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Assessment of upper respiratory and gut bacterial microbiomes during COVID-19 infection in adults: potential aerodigestive transmission

Hafez Al-Momani[1]✉, Andrew Nelson[2], Hadeel Al Balawi[3], Dua'a Al Balawi[3], Iman Aolymat[4], Ashraf I. Khasawneh[1], Hala Tabl[1], Ayman Alsheikh[5], AbdelRahman M. Zueter[6], Jeffrey Pearson[7] & Christopher Ward[8]

SARS-CoV-2 is the viral pathogen responsible for COVID-19. Although morbidity and mortality frequently occur as a result of lung disease, the gastrointestinal (GI) tract is recognized as a primary location for SARS-CoV-2. Connections and interactions between the microbiome of the gut and respiratory system have been linked with viral infections via what has been referred to as the 'gut-lung axis' with potential aerodigestive communication in health and disease. This research explored the relationship between the microbiomes of the upper respiratory and GI tracts in patients with COVID-19 and examined Extraesophageal reflux (EOR), a mechanism which could contribute to dysregulated communication between the GI and respiratory tract (as identified in COVID-19). 97 patients with a laboratory diagnosis of COVID-19 infection, and 50 age-matched controls were recruited and stool, saliva and sputum were obtained from each participant. ELISA Pepsin tests and Reflux Symptom Index scores (RSI) were conducted for EOR assessment. DNA sequencing of the V4 region of the 16 S rRNA gene was performed for microbiome analysis. No differences were observed between the fecal microbiome's alpha and Shannon diversity indices; however, a distinct microbial composition was observed in COVID-19 patients (when compared to the controls). The respiratory microbiota from individuals with COVID-19 demonstrated a statistically significant reduction in Shannon diversity and bacterial richness alongside an overall reduction in the prevalence of organisms from a typical healthy respiratory microbiome. Furthermore, the bacterial richness of the stool and sputum samples was significantly lower among COVID-19 patients admitted to ICU. A significantly higher RSI score and salivary pepsin level were detected among those with COVID-19. The data indicates that COVID-19 is associated with a dysregulation of both the gut and lung microbiome with a more marked perturbation in the lung, particularly among COVID-19 patients who had been admitted to the ICU. The presence of increased RSI scores, combined with elevated levels of Pepsin, suggests that increased micro-aspiration may occur, which is consistent with of under-recognized interactions between the GI and lung microbiomes in COVID-19 patients and requires additional study. Such studies would benefit from the insights provided by biological samples which reflect the continuum of the aerodigestive tract.

Keywords SARS-CoV-2, COVID-19, Human microbiota, Upper respiratory tract, Gut, Extraesophageal reflux, Aspiration

[1]Department of Microbiology, Pathology and Forensic Medicine, Faculty of Medicine, The Hashemite University, P.O box 330127, Zarqa 13133, Jordan. [2]Department of Applied Sciences, Faculty of Health and Life Sciences, Northumbria University, Northumberland Building, Newcastle Upon Tyne NE1 8ST, United Kingdom. [3]Faculty of Applied Medical Sciences, The Hashemite University, Zarqa 13133, Jordan. [4]Department of Anatomy, Physiology and Biochemistry, Faculty of Medicine, The Hashemite University, P.O box 330127, Zarqa 13133, Jordan. [5]Department of Medical Laboratory Sciences, Faculty of Allied Medical Sciences, Zarqa University, P.O.box 2000, Zarqa 13110, Jordan. [6]Department of Medical Laboratory Sciences, Faculty of Applied Medical Sciences, The Hashemite University, P.O box 330127, Zarqa 13133, Jordan. [7]Biosciences Institute, Newcastle University Medical School, Newcastle upon

Tyne NE2 4HH, GB, United Kingdom. [8]Translational and Clinical Research Institute, Newcastle University Medical School, Newcastle upon Tyne NE2 4HH, GB, United Kingdom. ✉email: Hafez@hu.edu.jo

Background

SARS-CoV-2 is the viral pathogen responsible for the contagious disease referred to as COVID-19. This virus was initially recognized in Wuhan (China), in December 2019, and subsequently gave rise to the global COVID-19 pandemic[1]. Statistics published in early April 2024 indicate that the pandemic extended to more than 200 nations and resulted in approximately 704 million documented cases of COVID-19 and more than seven million deaths. Infection with SARS-CoV-2 can give rise to long-term sequelae, the underlying mechanisms of which have yet to be delineated[2].

The virus gains access to cells by binding to the cell surface protein, angiotensin-converting enzyme 2 (ACE2)[3]. SARS-CoV-2 is typically introduced to the body via the respiratory tract, where it can have potent effects[4]. The clinical endpoints of COVID-19 infection are impacted by the interaction between the microbiome within the lungs and the host immune system[5]. Early studies indicate that microenvironments within the nose, mouth, pharynx, and lungs may be modified by the presence of SARS-CoV-2 and cause dysbiosis[5–7]which can make individuals vulnerable to secondary infections caused by bacterial pathogens and increase the likelihood of morbidity and mortality[8–13].

Although SARS-CoV-2 primarily targets the lung, it is evident that the virus affects other viscera[14,15]. A range of specimens (such as fluid from bronchoalveolar lavage, throat swabs, feces, serum and urine) has been found to contain viral particles and nucleic acids[16,17]and ACE2 expression has been demonstrated by single-cell RNA sequencing in numerous viscera and tissues[18–20]. In postmortem specimens, many organs have exhibited SARS-CoV-2 cell tropism[21].

Current research provides robust evidence that the gastrointestinal (GI) tract is a major focus of SARS-CoV-2[22,23]and causes symptoms such as sickness, diarrhea and anorexia[24,25]. A high SARS-CoV-2 load has been observed in the GI tract where the pathogen has exploited ACE2 as a viral receptor[26]. This finding implies that the GI tract can be infected by the virus[27,28]and suggests that there is a risk of fecal-oral dissemination[25].

In patients with SARS-CoV-2, the microbiota within the lower GI system is also affected, an outcome mediated via what is referred to as the 'gut-lung axis'[29,30]. Fluctuations in the bacterial population of the GI tract are related to the severity of COVID-19[8,9], and also appear to occur concurrently with microbiome changes in the respiratory tract[31].

Although the primary issue with COVID-19 infection concerns the management of its pulmonary manifestations, it is notable that the epithelial cells in the pulmonary alveoli exhibit only low levels of ACE2 expression[32]. A recent study has demonstrated that these modest levels of ACE2 expression restrict the permissiveness of the alveoli (for this viral pathogen)[33]. The authors surmised that the acute respiratory distress syndrome associated with SARS-CoV-2 reflects secondary immune dysfunction and not immediate injury to the alveoli[33,34]. Virions arising from SARS-CoV-2 (taken up by alveolar macrophages) are thought to contribute to this disease process[33].

In combination with micro-aspiration, Gastroesophageal reflux (GOR) (the backward regurgitation of gastric material into the esophagus) is another method via which the virions may become absorbed by pulmonary sentinel phagocytes[35,36]. Although common, GOR disease (GORD) may arise if GOR becomes pathological[37,38]. Research indicates similarities between the bacteria in the pulmonary and GI systems of healthy adults which is indicative of ongoing physiological micro-aspiration in healthy individuals[39].

When the retrograde flow of stomach contents extends into the upper esophagus, larynx, pharynx or nasal cavity, it is referred to as extra-esophageal reflux (EOR)[40]. Previous studies have identified a correlation between EOR and a range of respiratory tract problems such as post-nasal drip, cough, persistent throat clearing, throat discomfort, tight chest and bronchospasm[41]. Tooth decay and ear infections have also been associated with observations of reflux[42,43]. In children with cystic fibrosis, there is evidence that bacterial colonization of the smaller airways may be related to EOR which implies that bacteria may migrate from the gut into the lungs (and vice versa)[44]. This phenomenon may also arise in other pulmonary pathologies including SARS-CoV-2.

In ICU patients, a documented risk factor for the development of hospital-acquired pneumonia is a microorganism found in gastric samples[45]. Our previously published research draws attention to the role of the stomach as a reservoir for some bacterial pathogens[46,47]. These results substantiate the relationship between gastric and lower respiratory tract microorganisms[48](which could be pertinent to viral dissemination and infection) as well as the secondary bacterial infections associated with morbidity and mortality caused by viral infection[49].

Although the fluctuations in the GI microbiome have been confirmed via the collection of longitudinal fecal specimens in patients with COVID-19[50], there is a dearth of studies that have explored the possibility of any link between the microbiomes of the respiratory and GI tracts during infection with SARS-CoV-2.

Variations in the respiratory microbiome can be associated with a bidirectional shift between the gut and other organs which has been linked with several serious diseases[51]. These variations in the respiratory microbiome (i.e., the lungs and airways) can also impact the composition of the intestinal microbiome. The interactions in this bidirectional system (known as the 'gut-lung axis') suggest a direct correlation between several respiratory disorders and gastrointestinal indicators[52]. Animal-based research observed that *Pneumocystis murina*, *influenza*virus infection, or intratracheal instillation of lipopolysaccharide instigate modifications in the gut microbiome[53]; in mice, the *influenza* virus infection increases *Enterobacteriaceae* and reduces the amount of *Lactococci*and Lactobacilli in the gut microbiome[54]. Additionally, *Pseudomonas aeruginosa* and multidrug-resistant *Staphylococcus*induced pneumonia harms the gut by instigating reduced intestinal epithelial

proliferation[8]. Moderate lung damage dysregulates the microbiome of the airway resulting in increased short-term bacterial translocation into the circulation and an elevated bacterial load in the cecum[8].

Furthermore, dysbiosis in the intestinal microbiome is associated with respiratory issues and ailments[55,56] such as childhood asthma which has been linked with an increase in *Clostridia* and a decrease in the levels of *Bifidobacteria*in the gut[57]. Additionally, the gut-lung axis (in which immune cells migrate from the gut to the respiratory system via circulation) may augment immunity[58]and the stomach can regulate pulmonary responses via host-acquired inflammatory mediators in the circulation[59,60]. Additionally, respiratory viral infections can impact the gut microbiome which is crucial for facilitating the immune system's response to respiratory infections and its adaptive immune responses to lung-based pathogens[61,62]. When considering viral pulmonary infections, the macrophage response to respiratory infection is contingent upon gut bacteria[62,63]; therefore, the lung and gut are interconnected organs which can modify each other's homeostasis via immunological coordination involving the microbiome. In association with other ecological dynamics, microbes are indispensable in regulating immune responses in the lungs and gut[64]and COVID-19 cases frequently exhibit parallel crosstalk between the lungs and intestines[65]. Contemporary research indicates that gut microbiome manipulation plays a fundamental role in reducing enteritis and ventilator-associated pneumonia and reverses antibiotic effects to inhibit the replication of the influenza virus in the epithelium of the lungs[66–68]. Some studies have noted the possibilities of employing the gut microbiome as an auxiliary methodology for treating COVID-19 and the role of probiotics in negating GI symptoms (via preservation of the gut microbiome equilibrium and protection of the respiratory system)[65,69–71].

These findings indicate that gut-lung microbiome analyses of COVID-19 patients could enhance existing knowledge regarding the interactions between these microbiomes, facilitate the identification of biomarkers for disease progression and recovery, and shape individual treatment programs. Additionally, such research could ascertain the effect of comorbidities on microbiome composition, account for discrepancies in the severity of symptoms among patients and inform treatment programs and public health policies. This may be particularly for patients experiencing the long-term effects of COVID-19.

This research aims to explore the relationship between the microbiomes of the upper respiratory and GI tracts in patients with COVID-19 and control subjects. Furthermore, the current study aimed to examine the likelihood of EOR in patients with COVID-19 who required in-patient care. This research postulated that evidence of the symptoms of EOR, alongside the presence of the gastric protease (pepsin) in sputum or saliva would be consistent with possible SARS-CoV-2 transmission between the GI reservoir and the respiratory tract.

Methods

Ethical approval

The ethics committees Hashemite University and Prince Hamza Hospital granted full ethical approval for this research (ref. no. 5/3/2020/2021). Each of the participants submitted their informed written consent prior to their recruitment. The research was conducted in keeping with the appropriate recommendations and legislation and all of the methods employed were performed per the relevant guidelines and regulations.

Study population

147 participants were recruited for this study: 97 in-patients from the Prince Hamza Hospital (with a laboratory diagnosis of COVID-19 infection) and 50 age-matched controls. The control group consisted of adults attending routine physical assessments at primary care centers who were asymptomatic for COVID-19 and had a negative COVID-19 test following a routine screening polymerase chain reaction (RT-PCR) assay. The controls had not experienced any recent infections and had no history of anti-microbial, probiotic, chemotherapy, or other drug usage in the 14 days before the study. In the non-control cohort, SARS-CoV-2 infection was verified by two consecutive throat swabs using an assay based on RT-PCR. This group contained 26 patients with severe COVID-19 who were admitted to the ICU for close observation and monitoring; however, they did not require mechanical ventilation and intubation and, therefore, they were able to provide their informed written consent. The exclusion criteria for these patients included respiratory failure (or the need for mechanical ventilation), shock, or any visceral impairment which complicated the infection. The remaining patients with COVID-19 received treatment in the general ward.

Sample collection

Nursing staff provided each potential participant with an information sheet and guided those who chose to take part in the study through the consent process by marking each item on the form to indicate assent. Each patient and nurse were required to sign and print their name on the consent form. After consent had been obtained, and following their hospital admission, each participant (from both cohorts) was required to provide a specimen of feces, saliva, and sputum. Sterile containers were used for the collection of all the specimens, which were subsequently placed in storage at a temperature of −80 °C before processing. Pepsin tests were conducted on the saliva specimens to provide a quantitative measure of EOR. For the sampling process, all clinical personnel donned personal protection equipment (solid front-wraparound gowns, eye protection and N95 respirators) per the recommended biosafety guidelines.

To guarantee the accuracy and reliability of this study, a carefully designed sampling procedure was created for the collection of the sputum, saliva, and stool samples. Under the guidance of the research coordinator, a nurse-directed demonstration of saliva and sputum collection was conducted to illustrate the process to the participants. Additionally, each participant was asked to remove their dentures and braces (where applicable) and to thoroughly rinse their mouths. To collect the sputum samples, each patient was given a sterile container (labelled with their participant number and the date) and asked to expectorate deep cough sputum (after an overnight fast). In the period before sample collection, the participants were instructed to avoid consuming any

food or drink to enhance sample quality and facilitate the gathering of a more representative sample from the lungs. The sputum samples (which were obtained either on the day of admission or on the morning of the second day of admission) amounted to approximately 2 ml per patient. Following collection, the samples were stored in sterile containers at −80 °C in preparation for the DNA extraction phase.

To facilitate the collection of the saliva samples, 15 mL plastic centrifuge tubes were labelled with the participant number, dated, marked with a 4 mL measurement line, and distributed to the participants. To begin the saliva collection process, the sample tubes were placed in a cup containing ice and the participants were instructed to use their tongue to push the saliva into the tube. Following this process, they returned the tube to the cup, waited for more saliva to form, and added it to the tube. This process was repeated until 4 mL of saliva (excluding the frothy region) had been provided. Once the required sample had been obtained, the nurse was informed and the tube was sealed, sterilized with alcohol, and placed in the collection rack in the fridge.

To obtain the required stool samples, each participant was supplied with a sterile, leak-proof container and instructed on how to avoid contamination with urine or water and maintain the integrity of the sample. These specimens were stored in a freezer with dry ice and subsequently agitated in Dulbecco's phosphate-buffered solution for fifteen minutes and filtered through forty-micron filters. Throughout the sample collection phase, standard contamination protocols were in effect.

Reflux symptom index questionnaire

The Reflux Symptom Index (RSI) employs a ranking of 0 to 5 (5 being the most severe) to assess the presence and intensity of commonly reported EOR symptoms[72]. The questionnaire requests information regarding numerous symptoms including hoarseness (or dysphonia), throat-clearing, mucus in the throat, post-nasal drip, dysphagia (concerning solids, fluids or pills), post-prandial or supine cough, difficulty breathing, choking episodes, persistent or irritating coughing, feelings of a lump (or throat obstruction), dyspepsia, chest discomfort, and reflux of gastric acid. Clinically relevant EOR was suggested by a score > 13[73]. The RSI has demonstrated high test-retest reliability (rs = 0.921) and internal consistency reliability (α = 0.969)[74].

Demographic characteristics

This study recorded patient sociodemographic data (i.e. age and gender) and encompassed concurrent comorbidities such as diabetes mellitus, cardiovascular pathology, high blood pressure, hyperlipidemia, respiratory disorder, kidney or GI dysfunction, and thyroid disorder.

Salivary collection and pepsin management

Pepsin is one of the primary digestive enzymes that facilitate the digestion of proteins in food. It is a protease enzyme which is synthesized via its precursor pepsinogen in the stomach's gastric chief cells. The presence of pepsin in the esophagus (or proximal sites) is considered to be indicative of reflux[75,76]and it has been identified in the tissues of the throat and nasal sinuses, in fluid secreted in the trachea or collected by bronchoalveolar lavage, and in saliva. Pepsin is considered to be a non-invasive bioindicator for the diagnosis of GORD and reflux into the airways[76–78]. For the current study, a 2 mL sample of saliva was gathered from each participant into tubes containing 0.5 mL 0.01 M citric acid. These underwent centrifugation (at 4000 rpm for 5 min). A human pepsin ELISA kit (Catalog No. ELK8433; ELK Biotechnology, Wuhan, China), was employed to identify the presence of pepsin. The assay's range of detection and sensitivity were 3–200 ng/mL, and 1 ng/mL, respectively. Each specimen was encoded to ensure anonymity and the biochemical analysis was performed blind to any clinical or physiological data.

DNA extraction

Following the manufacturer's instructions, a QIAamp Power DNA Isolation Kit (QIAGEN, Hilden, Germany) was utilized to extract microbial genomic deoxyribonucleic acid (gDNA) from the sputum and fecal specimens. This process was conducted in the Category 3 facility at Hashemite University.

Molecular-based studies

Bacterial profiling of the variable region 4 (V4) of the 16 S rRNA gene was conducted by NU-OMICS (Northumbria University) and based on the Schloss wet-lab MiSeq SOP[79]. Briefly, PCR was conducted using 1x KAPA2G Robust HotStart ReadyMix, 0.5 µM each primer, and 1 µl of template DNA under the following conditions: 95^{0}C for 2 min, 30 cycles at 95^{0}C for 20s, 55^{0}C for 15s, 72^{0}C for 5 min and a final extension at 72^{0}C for 10 min. One positive (Zymobiomics Microbial Mock community DNA standard) and one negative control sample were included in each of the 96-well plates and carried through to sequencing. PCR products were quantified using Quant-iT™ PicoGreen™ dsDNA Assay (Invitrogen), each sample was normalized to 10nM, and each 96-well plate was pooled. Each pool was quantified using fragment size (as determined by BioAnalyzer (Agilent Technologies)) and concentration (as determined by Qubit (Invitrogen)). The pools were combined in equimolar amounts to create a single library then denatured using 0.2 N NaOH for 5 min; diluted to a final concentration of 4.5 pM; supplemented with 20% PhiX; and loaded onto a MiSeq V2 500 cycle cartridge.

DNA sequencing of the V4 region of the 16 S rRNA gene was performed at the NU-OMICs DNA sequencing research facility at Northumbria University (as previously described)[79]. One positive (Zymobiomics Microbial Mock community DNA standard) and one negative control sample were included in each 96-well plate and carried through to sequencing. The fastq files were processed in QIIME2 using the dada2 workflow (McCurdie 2017), the taxonomic information was assigned via the SILVA database (v138.1), and non-bacterial sequences were removed. The resulting data was rarefied to 4420 reads per sample for downstream analysis. The data was analyzed in R using the Agile Toolkit for Incisive Microbial Analyses software for calculating diversity metrics and statistical analysis. The significance of categorical variables was determined using the non-parametric

Mann-Whitney test for two-category comparisons (or the Kruskal-Wallis test when comparing three or more categories). All p values were adjusted for multiple comparisons with the FDR algorithm. Sequence data that supports the findings of this study has been deposited in BioProject-NCBI under the accession number PRJNA1162807 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1162807).

Statistical analysis

Univariate analysis was utilized to compare patients with COVID-19 to different degrees of severity while variable type dictated whether a t-test or chi-square test was performed. Independent risk factors for COVID-19 were established via the use of multivariate logistic regression analysis. A p-value of ≤ 0.05 was deemed to indicate statistical significance. GraphPad InStat 6.0 software was employed for data analysis.

Results

Patient characteristics

Of the 97 patients with COVID-19 recruited into the study, 26 were being cared for in the ICU and 71 in the medical unit. Additionally, this research included 50 age-matched controls recruited from patients attending primary care who had tested negative for COVID-19. The demographic characteristics of the subjects are presented in Table 1. The mean ± standard deviation (SD) (range) patient age was 55.9 ± 16.3 (17–92) years. 81/147 (55%) participants were men and 66/147 (45%) were women.

Gut microbiome in patients with COVID-19

Amplicon sequencing was performed on the stool specimens collected from each study participant to elucidate the influence of COVID-19 on the GI microbiome. When the results of the COVID-19 patients were compared with the age-matched control participants without COVID-19, no differences were observed between the fecal microbiome's alpha and Shannon diversity indices (see Fig. 1). However, a distinct microbial composition was observed in COVID-19 patients when compared with non-infected age-matched controls ($p = 0.05$, PERMANOVA) (see Fig. 2). Mann-Whitney FDR showed that taxa identified as significantly different between the COVID-19 and the control group (see Fig. 3).

The COVID-19 patients exhibited a greater prevalence of the following bacteria at phylum and genus levels: genus Lactobacillales (Enterococcus and Streptococcus) and Family Oscillospiraceae and Clostridalis genus (Lachnoclostridium) together with the bacterium Actinomyces (an opportunistic disease-inducing microorganism). Potentially advantageous butyrate-producing microorganisms (such as Fecalibacterium and Roseburia), as well as a number of Firmicutes (such as Lachnospiraceae genus Agathobacter, Anaerostipes and

Characteristic	All patients ($n = 147$)	COVID-19 patients (non-ICU) ($N = 71$)	COVID-19 ICU patients ($N = 26$)	Control ($N = 50$)
Patient age: mean ± SD (range)	**55.9 ± 16.3 (17–92)**	**58.5 ± 18.5 (18–92)**	**58.5 + 18.5 (39–88)**	**51.2 + 13.5 (17–83)**
Age group				
17–27	6	5	0	1
28–38	14	5	0	9
39–49	35	13	6	16
50–60	28	11	5	12
61–71	32	16	7	9
72–82	20	14	5	1
> 83	10	7	2	1
Gender				
Male	81 (55.1%)	37	11	27
Female	66 (44.9%)	29	15	23
Comorbidity				
Coronary artery disease	21 (14.3%)	12 (16.9%)	4 (15.3%)	5 (10.0%)
Congestive heart failure	11 (7.5%)	7 (9.8%)	3 (11.5%)	1 (2.0%)
Cardiac arrhythmia	6 (3.2%)	3 (3.2%)	3 (3.2%)	0 (0.0%)
Hypertension	45 (30.6%)	25 (35.2%)	10 (38.4%)	10 (20.0%)
Hyperlipidemia	24 (16.3%)	16 (22.5%)	4 (15.1%)	4 (8.0%)
Diabetes	27 (18.2%)	23 (18.2%)	8 (18.2%)	7 (18.2%)
Cerebrovascular accident	4 (4.2%)	2 (4.2%)	2 (4.2%)	0(4.2%)
Pulmonary disorders	15(4.4%)	8(4.4%)	5(4.4%)	2(4.4%)
Chronic renal insufficiency	13(9.5%)	3(9.5%)	2(9.5%)	2(4.0%)
Thyroid disorders	9(6.1%)	7(9.8%)	1(7.6%)	1(4.7%)
Irritable bowel syndrome	10 (6.8%)	6 (8.4%)	1 (3.8%)	3(6.0%)
Inflammatory bowel disease	4 (2.7%)	3 (4.2%)	0 (0.0%)	1 (2.0%)
Other GI disorders	8 (5.4%)	4 (5.6%)	2 (7.7%)	2 (4.0%)

Table 1. Demographic characteristics of study participants.

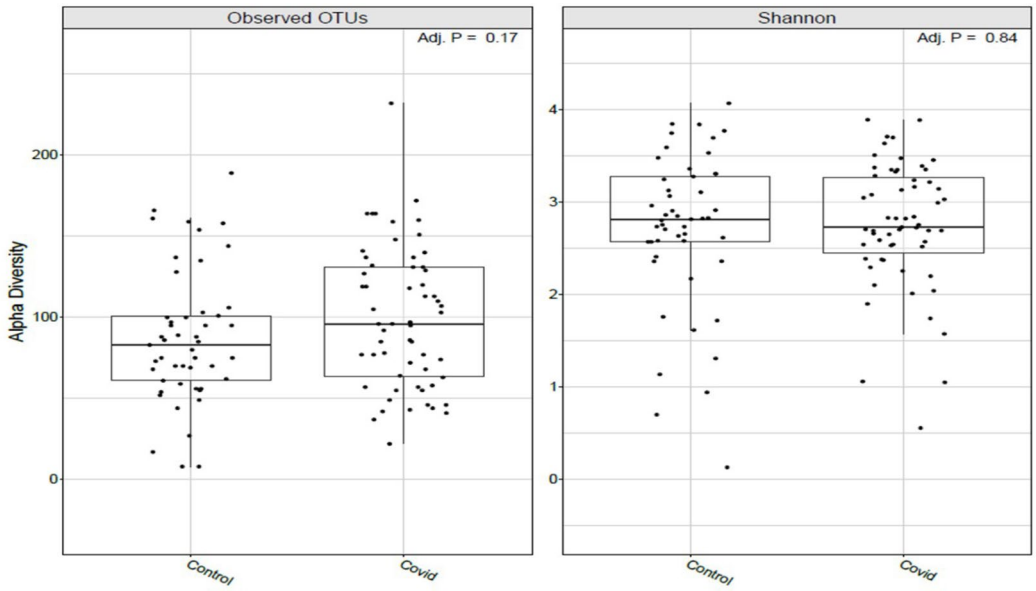

Fig. 1. Alpha and Shannon diversity calculations for non-COVID-19 (control) and diseased (COVID-19) stool samples. No significant differences between healthy and diseased samples were observed for various α-diversity measures.

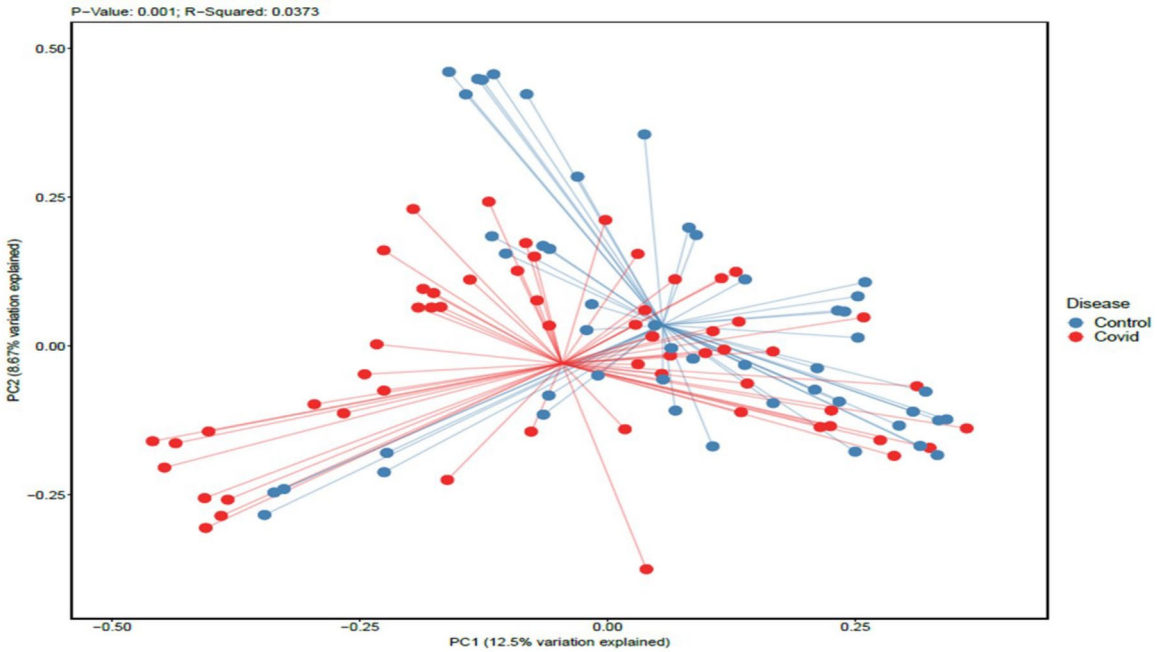

Fig. 2. Principal coordinate analysis (PCoA) plot with Bray-Curtis dissimilarity index. Healthy patient samples are shown in blue and diseased patient samples in red.

Roseburia), appeared to be less prevalent in this population. Additionally, COVID-19 patients demonstrated less microorganism heterogeneity and an increased relative abundance of commensal bacteria including Fecalibacterium, Clostridiaceae, Ruminococcus and Veillonella, and Dialister (from the phylum Firmicutes).

Variations in the gut microbiome between patients with mild and severe COVID-19

To understand the correlation between gut microbiota and COVID-19 severity, a comparison was conducted between the fecal samples of the COVID-19 ICU patients and those of the patients receiving treatment in the general ward. The alpha and Shannon diversity indices for the fecal microbiota were similar between the two

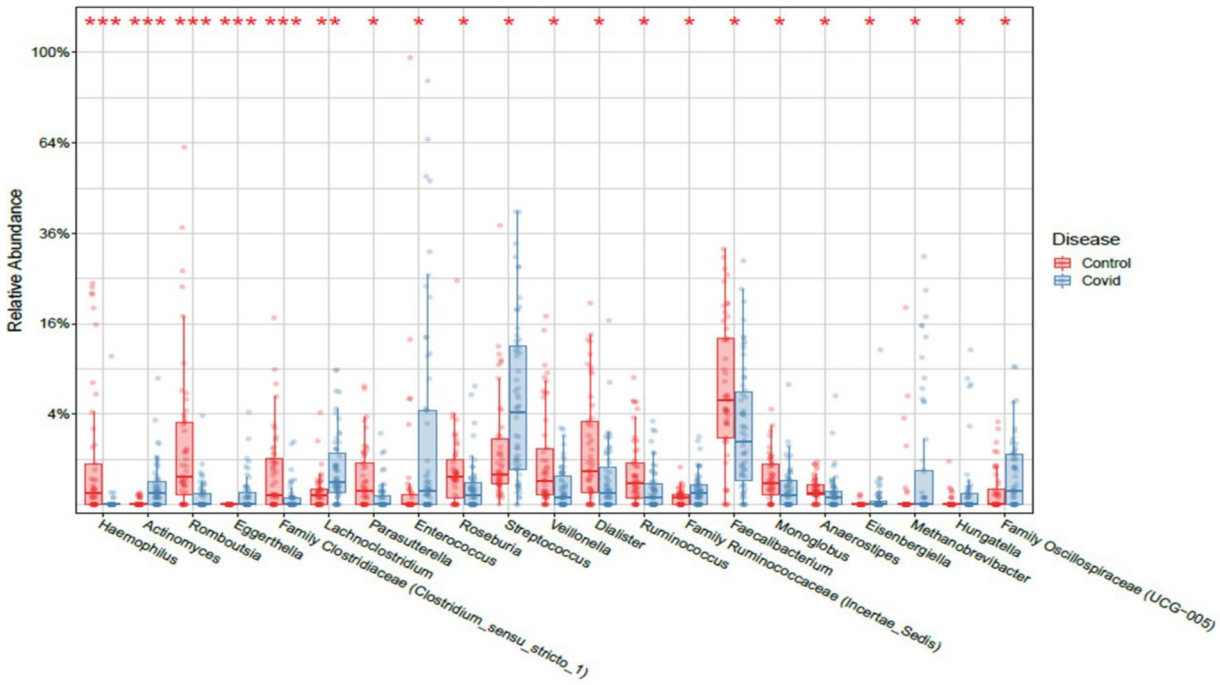

Fig. 3. Control and COVID-19-associated stool phyla by relative abundance with significant differences between health and disease are shown. Significance was assessed with a Mann-Whitney test ($P < 0.05$). COVID-19 abundances are shown in pale blue and control abundances are shown in red. *$0.01 < P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.001$.

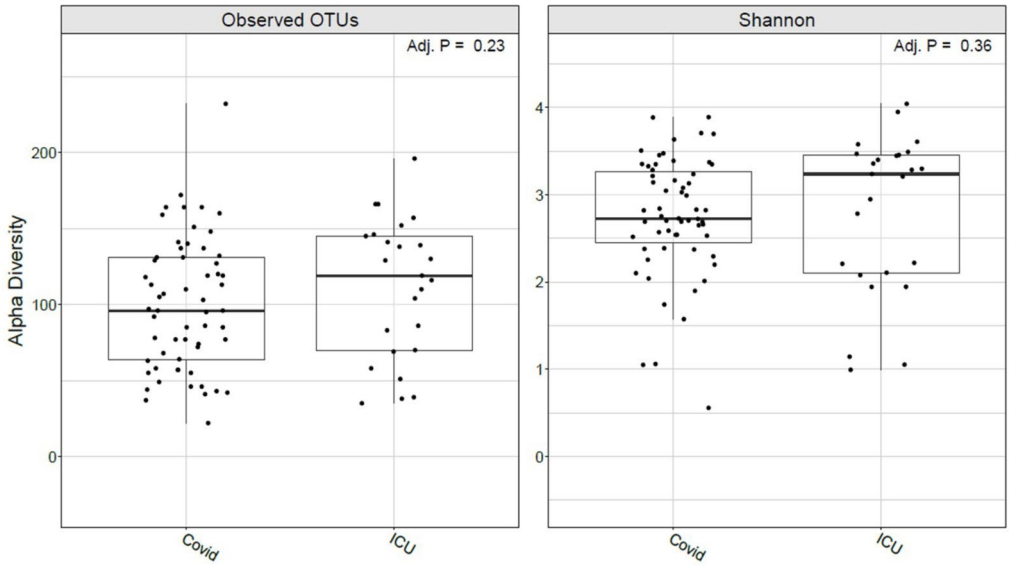

Fig. 4. Alpha and Shannon diversity calculations for stool samples from COVID-19 (non-ICU patients) and COVID-19 ICU samples.

cohorts (Fig. 4); however, the actual constituents of the two groups showed a significant difference ($P = 0.03$, PERMANOVA) (see Fig. 5).

The ICU patients displayed statistically significant amounts of Family Oscillospiraceae alongside a profusion of beneficial commensals such as Clostridiaceae, Haemophilus and Romboutsia. A higher abundance of both opportunistic pathogenic bacteria (Actinomyces) and Subdolgranulum were found among non-ICU COVID-19 patients (see Fig. 6).

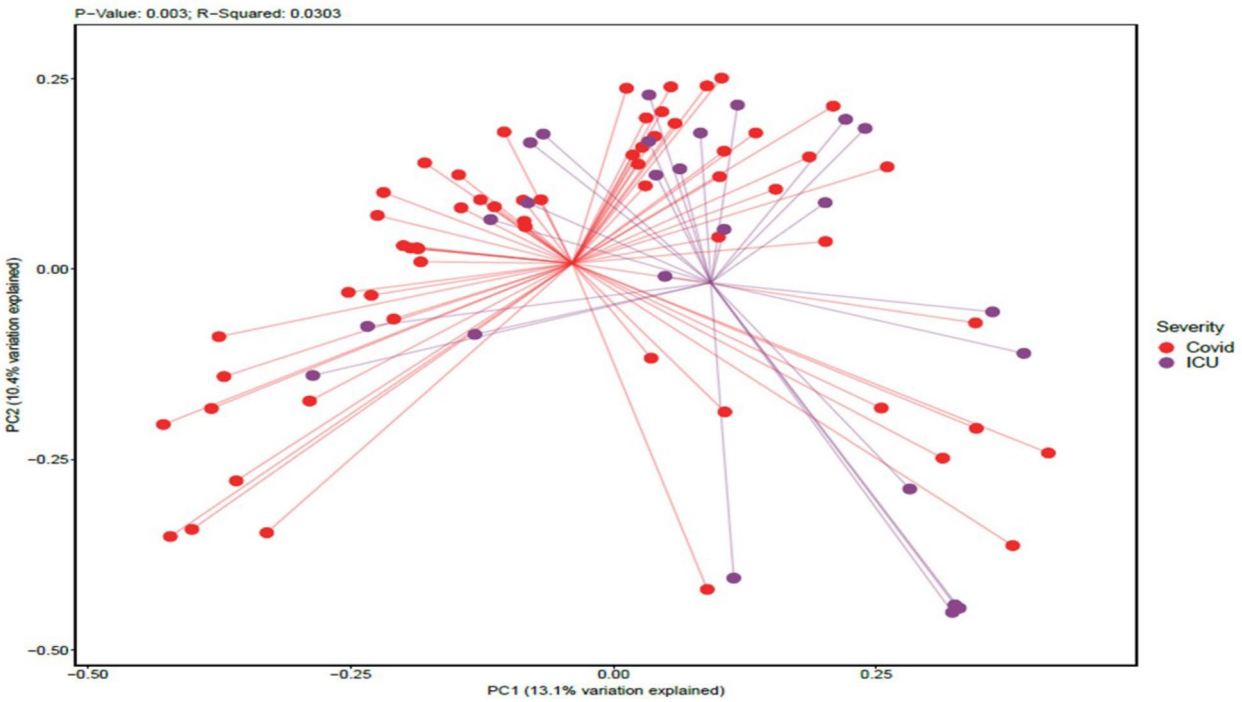

Fig. 5. Principal coordinate analysis (PCoA) plot with Bray-Curtis dissimilarity index. Non- ICU patient samples are shown in red and ICU patient samples are shown in purple.

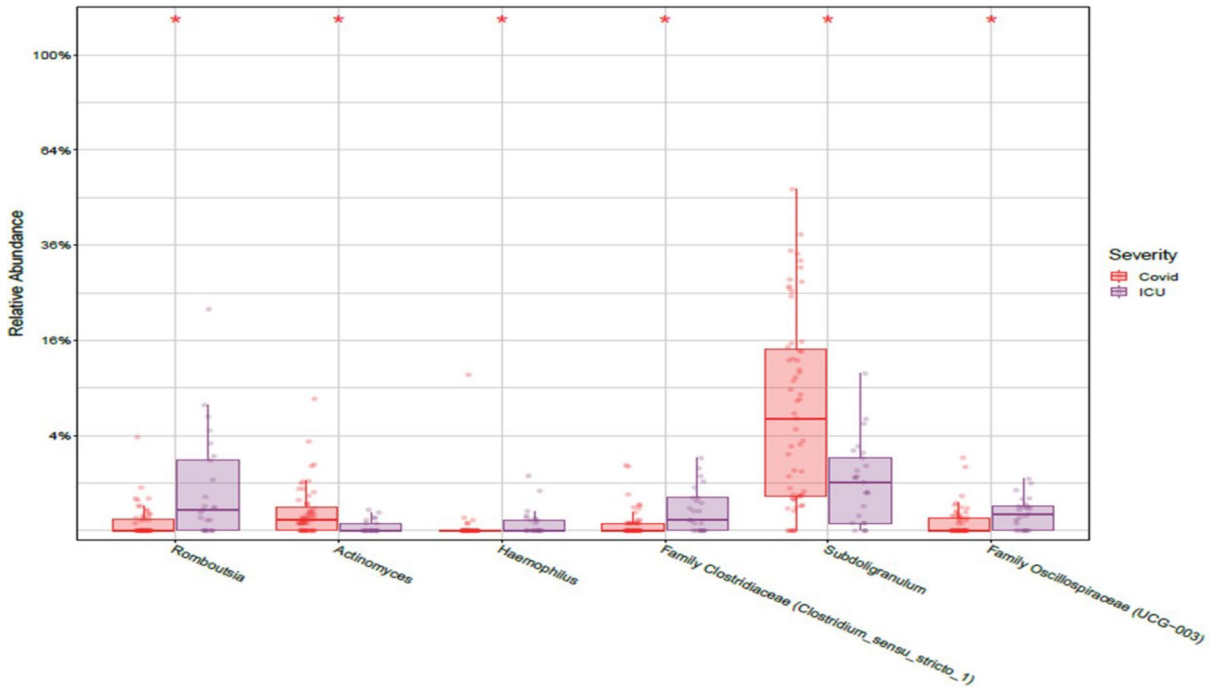

Fig. 6. ICU and non-ICU COVID-19 associated phyla by relative abundance with significant differences between ICU and non-ICU samples are shown. Significance was assessed with a Mann-Whitney test ($P < 0.05$). Non-ICU sample abundances are shown in pale blue, and ICU sample abundances in green. *$0.01 < P < 0.05$.

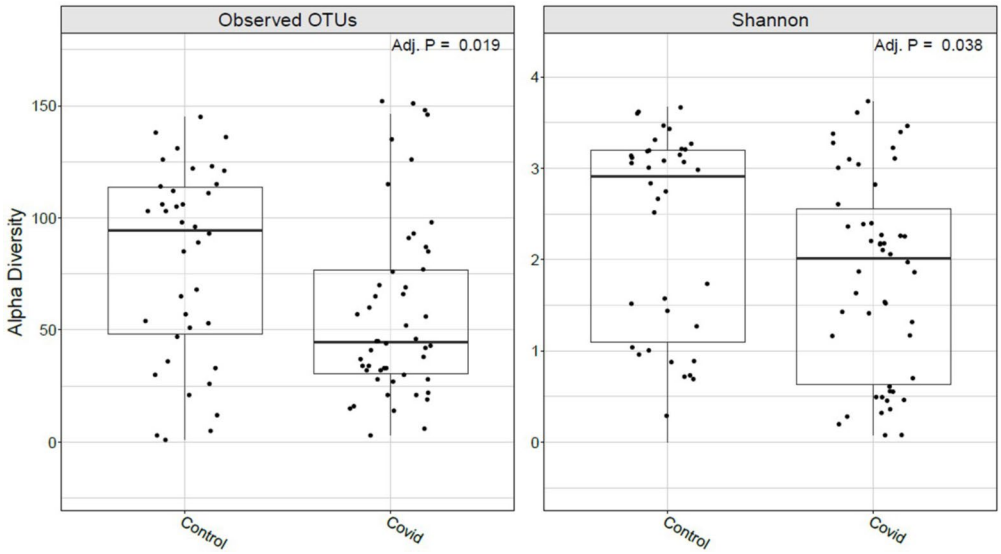

Fig. 7. Alpha and Shannon diversity calculations for non-COVID-19 (control) and diseased (COVID-19) sputum samples.

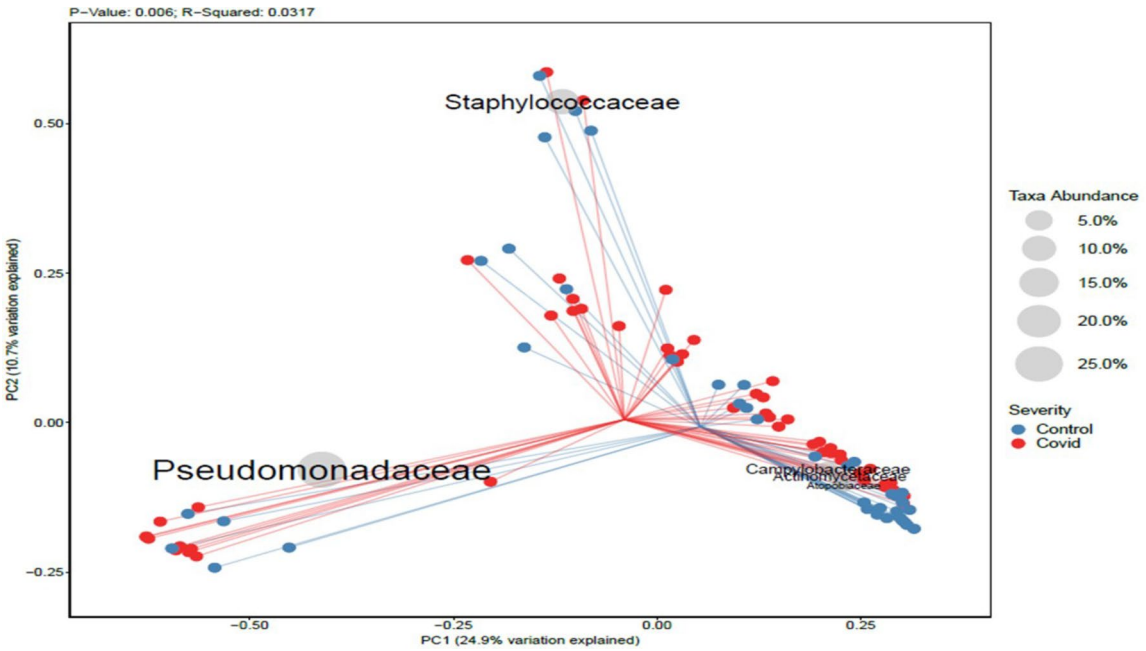

Fig. 8. Principal coordinate analysis (PCoA) plot with Bray-Curtis dissimilarity index. Healthy patient samples are shown in blue and diseased patient samples are shown in red.

Respiratory microbiome in patients with COVID-19

Compared to the control population, the respiratory microbiota from individuals with COVID-19 demonstrated a statistically significant reduction in Shannon diversity and bacterial richness, alongside a reduced frequency of the organisms typically associated with a healthy respiratory microbiome (see Fig. 7).

The patients with COVID-19 appeared to exhibit a distinct microbial composition (when compared to controls) ($P = 0.05$, PERMANOVA) (Fig. 8). When compared with the samples from the controls, the patients with COVID-19 evidenced a lower abundance of all taxa including *Firmicutes* (*Gemella* and *Lachnoanaerobaculum*), *Fretibacterium*, *Haemophilus*, *Oribacterium*, *Parvimonas*, and *Stomatobaculum* (see Fig. 9).

Variations in the respiratory Microbiome between patients with mild and severe COVID-19

The results from the sputum samples from the COVID-19 ICU group were compared with those of the patients managed in the general ward to explore the effect of SARS-CoV-2 infection severity on the respiratory

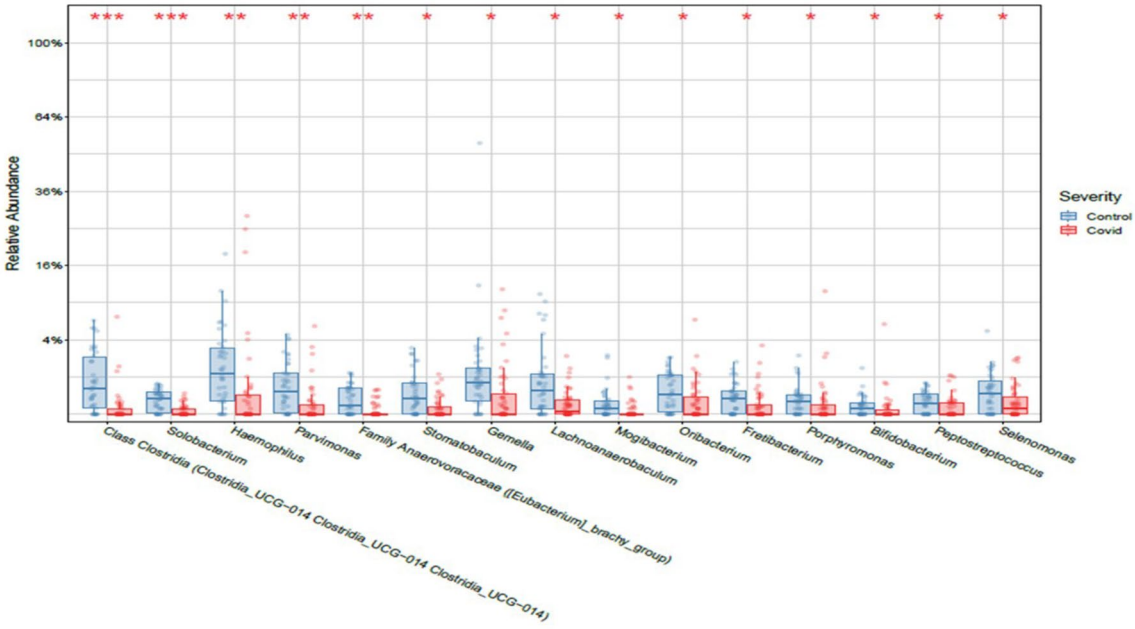

Fig. 9. Health and disease-associated phyla of sputum sample by relative abundance with significant differences between health and disease are shown. Significance was assessed with a Mann-Whitney test ($P < 0.05$). Disease abundances are shown in pale blue, and health abundances in red. *$0.01 < P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.001$.

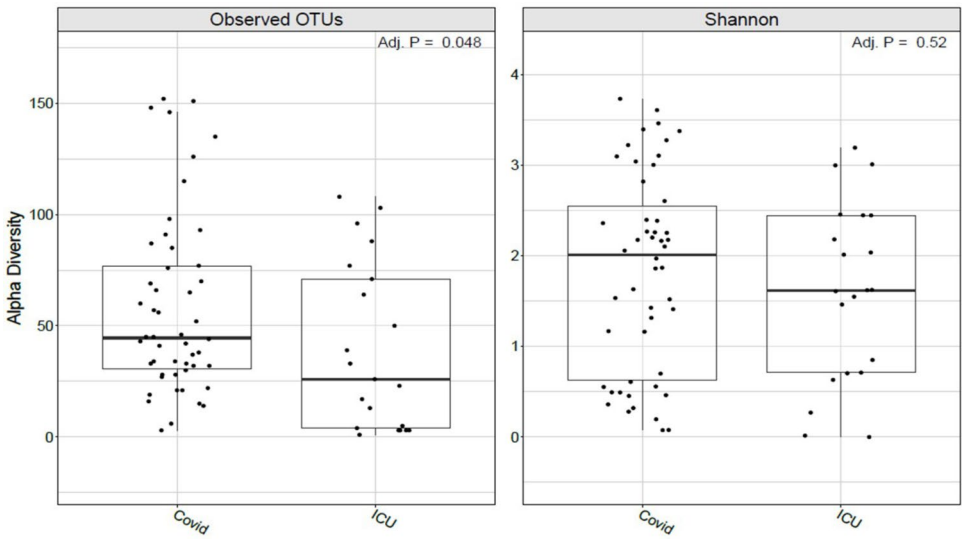

Fig. 10. Alpha and Shannon diversity calculations for respiratory samples from COVID-19 (non-ICU patients) and COVID-19 ICU samples. No significant differences between healthy and diseased samples were observed for alpha and Shannon diversity measures.

microbiota. The Shannon diversity index was analogous between the two cohorts; however, the bacterial richness was significantly lower in the ICU group ($p = 0.048$) (see Fig. 10). The bacterial constituents of the two patient groups were significantly different ($P = 0.001$, PERMANOVA) (see Fig. 11): the sputum samples from ward patients contained a greater abundance of *Pseudomonas* and *Veillonella* as well as *scardovia*; however, in the ICU group, *Staphylococcus* was more prevalent (Fig. 12).

Reflux symptom index score and salivary pepsin level

The RSI was employed to assess the presence and intensity of commonly reported EOR symptoms. For all participants, the RSI score range was 5–33 with a median of 12 (mean ± SD:13.9 ± 6.0) (see Table 2). In 96 of 147 subjects (65.3%), the RSI was < 13 and an RSI score > 13 was exhibited by the remaining 51 subjects (34.7%)

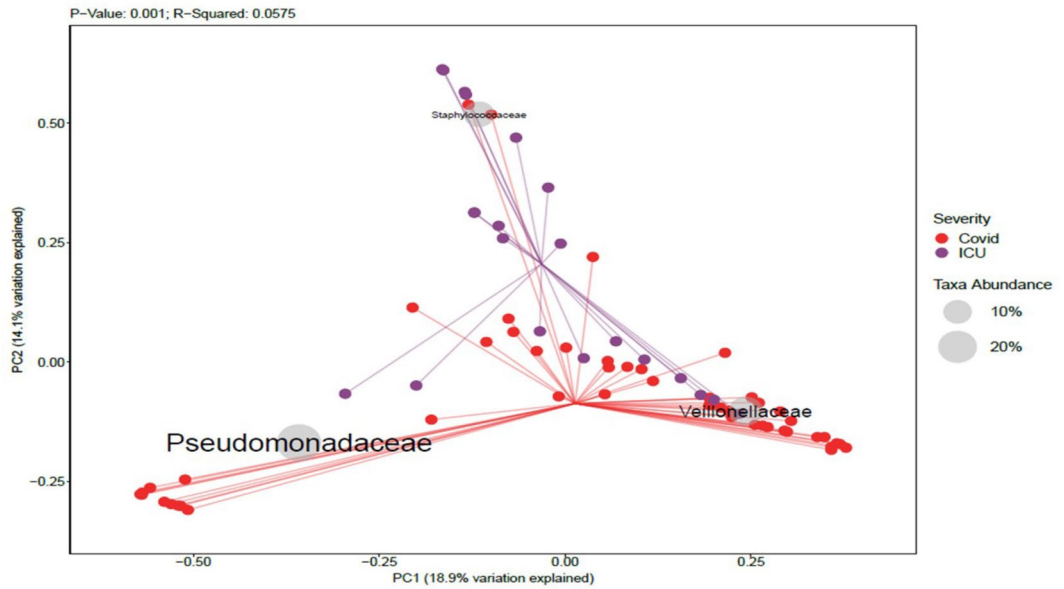

Fig. 11. Principal coordinate analysis (PCoA) plot with Bray-Curtis dissimilarity index. Results revealed that COVID-19 non-ICU samples are clustered separately from ICU samples which suggests that the COVID-19 community is relatively different according to disease severity. Non-ICU patient samples are shown in blue and ICU patient samples are shown in red.

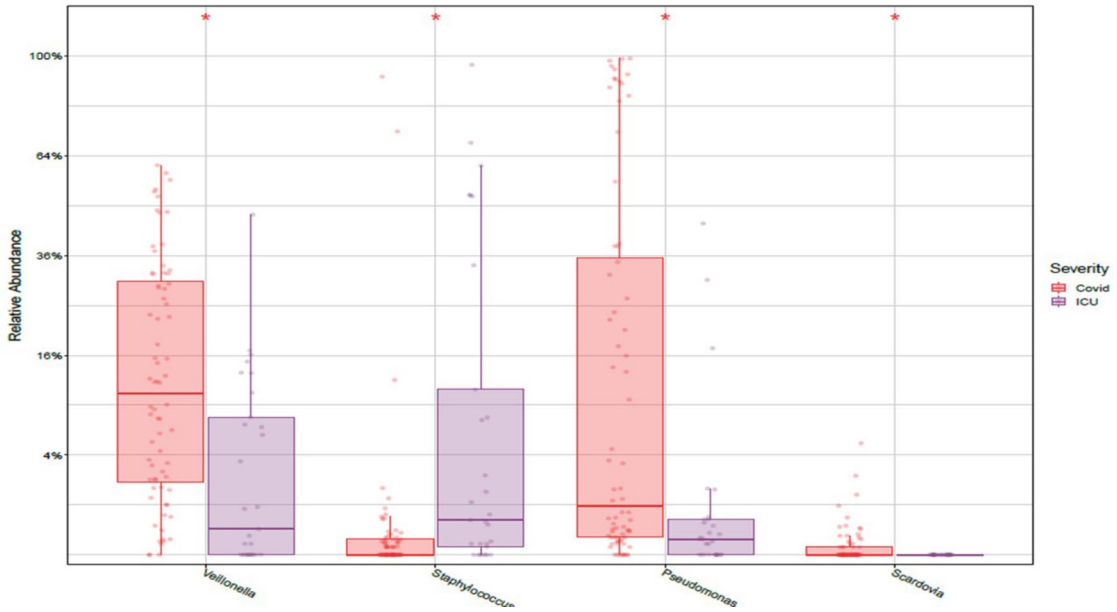

Fig. 12. ICU and non-ICU COVID-19 associated sputum phyla by relative abundance with significant differences between ICU and non-ICU samples are shown. Significance was assessed with a Mann-Whitney test ($P < 0.05$). Non-ICU sample abundances are shown in pale blue, and ICU sample abundances in red. *$0.01 < P < 0.05$.

indicating the presence of EOR in the latter group. The following median and range RSI scores were obtained for participant subgroups: the control group scored 30 (5–28); patients with COVID-19 managed on the ward scored 50 (4–33); and patients with COVID-19 managed in ICU scored 149 (9–33). A significant difference was observed between the RSI scores of the three groups ($F(3, 204) = 7.655$, $P = 0.0005$) with higher RSI scores among those with COVID-19 (see Fig. 13A).

When salivary samples were subjected to the ELISA pepsin assay, the mean ± SD (range, median) for the study population was 48.0 ± 31.0 (0–107, 48) ng/ml. Pepsin levels within the various patient groups with COVID-19 were demonstrated to be significantly different ($F(2, 206) = 23.84$, $P < 0.0001$) (see Fig. 13B) and those patients

	All patients ($n=147$)	COVID-19 patient (non-ICU) ($N=71$)	COVID-19 ICU patient ($N=26$)	Control ($N=50$)	P value
RSI Score					
0–13	96 (65.3%)	42 (59.1%)	16 (61.5%)	34 (68%)	$P=0.004$
> 13	51 (34.7%)	29 (40.8%)	10 (38.4%)	16 (32%)	
Mean + SD	13.9 ± 6.0	14.9 ± 6.1	15.8 ± 6.8	12.2 ± 5.2	$P=0.0005$
Median	48	50	49	30	
range	5–33	4–33	9–33	5–28	
Pepsin level					
Mean + SD	48.0 ± 31.0	55.8 ± 31.2	49.3 ± 32.8	36.4 ± 28.3	$P<0.0001$)
Median	48	50	49	30	
Range	0–107	0–107	0–105	0–105	
Below median (N)%	85(57.8%)	38 (53.5%)	15 (53.8%)	33 (66.0%)	< 0.0001
Above median (N)%	62 (42.1%)	33 (46.4%)	11 (46.1%)	17 (34%)	

Table 2. RSI score and pepsin level.

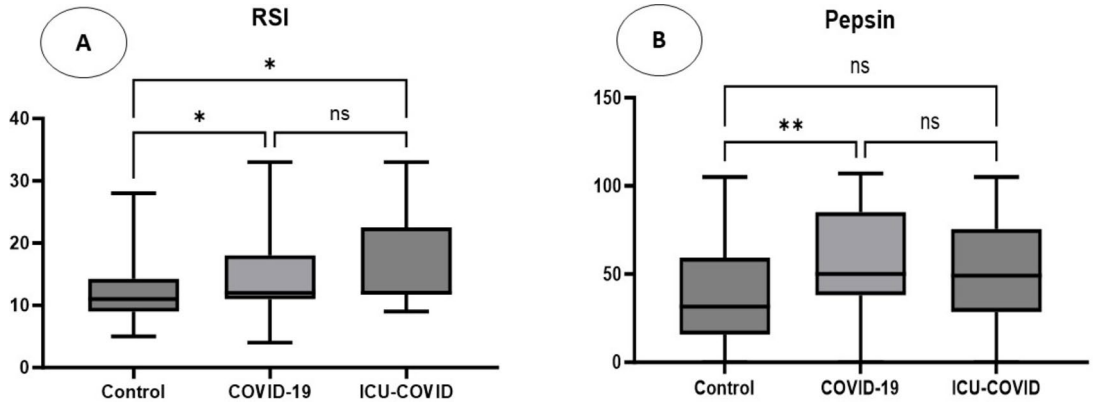

Fig. 13. RSI score among distinct groups. B: level of salivary pepsin detected by ELISA pepsin assay. *$0.01 < P < 0.05$; **$0.001 < P < 0.01$.

managed in ICU and on the ward exhibited higher pepsin titers. These data indicate that patients with SARS-CoV-2 infection exhibit increased pepsin concentrations in their saliva and that an RSI score > 13 was more common in patients with SARS-CoV-2 infection.

Discussion

In this study, concurrent alterations in bacterial populations from respiratory and stool samples were identified in patients with COVID-19. The presence of EOR and elevated levels of pepsin are potential bioindicators of micro-aspiration in such individuals, which is potentially consistent with the translocation of microorganisms including viruses. The analyses of gut and sputum profiles did not reveal an associative relationship in stool samples; however, stool samples which tend to be representative of the lower GI tract may not be an optimum method for predicting micro-aspiration of gastric content.

Changes in the alpha and beta diversities of microbiota in the respiratory tract of patients with COVID-19 have been demonstrated in several studies[80,81]. The current study is one of the first to investigate and compare the microbiomes within the respiratory and GI tracts in patients with acute SARS-CoV-2 infection of varying severity (i.e., those requiring intensive care versus general ward management and age-matched control subjects without COVID-19). This study demonstrated that compared with the control subjects, patients with COVID-19 experienced elevated levels of EOR which is a recognized precursor to micro-aspiration. The Shannon and alpha indices of diversity among sputum samples were significantly lower in patients with SARS-CoV-2 infection. However, no dissimilarities were observed concerning stool sample diversities which is in contrast to previous studies in which patients with COVID-19 were found to have decreased Shannon and alpha diversity indices[82,83]. Nevertheless, when stool samples from patients with COVID-19 and the controls were compared, there was a notable shift in the GI microbiota. This finding was also noted when conducting a comparison of results between patients with COVID-19 managed in the ICU and those patients treated on the ward. These observations imply that the extent of microbial dysbiosis might be correlated with COVID-19 infection as well as with the severity of the disease.

This study observed a reduction in the prevalence of healthy bacterial taxa in patients with COVID-19 when compared to the controls. The respiratory microbiome can cause individuals to be predisposed to infections

of the respiratory tract, such as bronchiolitis, and has been associated with the severity of symptoms and clinical endpoints[84]. The data presented within this study indicate marked alterations in the respiratory tract between ICU- and ward-managed patients with COVID-19, which infers that such changes may represent a proxy indicator of the severity of the SARS-CoV-2 infection. The reduced prevalence of the common respiratory components of the microbiota may occur due to the entry of SARS-CoV-2 into the lungs, and potentially the excessive growth of competing bacteria, an exaggerated response of the immune system to the viral infection, or both mechanisms[85].

Earlier research on human subjects has revealed changes in GI microorganism populations in individuals testing positive for SARS-CoV-2 both at the onset and during the infection[86]. Bacteria which synthesize short-chain fatty acids, e.g. Lachnospiraceae and Fecalibacterum, were found to be significantly lower in previous studies[83,87]which aligns with the outcomes of the current study. Additionally, enrichment of the fecal microbiome with opportunistic disease-inducing microorganisms has been observed in patients with COVID-19[88]. Ruminococcus[82,89], and Roseburia[82,89]are less prevalent while Veillonella, Enterococcus, Streptococcus and Lactobacillus were more prevalent[10,90]. Comparable results were obtained in the current study i.e., a greater abundance of *Enterococcus* and *Streptococcus* was identified in the gut microbiome of patients with COVID-19. The pathogenic nature of these bacteria may contribute to the severity of the SARS-CoV-2 infection. The GI tract in such patients acts as a reservoir of pathogenic microorganisms which may opportunistically traverse the compromised intestinal epithelial cells and enter the bloodstream.

In the current study, hospitalized patients with COVID-19 evidenced EOR, demonstrated by elevated pepsin levels in the saliva samples and an RSI score > 13. This finding implies that this condition is common amongst patients with COVID-19 and represents a risk for elevated levels of micro-aspiration. It is postulated that GI tract peristalsis or the competence of the esophageal sphincter may be adversely impacted by the viral infection. Live virus has been described in stool samples[91]. Another study has observed that despite respiratory tract specimens being virus-free, the stool samples of up to 50% of patients with COVID-19 contained viral RNA[92]. This finding suggests that the GI tract forms a significant entry point and reservoir for viable SARS-CoV-2 and is an observation which supports the potential for COVID-19 to be spread via either the fecal-oral route or between the GI tract and the lung[25].

An oral-lung aspiration axis is widely recognized as a significant factor in the development of several viral illnesses affecting the lower airways[93–95]. It is anticipated that a concentrated amount of virus could be aspirated into the lower region of the lungs and potentially attain a level that surpasses the required threshold needed to initiate infection[96–98]. This research indicates that individuals with COVID-19 infection have a significant occurrence of EOR which implies that small-volume micro-aspiration may be a potential mechanism for viral transmission. Additionally, EOR aspirates include enzymes and/or inflammatory mediators that may prime alveolar cells for infection. The aspiration of SARS-CoV-2 into the lung is consistent with the patchy, bibasilar infiltrates observed by chest CT in COVID-19[99]. Notably, individuals who are elderly, diabetic, and obese are more likely to experience frequent micro-aspiration and gastroesophageal aspiration, which is in accordance with their consistently documented higher risk for developing severe lower respiratory illness from COVID-19[100,101].

Earlier work from this group has indicated a potential association between the microflora in gastric fluid and sputum specimens in respiratory disease which may signify an underlying gastric etiology in such patients and the presence of a possible microorganism reservoir[102,103]. In children with cystic fibrosis, a correlation was reported between *Pseudomonas aeruginosa*infection of the smaller airways and GOR which supports the hypothesis that microorganisms can cross between respiratory and GI tracts. An additional study demonstrated that this possibility may arise autonomously from the bacteria present in the oropharynx[104]. The findings by Wölfel, Corman[105]indicate that COVID-19-positive individuals experience initial infection in the upper respiratory tract during the first 5 days, followed by subsequent aspiration and infection of the lower lung. Wölfel, Corman[105] specifically investigated the oropharynx as a probable location for the first establishment of the virus. As previously mentioned, there is also a gastro-oropharyngeal axis involving EOR: the data obtained by this study indicates that oropharyngeal secretions in COVID-19 can reflect a complex mixture of local secretions admixed with a contribution from EOR.

This study was observational in design and is subject to several limitations. The study was conducted in a single center with in-patients with COVID-19; therefore, its study population is not representative of every individual with SARS-CoV-2 infection. Only a small patient population was included; therefore, this study recommends that future studies include larger sample populations to enhance the generalizability of the findings. Additionally, this study only included patients with COVID-19 who required in-patient care which resulted in selection bias towards individuals with the disease at the more severe end of the spectrum.

There is increasing evidence of complex aerodigestive interrelationships between health and lung disease. Micro-aspiration may be an important determinant of physiological immune function in the lung whereby viable organisms produce metabolites with local immunomodulatory capacity. Limited micro-aspiration may therefore be a normal input into the lung microbiome and a determinant of innate immunity[106–108]. In patients with idiopathic pulmonary fibrosis, it has been demonstrated that commensal oral microbiota, disease severity, and mortality are related and that, notably, a greater proportion of streptococcus mitis was associated with a reduced risk[109].

This study has demonstrated that an integrated understanding of the aerodigestive microbiome is relevant to the diagnosis and treatment of chronic lung diseases such as smoking-related chronic obstructive pulmonary disease, progressive pulmonary fibrosis, cystic fibrosis, bronchiectasis, and lung allograft injury[110–113]. Crucially, an enhanced understanding of aerodigestive pathophysiology can be employed to inform personalized treatment approaches to lung disease and emphasizes the benefits of a multi-disciplinary team methodology consisting of respiratory, gastrointestinal, and microbiology professionals[112,114].

The gut and respiratory microbiome profile of COVID-19 patients might therefore be impactful in influencing targeted medicine administration via approaches which prevent adverse microbial shifts such as gut microbiome modulation and the introduction of beneficial bacteria into the body. To fully understand the pathophysiology of SARS-CoV-2, this study recommends additional research regarding microbial activity, host-microbiome interactions during infection, the potential benefits of anti-reflux strategies, and the use of probiotics and prebiotics. This research contains real-world implications. For example, despite having established benefits and uses, a default approach or reliance on proton pump inhibitors may not be beneficial as a sole approach to managing reflux in respiratory patients at risk of extraesophageal reflux. It is known that gastric acid has a homeostatic microbicidal function and that the stomach can be a reservoir of disease-relevant organisms, particularly at higher pH[115–117]. The exclusive use of PPI therapy would not benefit injury from non-acid aspiration caused by bile, pepsin, and a dysregulated microbiome. Therefore, this research recommends that further studies and patient management could benefit from a mixed-team approach because the considerations are interdisciplinary.

The results of this study provide original insights and added information regarding the treatment of COVID-19 patients. The degree of non-commensal microorganism richness may have the utility to predict the severity of SARS-CoV-2 infection which is suggestive of their potential use as biomarkers. To determine the true significance of gut bacteria in SARS-CoV-2 pathogenesis, additional research must be conducted regarding microbial shift and host-immune responses between patients with COVID-19 and healthy controls.

Moreover, if future research indicates that the gut microbiota profile corresponds with illness severity, the microbiome could be employed as a predictive indicator for the course of a disease. In such a scenario, early management of the gut microbiota might be beneficial for prevention or treatment (e.g., by fecal transplant, fermented food, probiotics, and symbiotics).

The use of probiotics for treating a range of ailments has been documented and includes the therapeutic benefits of probiotics for respiratory conditions. Probiotics may be used as an adjuvant therapy to reduce the risk of mortality and symptoms in COVID-19 cases. This is corroborated by a meta-analysis that indicated probiotics minimized hospital stays, reduced recovery times, and decreased the chance of death[118]. According to the gut-lung axis theory, the gut microbiota and lung interact in both directions. Via this axis, probiotics could relieve respiratory symptoms, decrease hospital stays, and lower blood C-reactive protein levels. Furthermore, probiotics can control Treg cell production and activity which further modulates immune function and, additionally, they can control the concentration of associated metabolites and the makeup of the gut flora[119]. More research should be conducted regarding the ability of probiotics to control gut microbiota, preserve intestinal homeostasis, and function as an antiviral defense[120].

The authors of this research believe that our novel aerodigestive observations in COVID-19 cases warrant additional research to identify the contributory factors that impact alterations in the microbiota (as seen in response to COVID-19 severity). Such studies should include lung, oropharyngeal, gastric, and lower GI samples to map the dysregulated microbiome associated with COVID-19 and facilitate an increased understanding of the potential bidirectional exchange of aerodigestive organisms. Such work may indicate that an overall mixed disciplinary consideration of GI and lung systems would be beneficial for future therapeutic management paradigms.

Data availability

Sequence data that supports the findings of this study has been deposited in MG-RAST under the accession numbers PRJNA1162807 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1162807).

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Author contributions
Conceptualization, H. Al-momani, A.N and C.W; resources and data curation, I.A, A.I.K and H.Tmethodology, H. Al-momani, A.A and A.M.Z. Patient sampling, H.A and D.A , writing—original draft preparation, H. Al-momani, C.W and J.P. writing—review and editing, H-Al-momani, A.N, C.W and J.P. supervision, H. Al-momani. project administration, H-Al-momani and C.W. All authors have read and agreed to the published version of the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

Additional information
Correspondence and requests for materials should be addressed to H.A.-M.

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