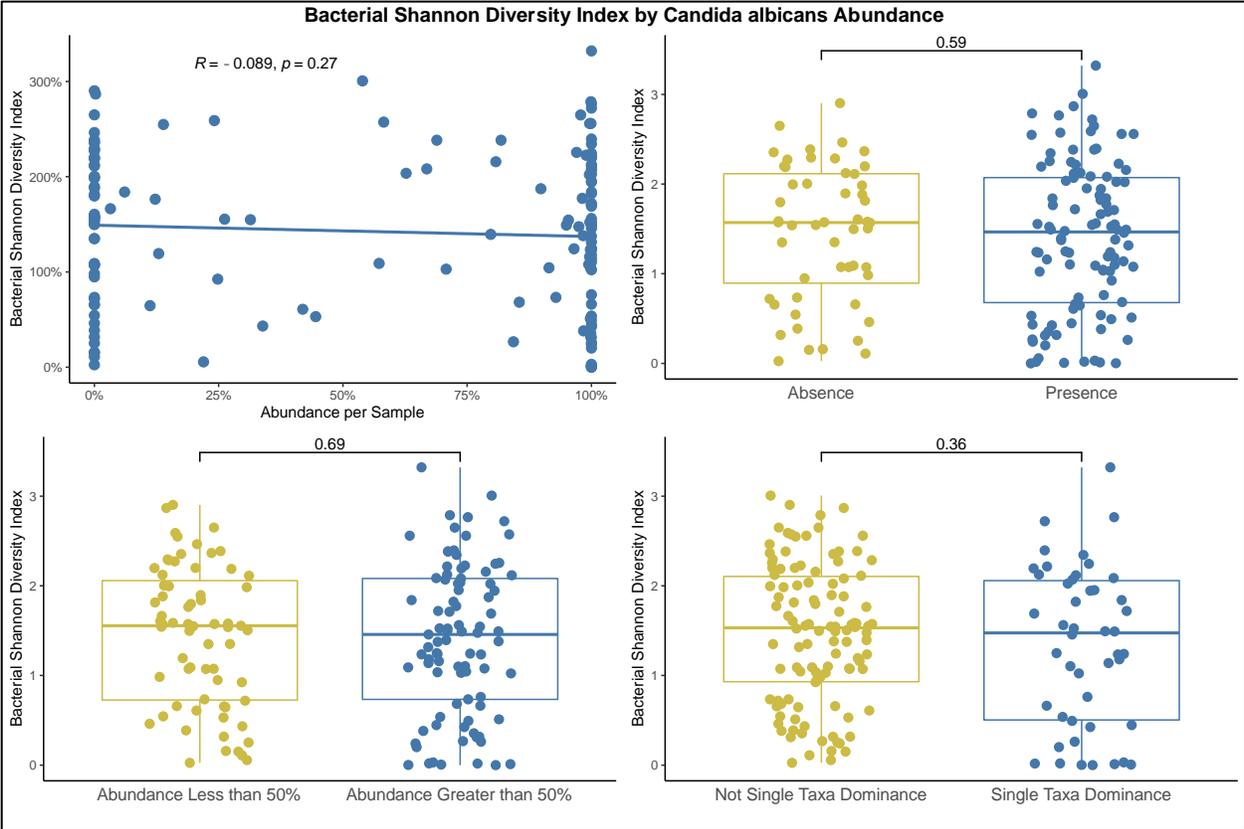
Appendix Figure 14: Bacterial Load by Mycobiome Characteristics



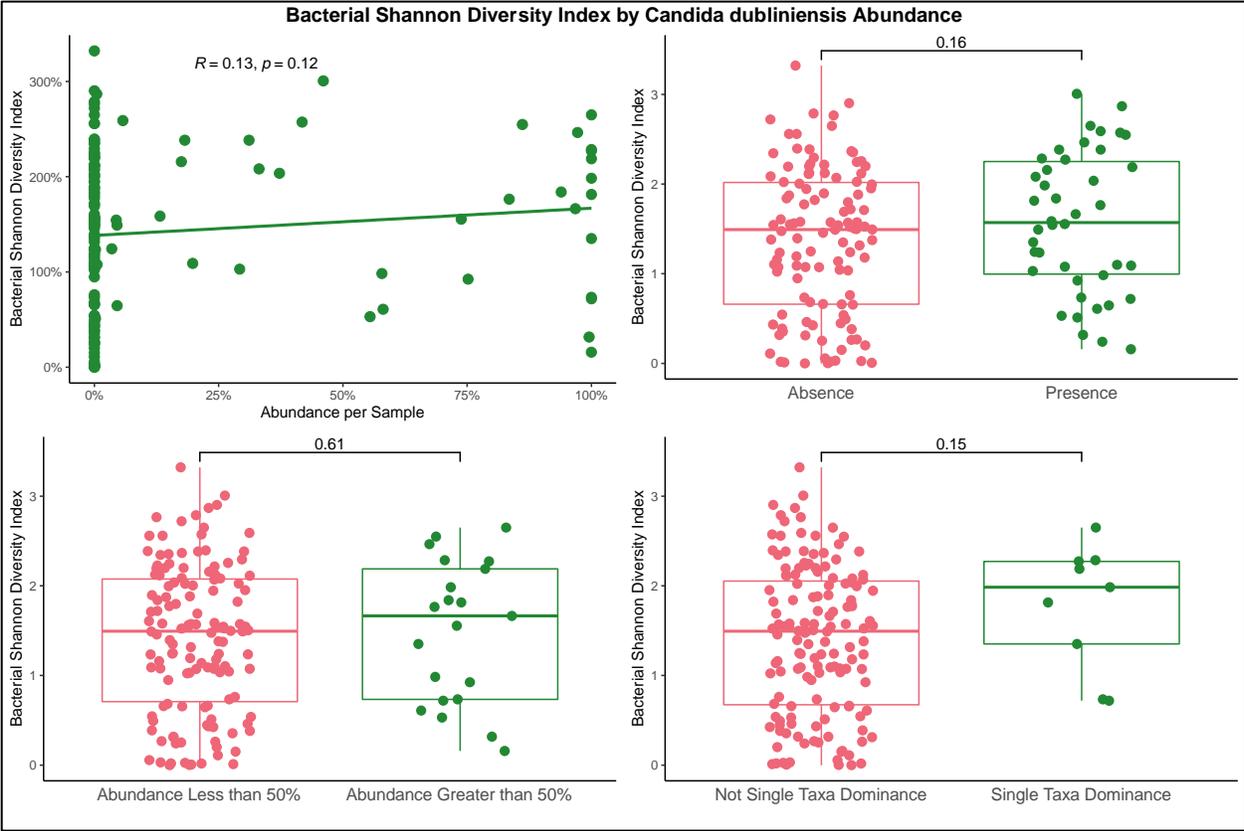
Appendix Figure 15: Bacterial Shannon Diversity Index by Mycobiome Characteristics



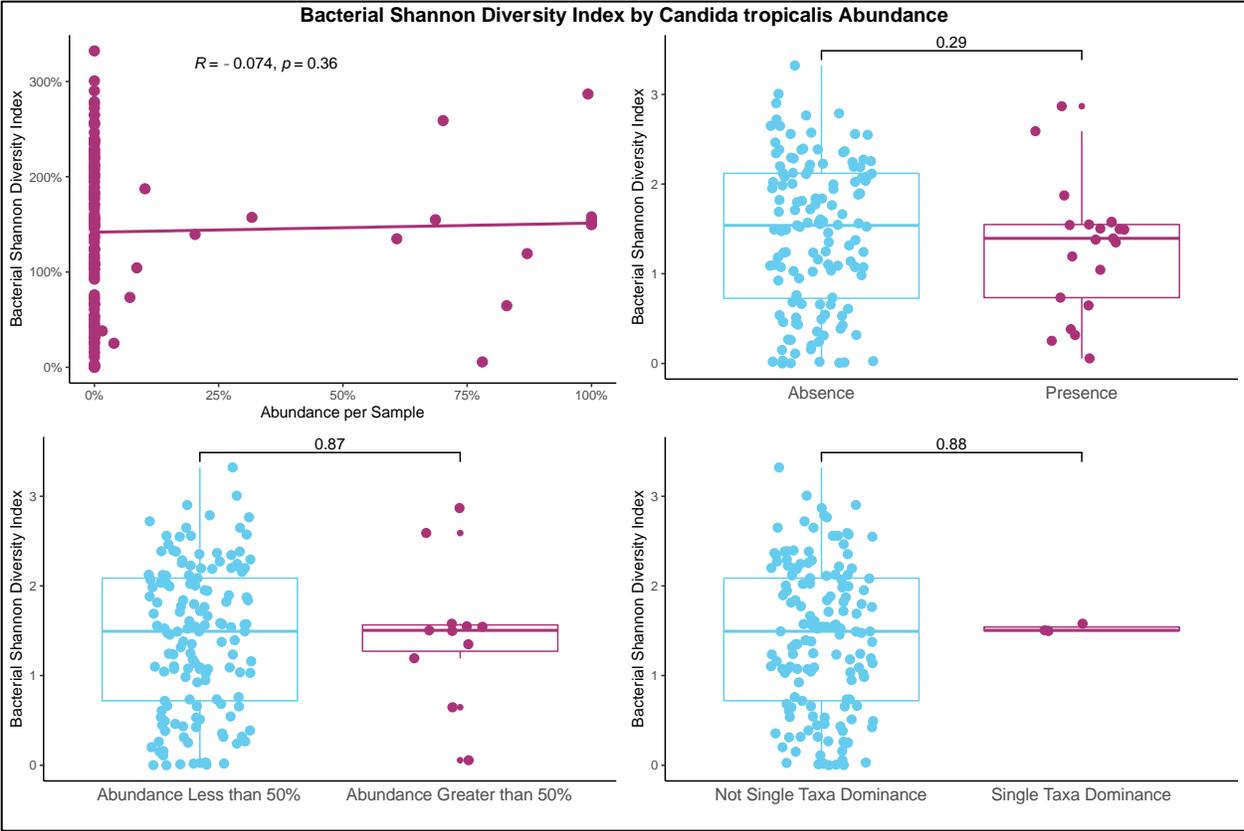
Appendix Figure 16: Bacterial Simpson's Diversity Index by Mycobiome Characteristics



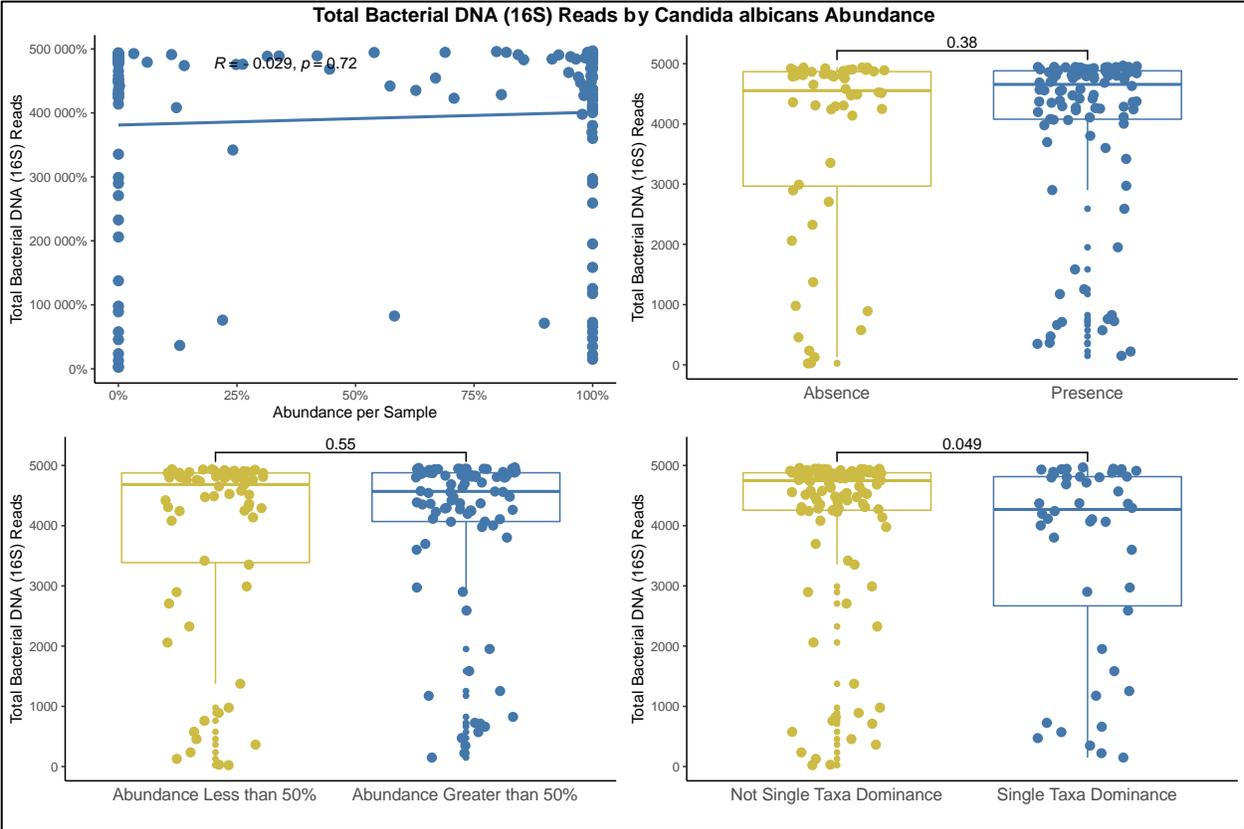
Appendix Figure 17: Bacterial Shannon Diversity Index by *Candida albicans* Abundance



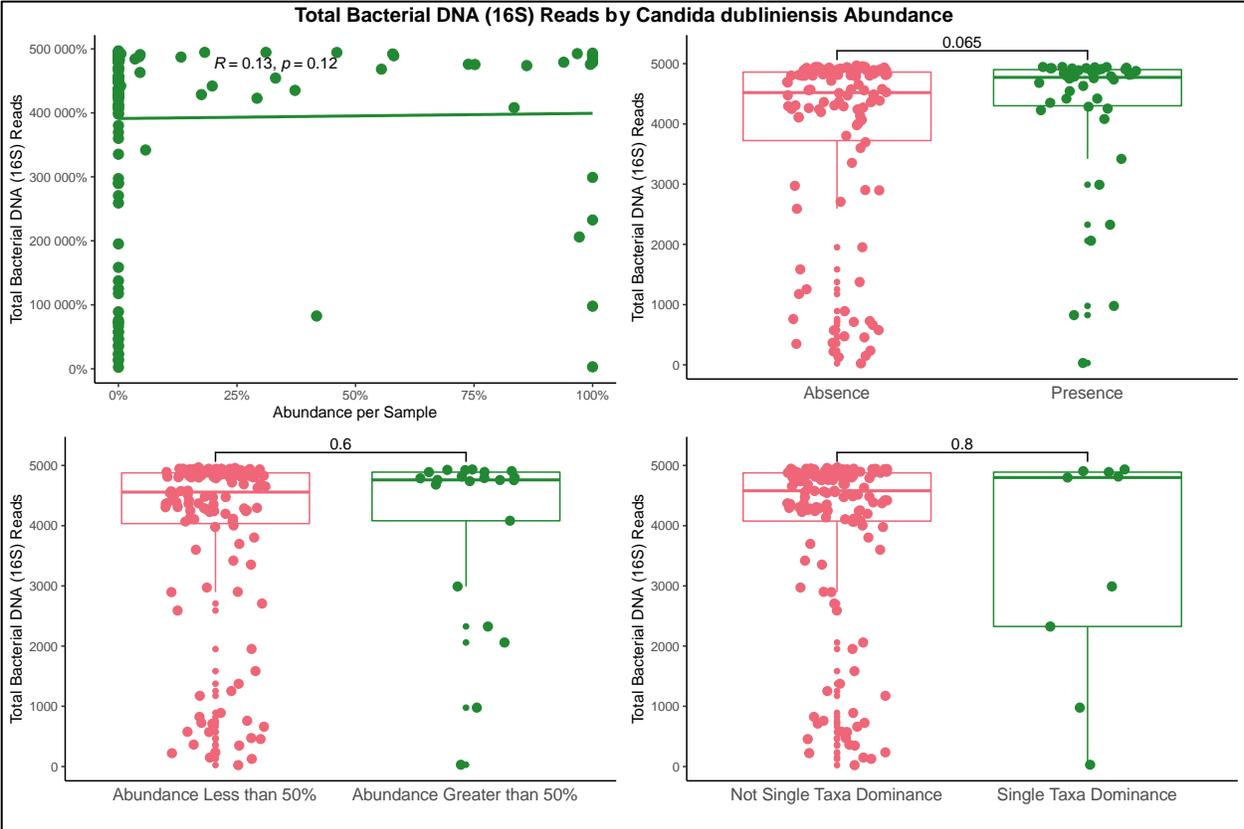
Appendix Figure 18: Bacterial Shannon Diversity Index by *Candida dubliniensis* Abundance



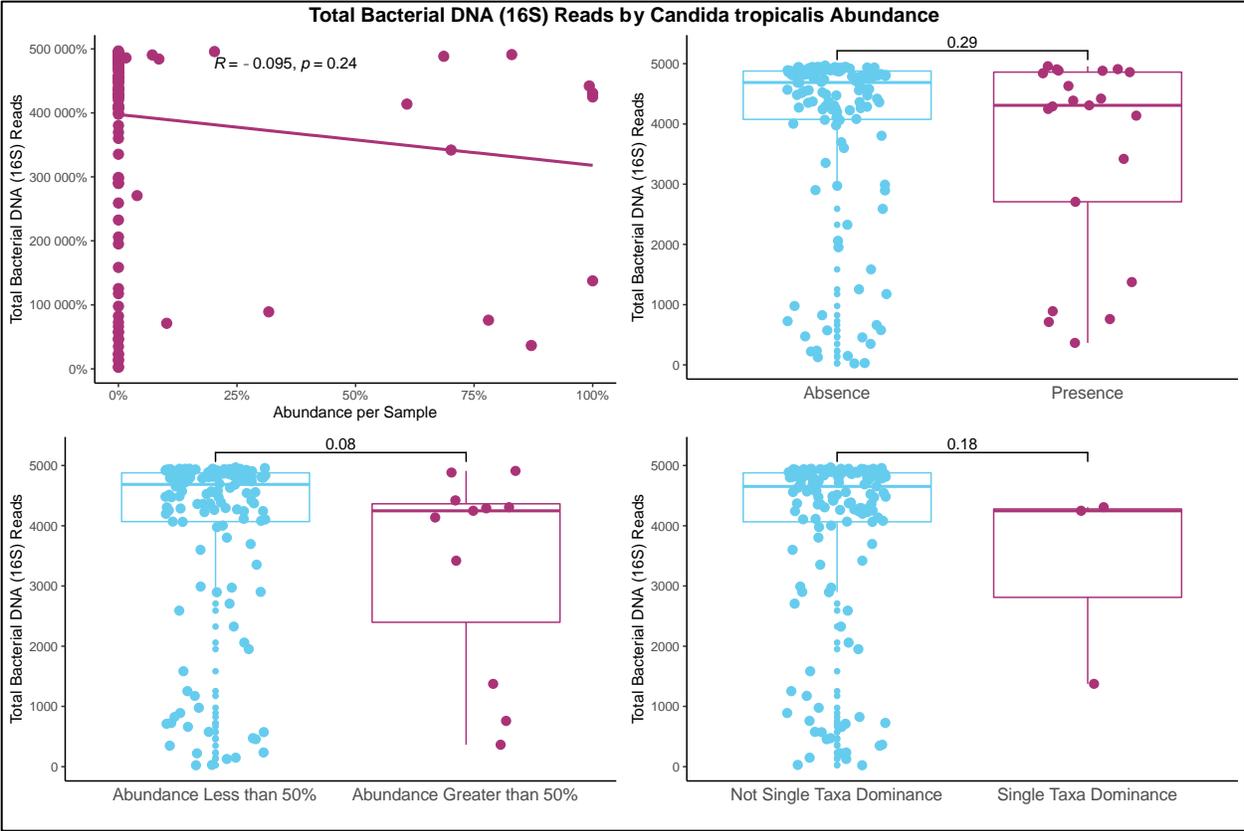
Appendix Figure 19: Bacterial Shannon Diversity Index by *Candida tropicalis* Abundance



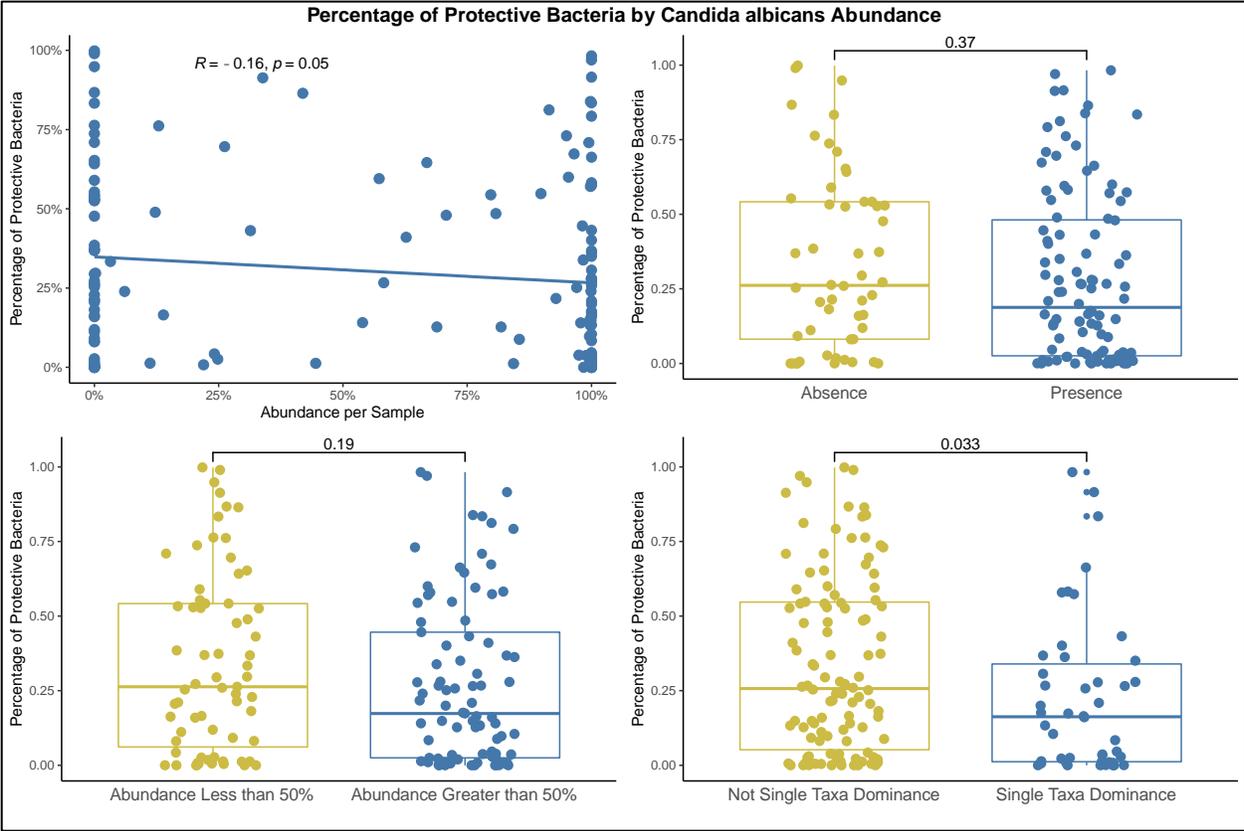
Appendix Figure 20: Total Bacterial DNA Reads by *Candida albicans* Abundance



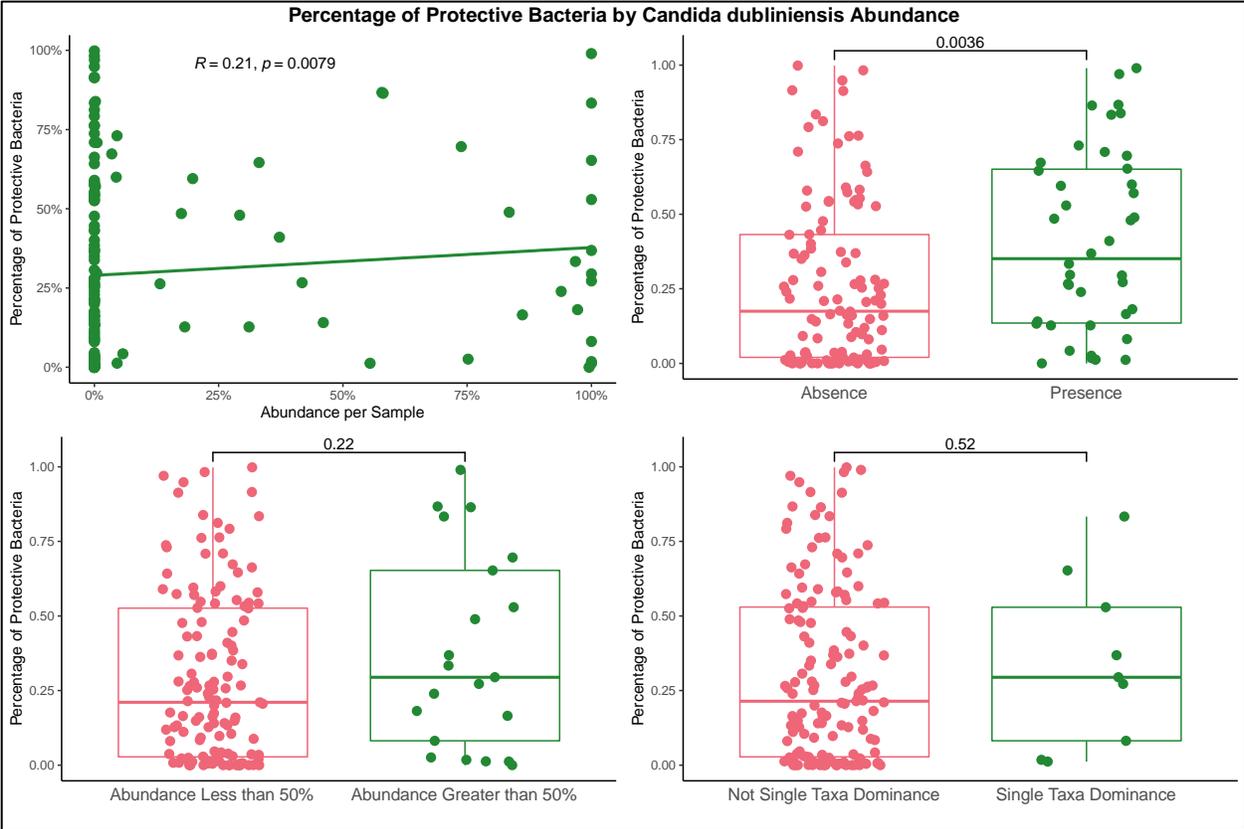
Appendix Figure 21: Total Bacterial DNA by *Candida dubliniensis* Abundance



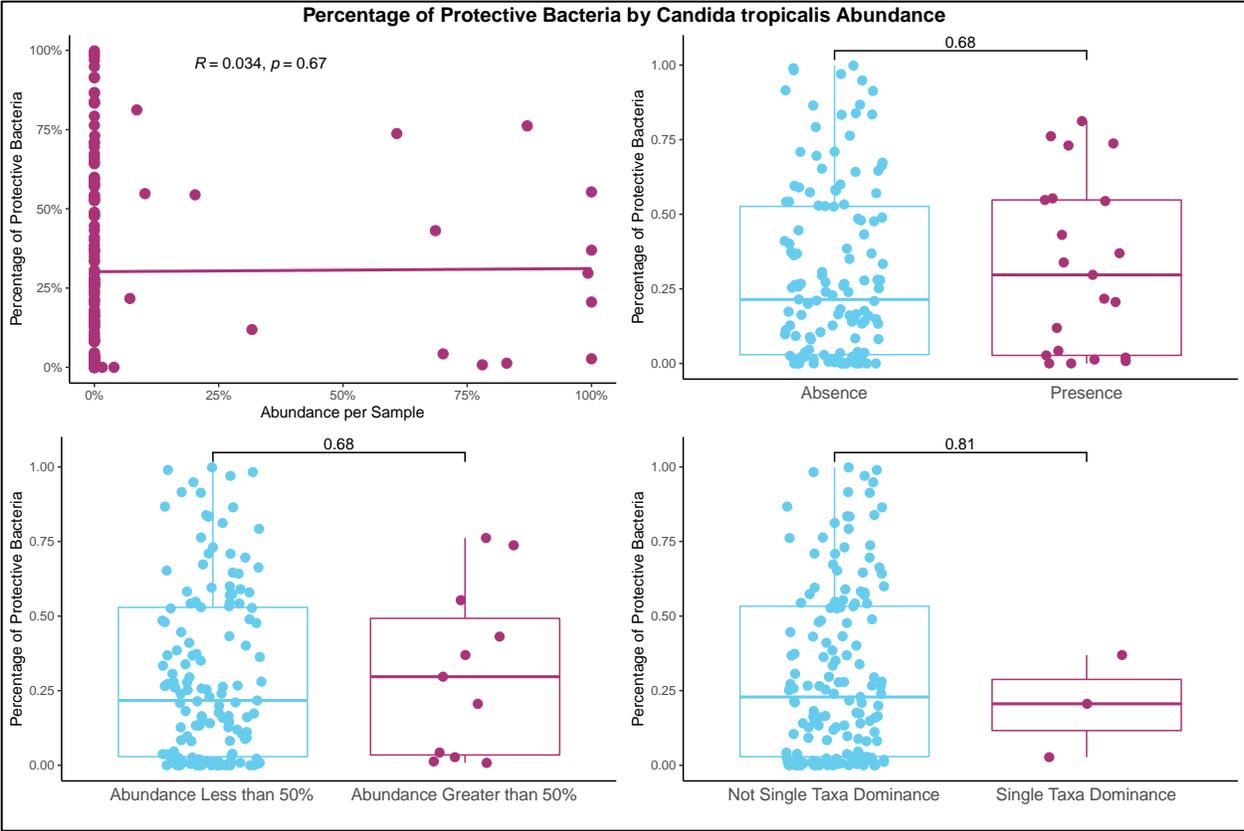
Appendix Figure 22: Total Bacterial DNA Reads by *Candida tropicalis* Abundance



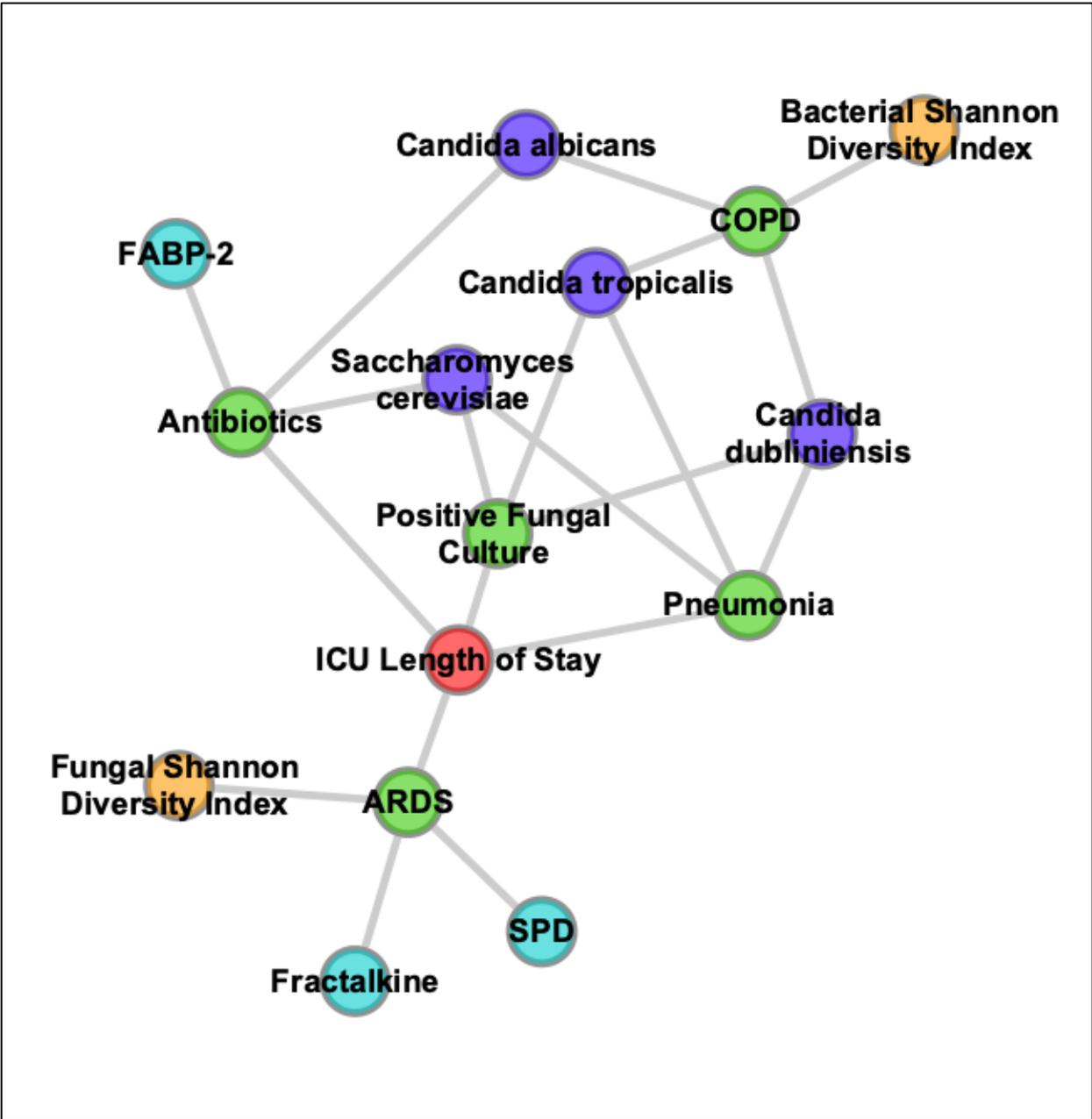
Appendix Figure 23: Percentage of Protective Bacteria by *Candida albicans* Abundance



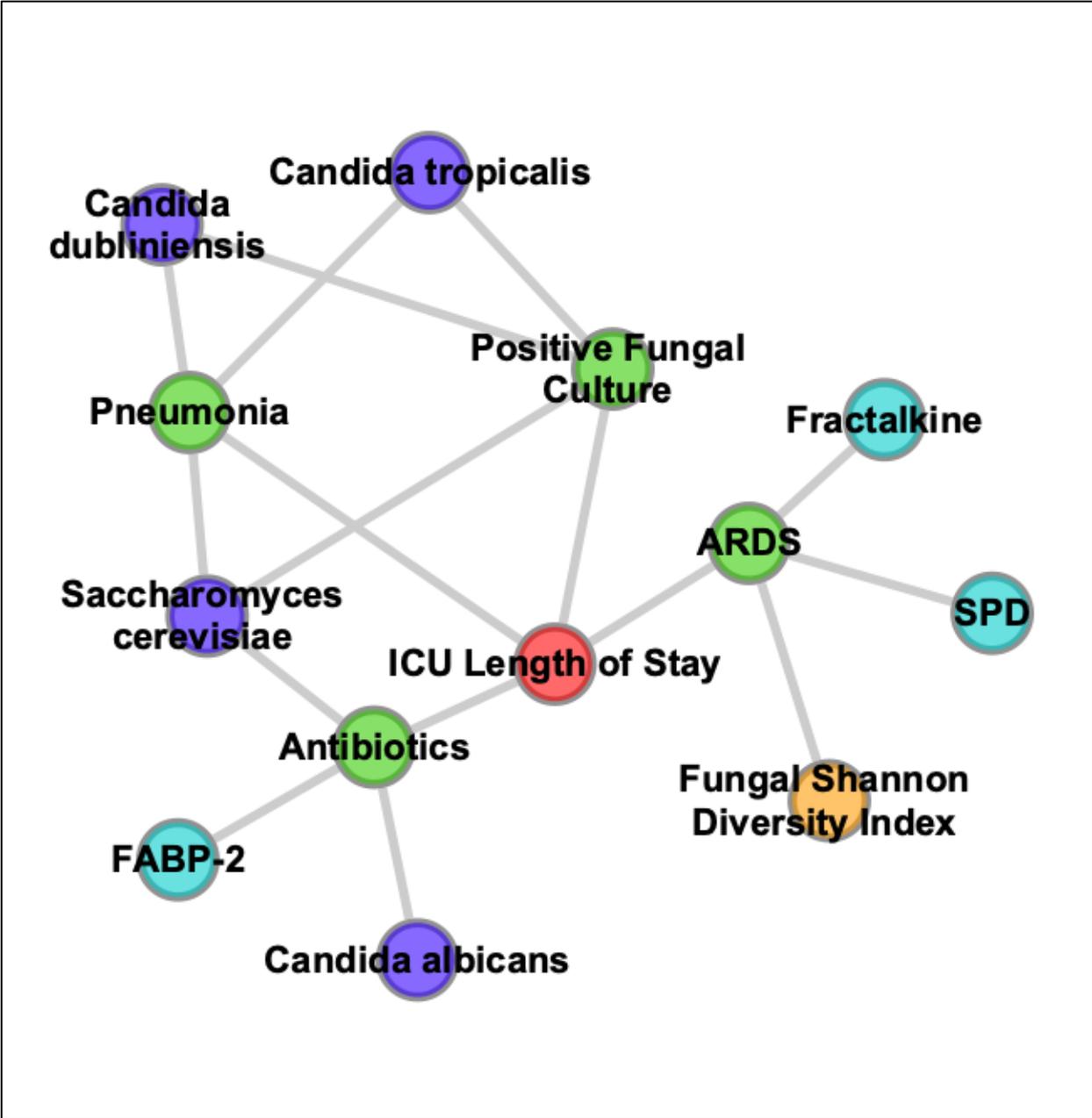
Appendix Figure 24: Percentage of Protective Bacteria by *Candida dubliniensis* Abundance



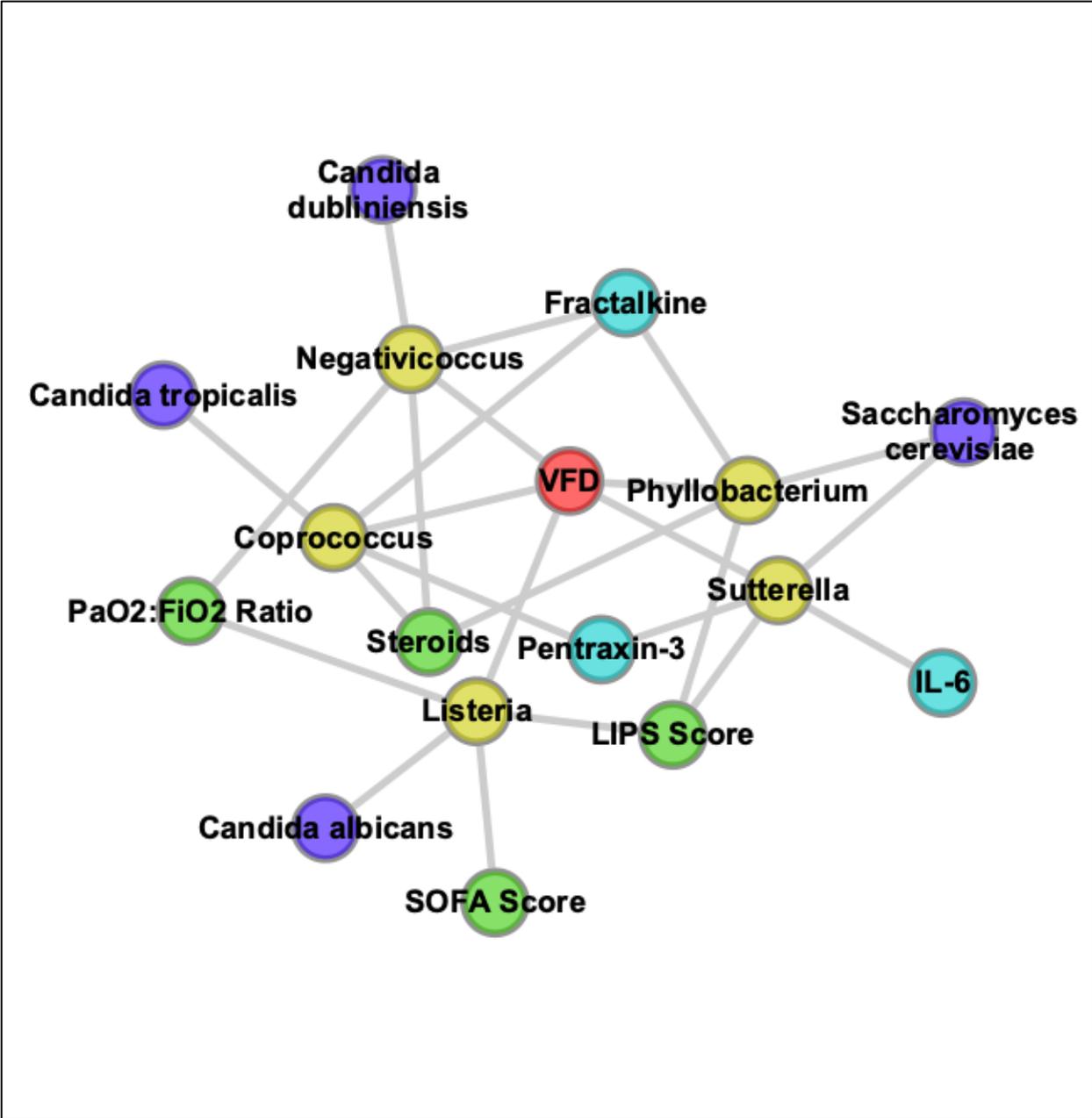
Appendix Figure 25: Percentage of Protective Bacteria by *Candida tropicalis* Abundance



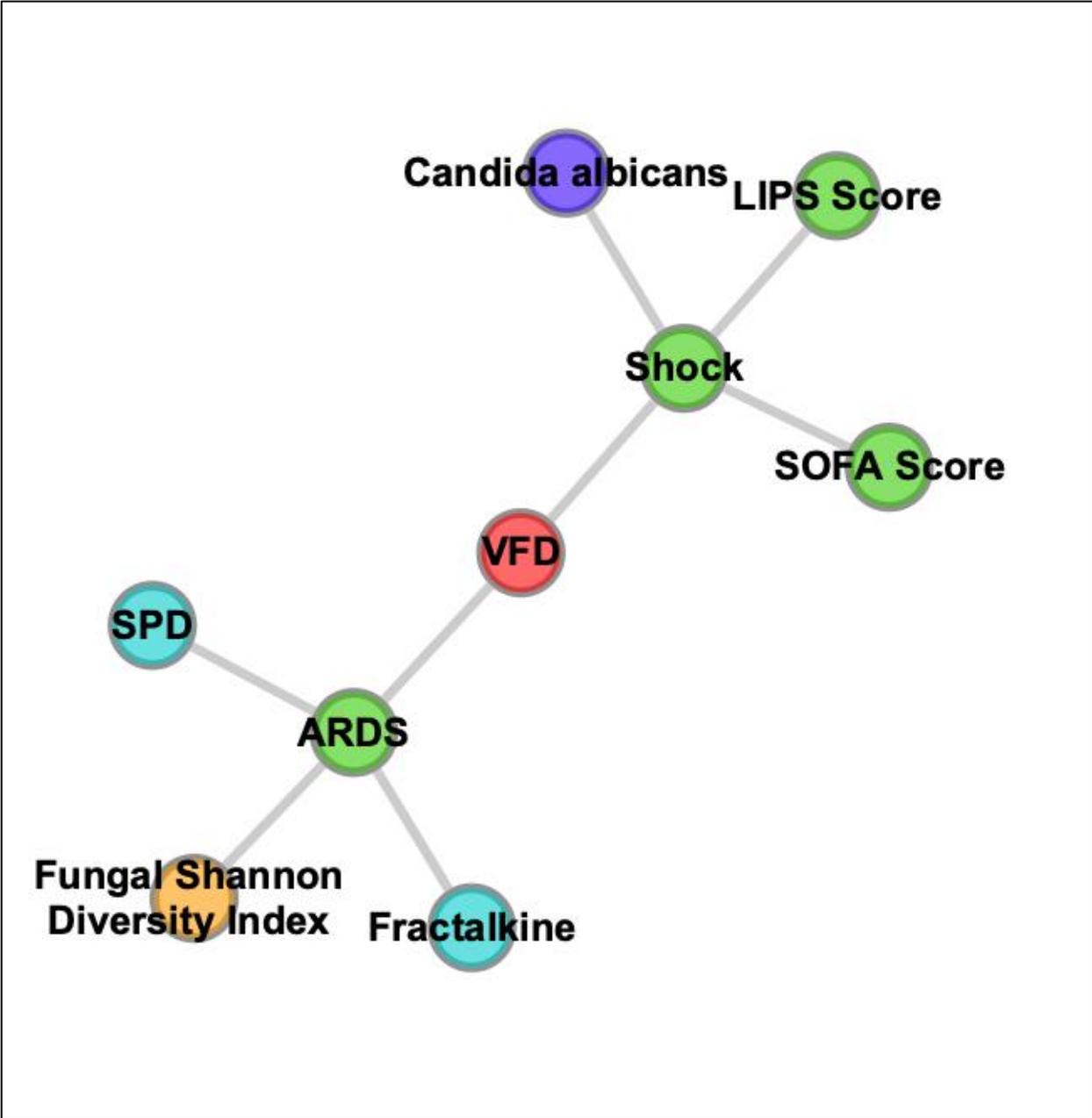
Appendix Figure 26: Probabilistic Graphical Model Network Analysis Demonstrating First and Second Neighbors of ICU Length of Stay Including Bacterial Taxa



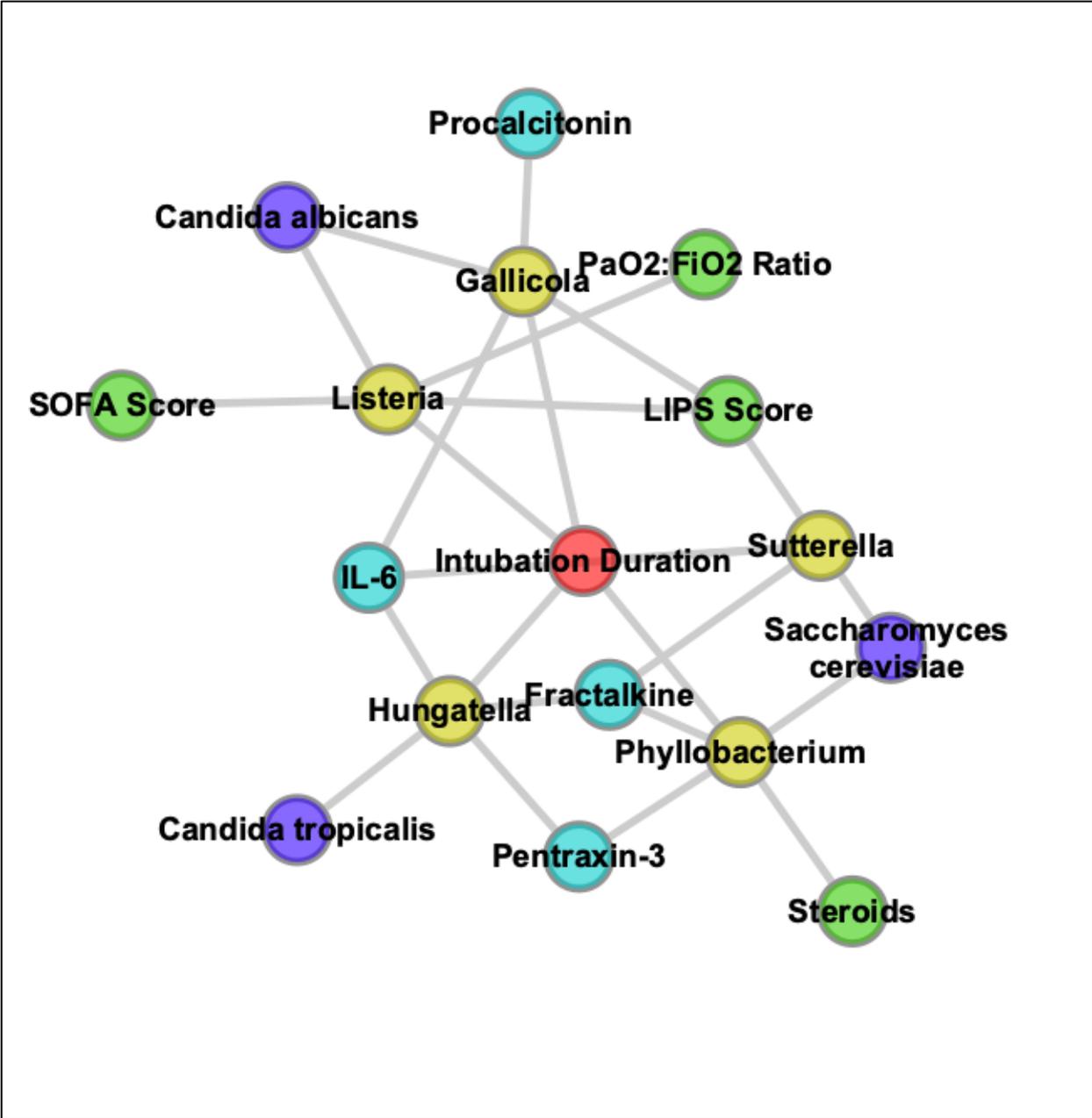
Appendix Figure 27: Probabilistic Graphical Model Network Analysis Demonstrating First and Second Neighbors of ICU Length of Stay



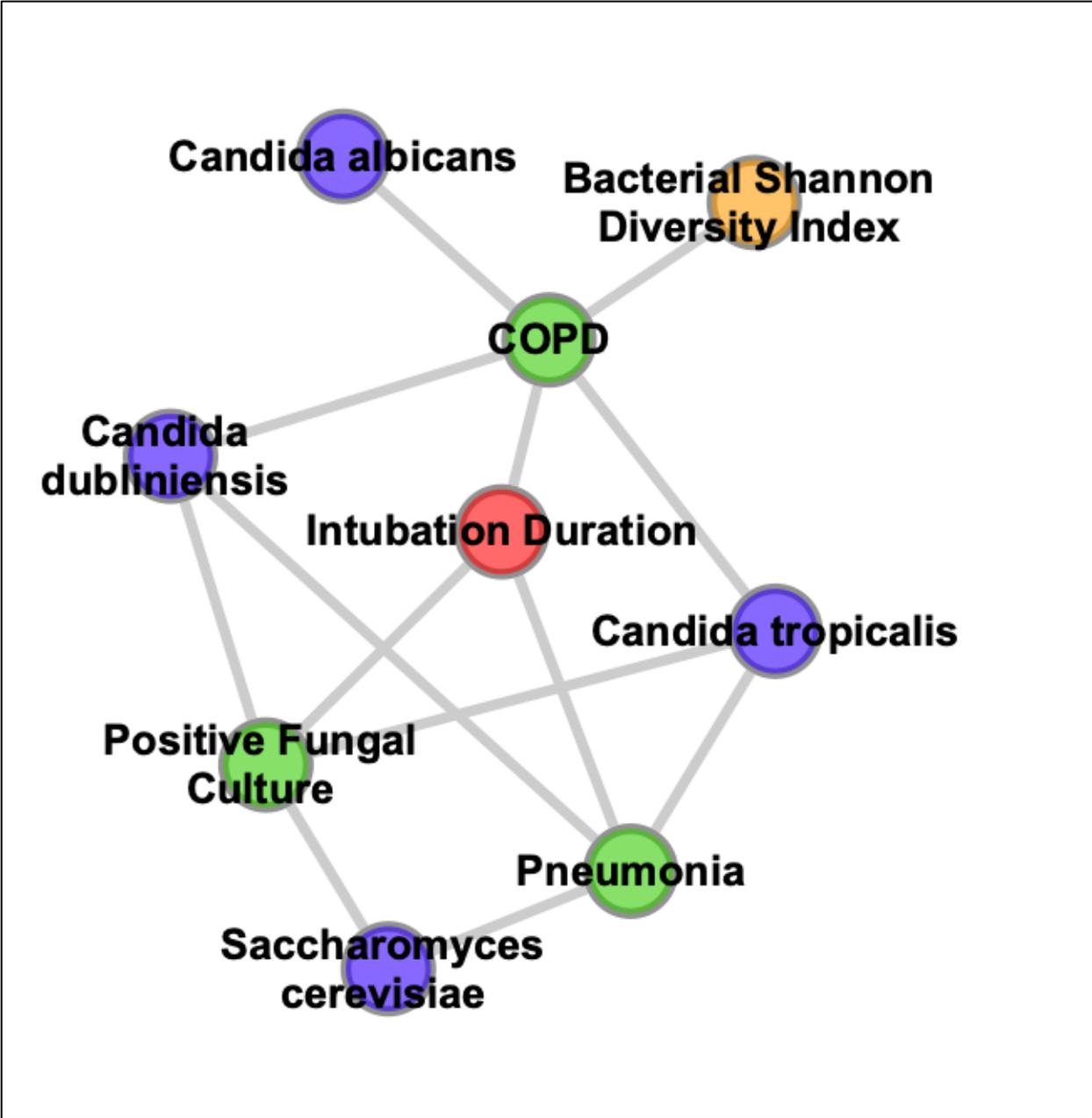
Appendix Figure 28: Probabilistic Graphical Model Network Analysis Demonstrating First and Second Neighbors of Ventilator Free Days Including Bacterial Taxa



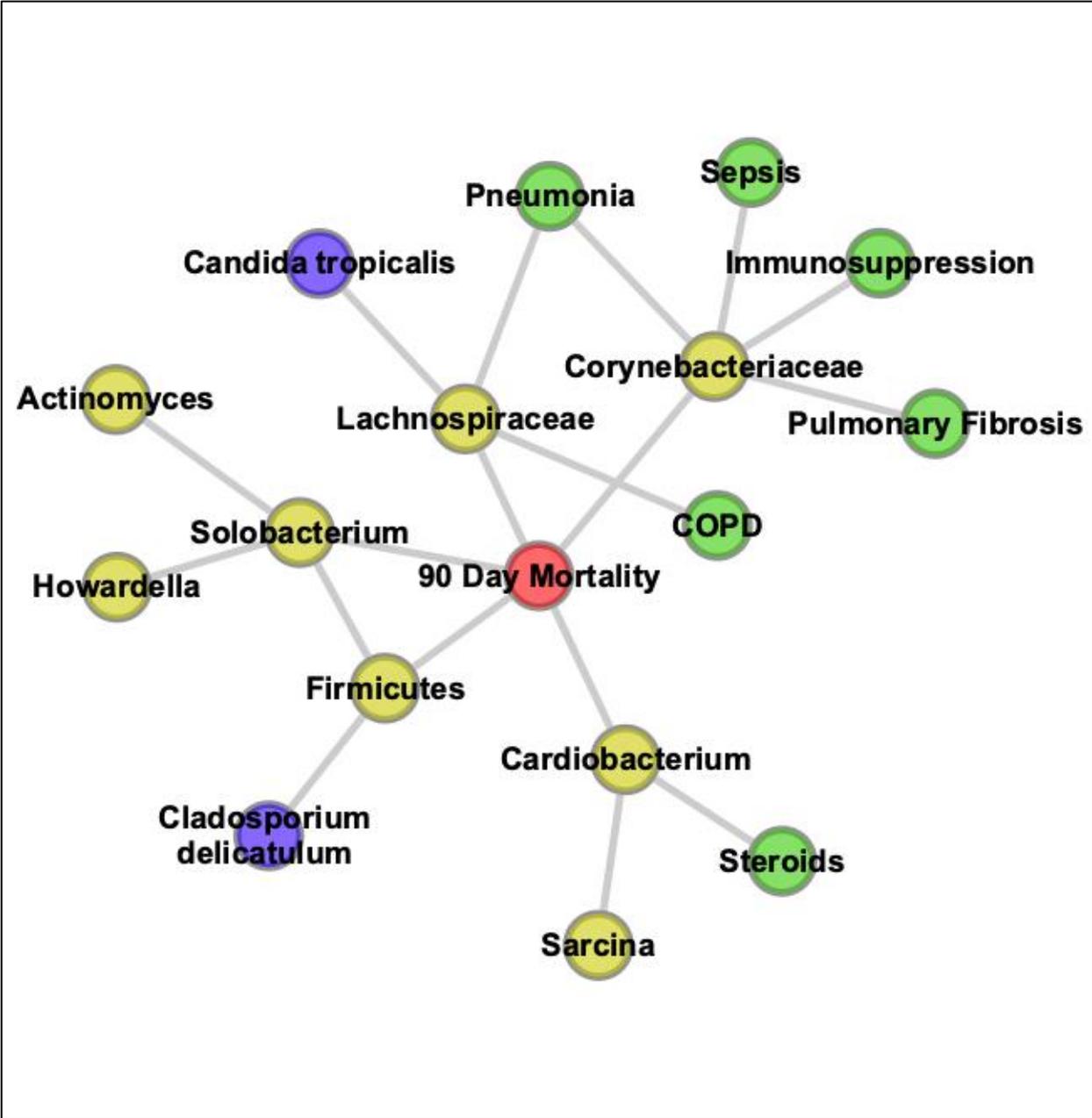
Appendix Figure 29: Probabilistic Graphical Model Network Analysis Demonstrating First and Second Neighbors of Ventilator Free Days



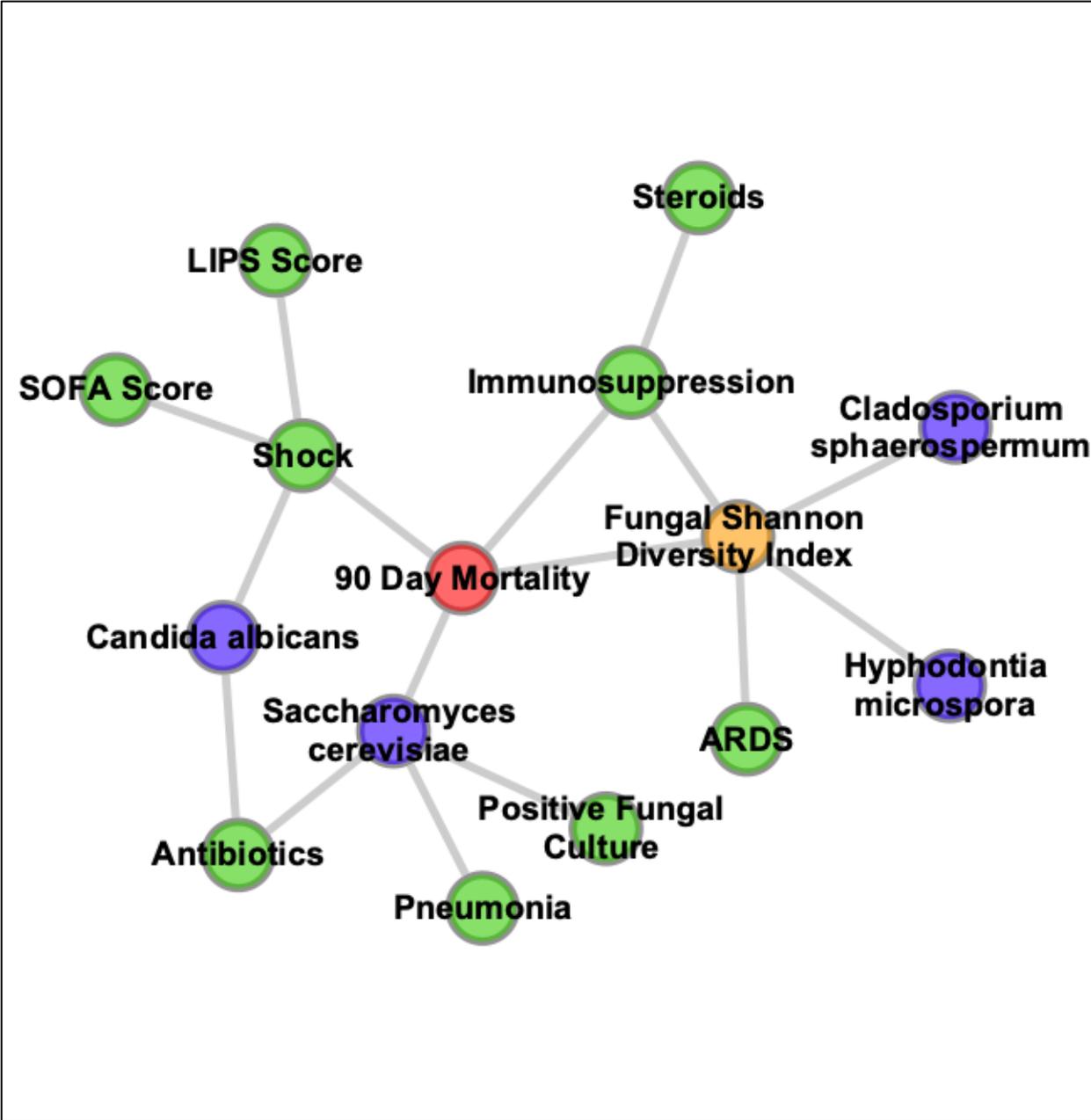
Appendix Figure 30: Probabilistic Graphical Model Network Analysis Demonstrating First and Second Neighbors of Time to Liberation from Mechanical Ventilation Including Bacterial Taxa



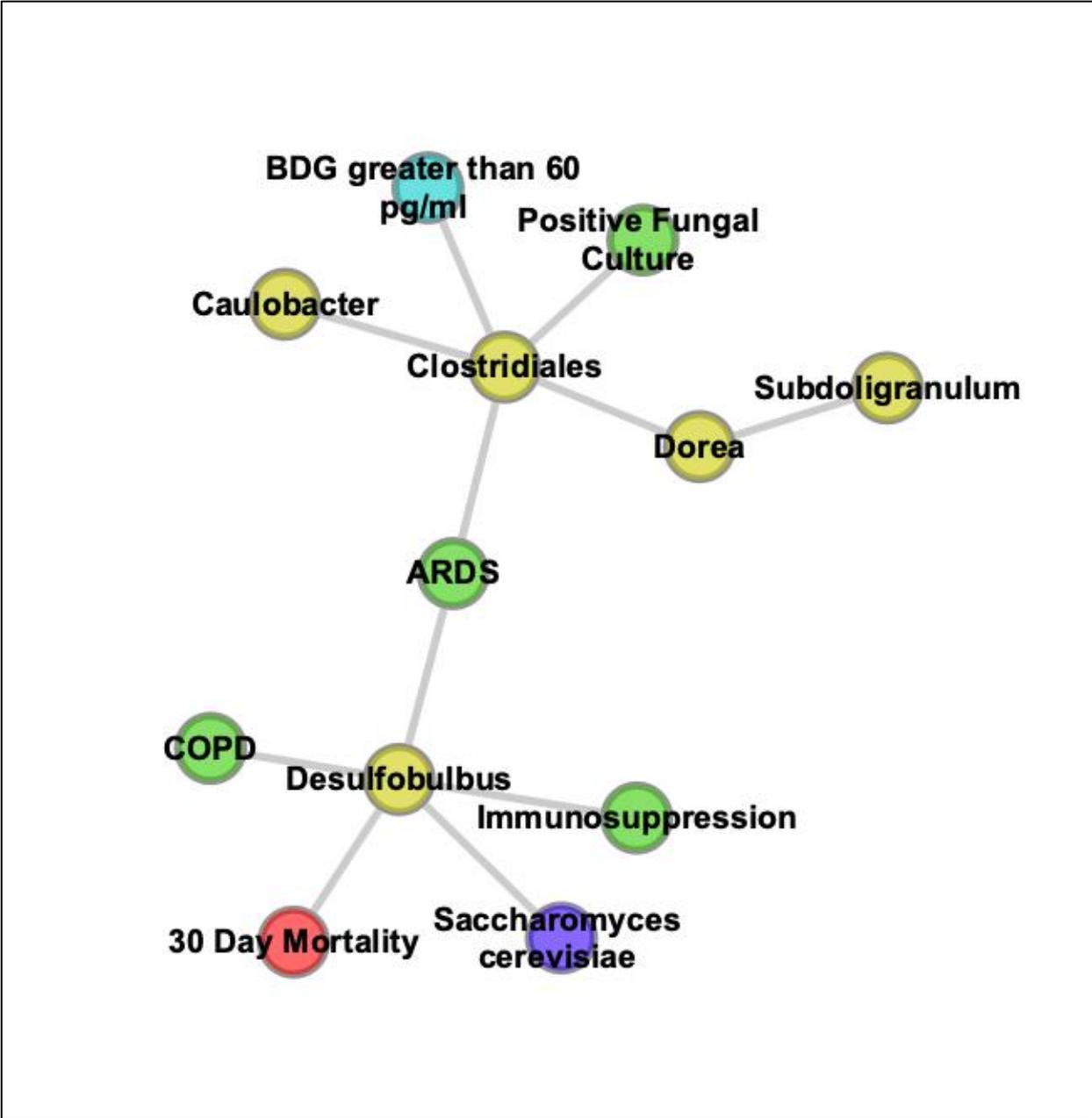
Appendix Figure 31: Probabilistic Graphical Model Network Analysis Demonstrating First and Second Neighbors of Time to Liberation from Mechanical Ventilation



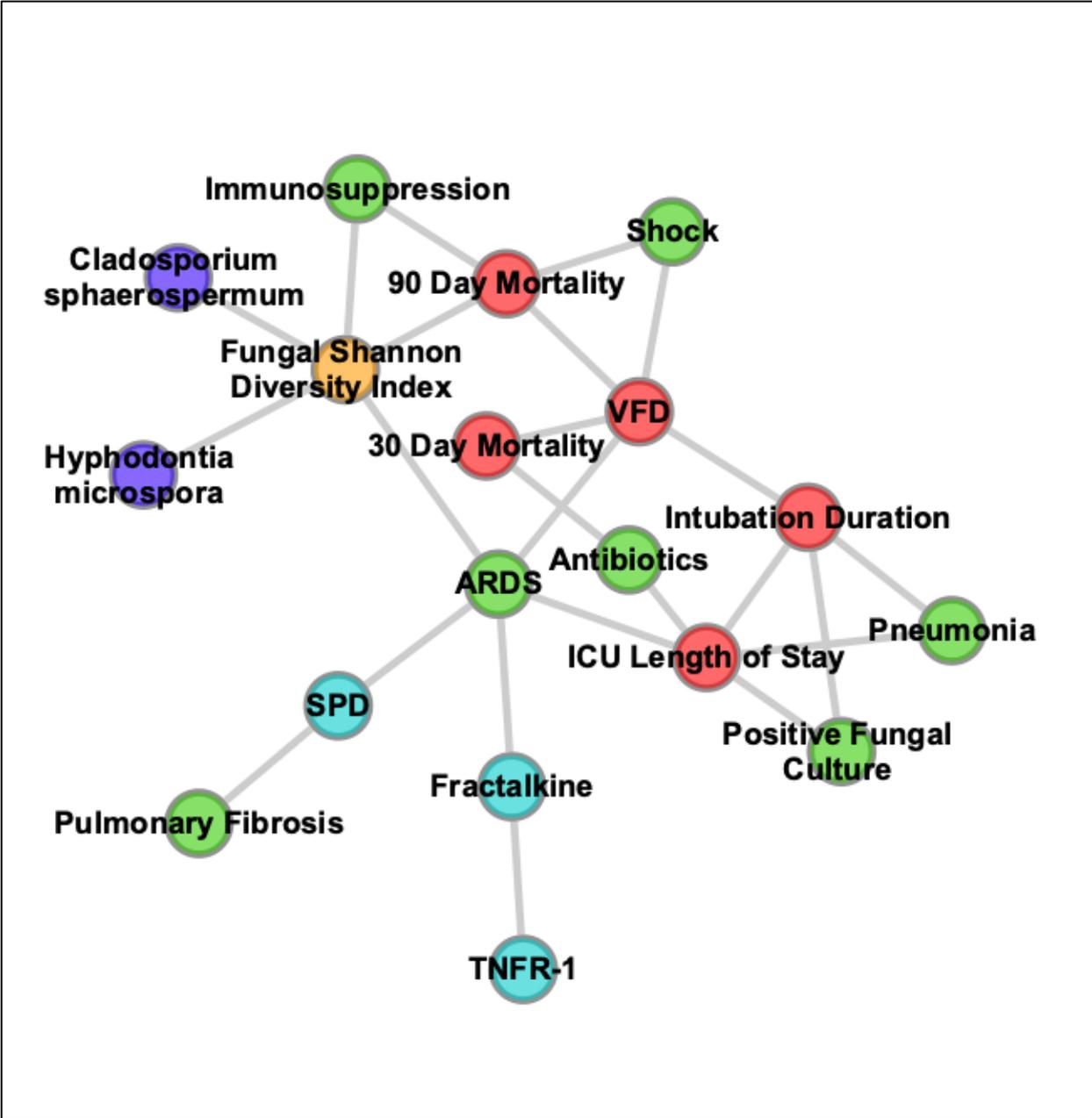
Appendix Figure 32: Probabilistic Graphical Model Network Analysis Demonstrating First and Second Neighbors of Mortality at 90 Days Including Bacterial Taxa



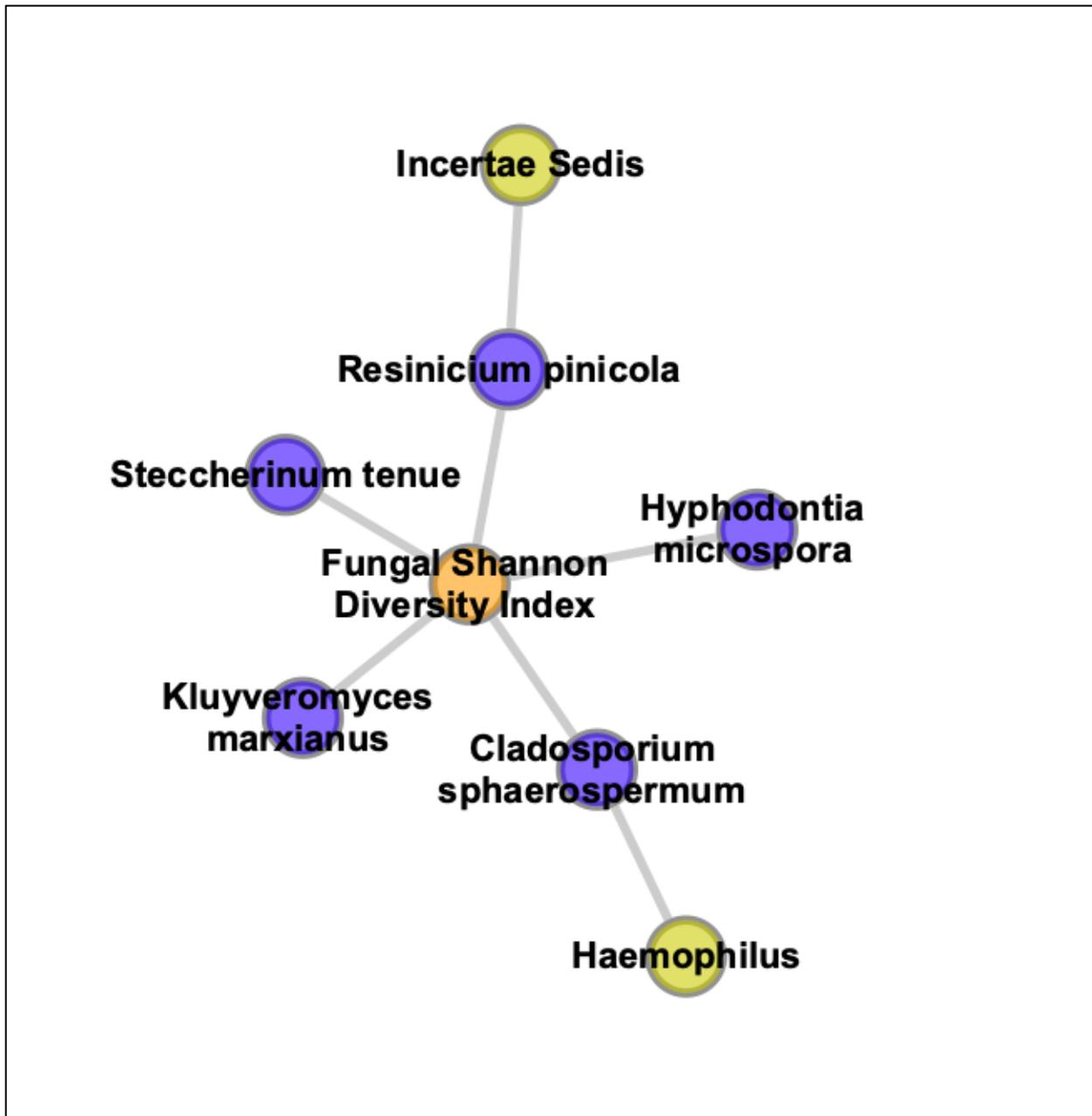
Appendix Figure 33: Probabilistic Graphical Model Network Analysis Demonstrating First and Second Neighbors of Mortality at 90 Days



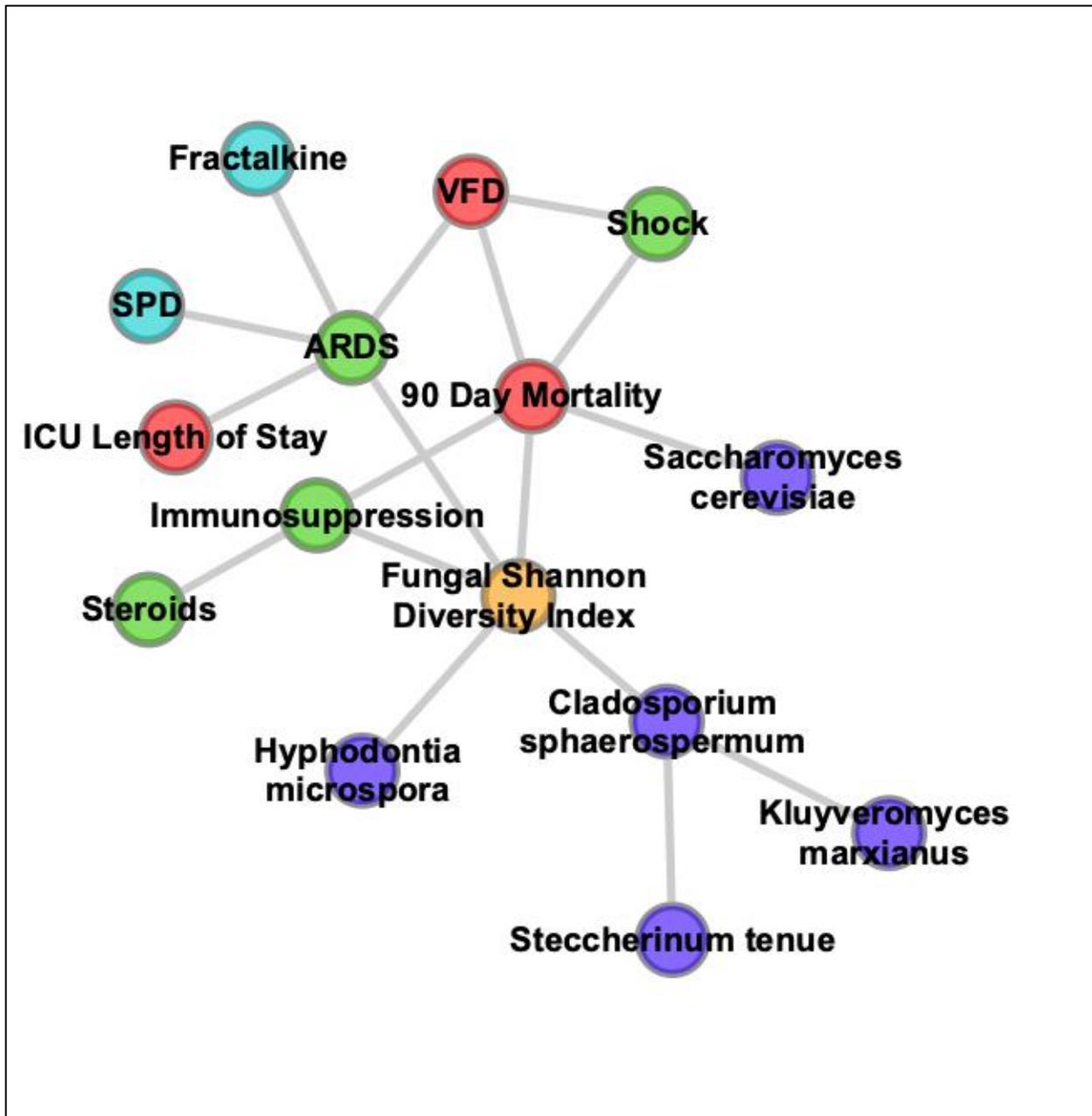
Appendix Figure 34: Probabilistic Graphical Model Network Analysis Demonstrating First and Second Neighbors of ARDS Status Including Bacteria



Appendix Figure 35: Probabilistic Graphical Model Network Analysis Demonstrating First and Second Neighbors of ARDS Status



Appendix Figure 36: Probabilistic Graphical Model Network Analysis Demonstrating First and Second Neighbors of the Shannon Index Including Bacteria



Appendix Figure 37: Probabilistic Graphical Model Network Analysis Demonstrating First and Second Neighbors of the Shannon Index

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