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GUT MICROBIOME FEATURES IN COVID-19: ANALYSIS OF A COHORT OF HOSPITALIZED PATIENTS

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Abstract – *Objective:* Emerging evidence suggests a direct involvement of the gastrointestinal tract in COVID-19. Although the specific immune response is of paramount importance in the SARS-CoV-2 virus elimination process, aberrant immune activity could lead to severe disease and late inflammatory forms. In this context, the intestinal microbiota plays a primary role in the maturation and maintenance of the immune system. This study investigates whether the SARS-CoV-2 infection can be associated with alterations in the gut microbiome composition and if such variations could correlate with the severity of symptoms and disease outcomes.

Patients and Methods: We performed shotgun metagenomic sequencing of stool samples of 45 patients, aged between 30 and 95 years, hospitalized with COVID-19. Patients were grouped by clinical severity (i.e., non-critical or critical), type of hospitalization (non-intensive care or intensive therapy unit) and outcome (survival or deceased) to explore the impact of the gut microbiome changes on patients' health.

Results: COVID-19 severity is associated with alterations in the intestinal microbiome, reduced microbial biodiversity and increased *Escherichia* and *Bacteroides genera*. No statistical significance was found between the extent of dysbiosis and clinical severity. We found an enrichment of micro-eukaryotic species, e.g., Candida albicans, Candida tropicalis, Saccharomyces cerevisiae and bacterial species previously associated with diseases as well as unhealthy cardiometabolic markers, e.g., *Escherichia coli*, *Bacteroides fragilis*, *Clostridium bolteae*, *Clostridium innocuum*, *Clostridium symbiosum*, *Eggerthella lenta*, *Enterococcus faecium*, and *Flavonifractor plautii*.

Conclusions: Our findings suggest a trend of correlation between the degree of intestinal dysbiosis and the severity of the disease, likely depending on the depletion of some microorganisms with immunomodulatory effect. Furthermore, gut dysbiosis could explain the inflammatory outcome, which persists after viral negativization and might justify possible future complications.

Keywords: COVID-19, Dysbiosis, Gut microbiota, SARS-CoV-2.

INTRODUCTION

The pathophysiology of COVID-19 is characterized by exacerbated inflammatory responses, strongly implicated in the multiorgan failure observed in some patients. Moreover, the post SARS-CoV-2 infection could lead to multisystem inflammatory syndrome and Kawasaki-like disease in children[1-4].

Compelling evidence suggests a direct involvement of the lung-gut axis in the disease pathogenesis. In particular, the virus per se replicates in human small intestine enterocytes[5] and has been detected in the patient's feces[6-8]. Thus, the gastrointestinal system is directly involved in the COVID-19 inflammatory phenotype and complications[9-11]. Additionally, altered gut microbiota correlates with poor clinical outcomes in mechanically ventilated COVID-19 patients[12-15]. Therefore, a close cross-talk between viral infection and the alteration of the gut commensal might contribute to disease severity through its influence on the host immune system[16]. Indeed, the gut microbiota directly modulates the activation of the immune system by providing nutrients and metabolites (i.e., vitamins and short-chain fatty acids) and through continuous exposure to non-self-antigens[17-20]. On the other hand, the immune cells inhabiting the intraepithelial space, the lamina propria and the secondary lymphoid structures of the intestinal mucosa protect the integrity of the barrier and influence bacterial growth and biodiversity[17]. However, the balance of this symbiotic relationship is impaired following systemic viral infection, leading to alteration of the intestinal permeability, reduction of the immune tolerance, production of inflammatory cytokines, skewing the adaptive immune response and, ultimately, inflammatory dysfunctions. Thus, COVID-19 disease could be a central focus in the interactions, most likely multi-directional, between the virus, inflammation, gastrointestinal tract, and intestinal microbiota.

This study aimed at investigating whether any correlation occurs between the severity of COVID-19 and alterations of gut microbiota composition.

PATIENTS AND METHODS

Cohort Description

This study was conducted according to the Helsinki Declaration for Clinical Trials and approved by the local Ethics Committee (code: 286/2020/Oss/AOUFe). Written informed consent was obtained from all subjects. Patients recruited for the study fulfilled the following requirements: 1) hospitalization for COVID-19; 2) COVID-19 diagnosis established according to the WHO definition (i.e., detection of SARS-CoV-2 infection by nasal/oropharyngeal swab via PCR technique[21]); 3) radiographic findings of bronchopneumonia, by chest X-ray or computed tomography; 4) aged ≥18 years. The study population was grouped into two subpopulations according to clinical severity (non-critical or critical patients). The clinical definition of critical patients was based on one of the following clinical signs: i) a respiratory rate greater than 30 acts/minute; ii) arterial oxygen saturation ≤ 93% in ambient air; iii) a PaO_2/FiO_2 ratio ≤ 300 mmHg. For each patient, data collected during the entire duration of the study were: a) patients' background, b) clinical details, c) comorbidities and pre-admission therapy, d) symptoms at the time of admission to the hospital, e) relevant laboratory data and symptoms during the hospitalization, f) possible transfer to intensive care / treatment unit (ITU) as well as hospital therapy, with particular focus on antibiotics and g) outcome.

Fecal samples were collected from each patient at the admission to the recovery ward before any antimicrobial treatment was started. The DNA and RNA were extracted from feces and analyzed to reconstruct the composition of the gut microbiome and SARS-CoV-2 genome. In order to evaluate a possible link between the gut microbiota and the manifestations of COVID-19, the following comparisons were performed: non-critical *vs.* critical patients, non-intensive care *vs.* ITU, recovered *vs.* deceased. In addition, the metatranscriptomic analysis from stool was performed to reconstruct SARS-CoV-2 genome with the aim identify the virus lineage of the study cohort.

DNA EXTRACTION AND SHOTGUN METAGENOMIC SEQUENCING

DNA was extracted from 0.25 g of frozen stool. Briefly, samples were weighed and put in the PowerBead Pro tube of the DNeasy PowerSoil Pro Kit (Qiagen, Shanghai, China) that was used to purify DNA following the manufacturer's instructions. Quality and quantity of DNA, eluted in 50 µl, were assessed by Nanodrop and Qubit assay (Thermo Fisher Scientific, Waltham, MA, USA). Sequencing libraries were prepared using the Nextera DNA Flex Library Preparation Kit (Illumina, San Diego, CA, USA), and sequenced on the NOVAseq 6000 platform (Illumina) using the NovaSeq Xp 4-Lane, with a target sequencing depth of 7.5Gb (2x150bp reads).

RNA EXTRACTION AND SHOTGUN METATRANSCRIPTOMIC SEQUENCING

RNA extraction was performed on frozen feces using the RNeasy Power Microbiome Kit following the manufacturer's instruction. Briefly, a stool sample (0.25 g) was chemically and mechanically lysed. The RNA was then purified on spin columns and DNase treatment was implemented to remove DNA contamination. Quality and quantity of the eluted RNA were assessed by Bioanalyser and Qubit, respectively. 100 ng of RNA were used for RNA library preparation using the Illumina Stranded Total RNA Prep Ligation with Ribo-Zero Plus kit, following the manufacturer's guidelines and sequenced (2 x 50 pb) on the Illumina NovaSeq 6000.

METAGENOME QUALITY CONTROL AND PREPROCESSING

All metagenomes were quality controlled using the preprocessing pipeline available at https://github.com/SegataLab/preprocessing. Briefly, preprocessing consisted of three main steps: i) read-level quality control; ii) removal of host sequences contaminants; and iii) split and sorting of cleaned reads. Read-level quality control removes low-quality reads (quality score <Q20), fragmented short reads (<75 bp), and reads with ambiguous nucleotides (>2 Ns), using trim-galore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Host sequences of contaminant DNA were identified using Bowtie 2[22] with the "sensitive-local" parameter to remove both the phi X 174 Illumina spike-in and human-associated reads. Splitting and sorting allowed for the creation of standard forward, reverse, and unpaired reads files for each metagenome.

TAXONOMIC AND FUNCTIONAL PROFILING

The metagenomic analysis was performed using the bioBakery 3 suite of tools[23]. In particular, taxonomic profiling and estimation of species' relative abundances were performed with MetaPhlAn 3 (v. 3.0.7 with default parameters)[23,24]. MetaPhlAn 3 taxonomic profiles were used to compute three alpha diversity measures: 1) the number of species with positive relative abundance in the microbiome ("Richness"); 2) the Shannon entropy estimation ("Shannon"); and 3) the Simpson diversity index ("Simpson").

STATISTICAL ANALYSIS

The statistical analyses were performed using the R software (v. 4.0.5). The significance differences between the alpha diversity distributions of the groups were assessed using the Wilcoxon rank-sum test from the "wilcox.test" function of the "RClimMAWGEN" package (p <0.05).

RECONSTRUCTION OF THE SARS-COV-2 GENOME

The SARS-CoV-2 metatranscriptomic reads were identified using Bowtie 2 against the SARS-CoV-2 Wuhan-Hu-1 reference genome (NCBI accession: NC_045512). Mapping results in SAM

format were converted to BAM files using SAMtools (v. 1.12)[25]. The genome sequence of the SARS-CoV-2 was reconstructed from the BAM files using the "consensus.py" script from CM-Seq (v. 1.0.3)[26] with parameters "sortindex dominant_frq_thresh 0.8 mincov 4". Epidemiological lineage was assessed using the Pangolin web tool (https://pangolin.cog-uk.io/; accessed 12th October 2021)[27].

RESULTS

Hospitalized Patients with Severe COVID-19 Symptoms Showed an Altered Gut Microbiome Composition

A total of 46 COVID-19 patients (27 males and 19 females; age range: 30-95 years) were recruited at the St. Anna University Hospital of Ferrara (Italy) between March and June 2020 (**Supplementary Table I**). In this study-cohort, 34 patients were non-critical, while 12 patients were in critical condition. Eleven patients were recovered in the intensive care/treatment unit (ITU). Four patients died during the study.

To investigate the alterations that a severe SARS-CoV-2 infection can produce in the gut microbiome composition of hospitalized patients, shotgun metagenomic sequencing was performed on patients' stool samples. Microbiome profiles were compared within patient grouped for clinical severity (non-critical *vs*. critical), type of hospitalization (non-intensive care *vs*. ITU) and outcome (survival *vs*. deceased). Within each comparison, the analysis highlighted a common trend of low microbial richness in the most severely affected COVID-19 group (Figure 1A-C). However, no statistically significant differences were observed in terms of alpha diversity between groups of patients, either when comparing those in critical condition with non-critical ones, or in ITU *vs*. those in non-intensive care unit, or in deceased patients *vs*. those who recovered (Wilcoxon signed-rank test, $p>0.05$; Figure 1A-C). Compared to the non-critical cohort, the evaluation of the relative abundance of micro-eukaryotic species in critical condition, ITU and deceased patients showed an enrichment of *Candida albicans*, *Candida tropicalis* and *Saccharomyces cerevisiae* ranging from 50 to 94% among patients. Moreover, the relative abundance analysis on microbiota showed the enrichment of *Escherichia coli*, *Bacteroides fragilis*, *Clostridium bolteae*, *Clostridium innocuum*, *Clostridium symbiosum*, *Eggerthella lenta*, *Enterococcus faecium*, or *Flavonifractor plautii* (Figure 1D). Interestingly, these bacterial species were previously associated with diseases and unhealthy cardiometabolic markers[28-30]. However, the gut microbiome composition showed no clustering patterns in agreement with the patients' condition.

RECONSTRUCTION OF THE SARS-COV-2 GENOME FROM FECAL SPECIMENS

Shotgun metatranscriptomic sequencing of 10 stool samples allowed to reconstruct the full SARS-CoV-2 genome with no polymorphic bases in 99.87% of the genome length, from a 76-year-old male patient who died two weeks after hospitalization (**Supplementary File 1**). In agreement with the epidemiological lineage and spread data collected during the Northern Italian outbreak early in 2020, the genome reconstructed belongs to the B.1 variant[31]. In comparison to strains from the B.1 lineage retrieved from other Italian COVID-19 patients during the same time period (Genbank accessions: MW134560.1, MT925569.1 and MW468415.1) the SARS-CoV-2 genome reconstructed carried three single nucleotide polymorphisms in positions 21,658, 23,520 and 25,407 respectively.

DISCUSSION

In patients with COVID-19, we observed a reduced microbial biodiversity lending support to the occurrence of dysbiosis[32] characterized by an increase of bacteria usually defined as disease markers. Among these, the genera *Escherichia* and *Bacteroides*, which we have found to be more abundant in COVID-19 patients in critical conditions or with death outcome. Inter-

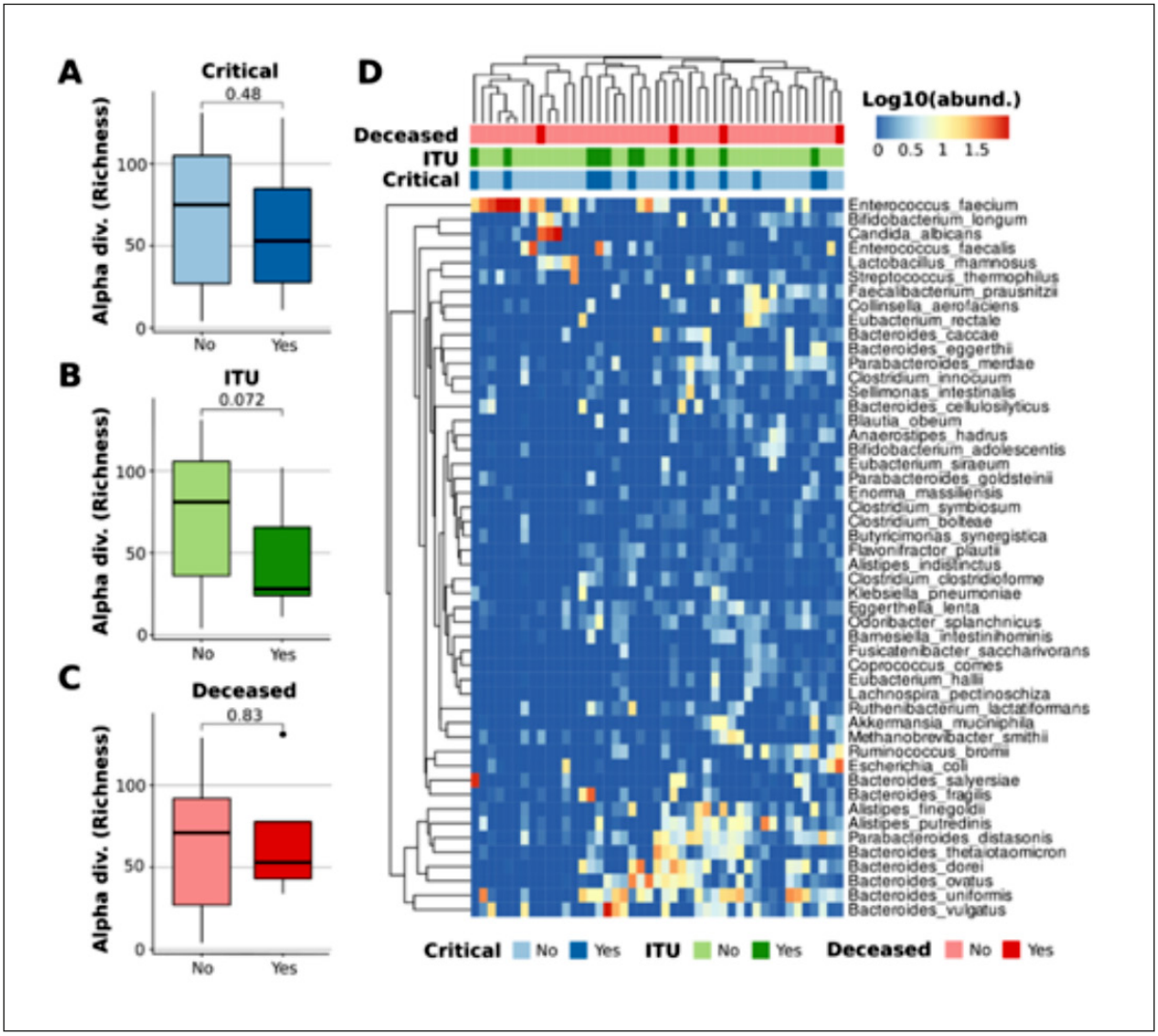

Figure 1. The gut microbiome composition of hospitalized patients suffering severe COVID-19 symptoms do not differ regardless of the patient condition. (**A-C**), Microbiome species' richness, measured as the number of detected species, shows no differences when comparing (**A**) patients under critical condition compared to those who were not, (**B**) patients on an intensive treatment unit compared with those who were not, (**C**) or patients who passed away compared to those who recovered (Wilcoxon signed-rank test, $p>0.05$). **D**, Hierarchical clustering of the microbiome composition considering only species' level relative abundances shows no clustering patterns in agreement with the patient condition. The top 50 species based on the 90th percentile of the relative abundances are shown.

estingly, these bacteria genera are known to be associated with high levels of inflammatory markers, such as C-Reactive Protein (CRP) a marker associated with a variety of diseases including cardiovascular disease, type 2 diabetes mellitus, and obesity[33]. Moreover, a systematic review and meta-analysis of 32 studies on 10,491 patients highlighted CRP as an early biomarker of the disease severity in COVID-19 hospitalized patients, suggesting that this index levels can be useful to stratify patients in non-critical *vs*. critical in order to improve their management[34]. Consistently, CRP levels were significantly increased in about 80% of our patients and also were significantly higher in critical patients *vs*. non-critical ($p=0.0274$) and in patients admitted to ITU *vs*. normal ward ($p= 0.0074$) (**Supplementary Figure 1**). Therefore, our data support the use of CRP as a biomarker for the early identification of the high-risk COVID-19 patients and for the correct allocation in the care setting.

Regarding the micro-eukaryotic species, we observed a relative increase in *Candida albicans*, the most abundant fungi in the intestine of healthy individuals[35] and *Saccharomyces cerevisiae*. Some evidence suggests that *Candida albicans* could be implicated in changing

the integrity of body barriers (e.g., the intestinal barrier) by reducing E-cadherin synthesis in epithelial cells. Moreover, *Candida albicans* has also been related to some tumors and inflammatory diseases (e.g., IBD, psoriasis, asthma) so far considered to have a stochastic onset[36]. In our case, the relationship between the extent of dysbiosis and the clinical severity did not lead to statistically significant results; nonetheless, our data showed a tendency towards the reduction of alpha-richness in patients with more severe clinical manifestations in the three observed categories (critically ill, ITU hospitalized and deceased patient).

Other studies have already explored the correlation between microbiota composition and COVID-19 severity. In line with our work, Yeoh et al[1] investigated the possible link between dysbiosis and COVID-19 infection in 100 hospitalized patients compared with a control cohort of 78 adults. Patients were grouped by severity of symptoms into four classes (mild, moderate, severe, and critical disease) and microbial composition of fecal samples and the levels of some of the plasma inflammatory cytokines were analyzed. In agreement with our results, Yeoh et al[1] study documented a marked difference between patients and controls regardless of the use of antibiotics during hospitalization, which were surprisingly less relevant. Specifically, the SARS-CoV-2 infected patients were characterized by a higher prevalence of *Bacteroidetes* and a reduced population of *Actinobacteria*. Intriguingly, a further finding that emerged from that study was that the microbiota composition of COVID-19 patients was primarily linked to the severity of the disease more than antibiotic therapy, as one would expect. Additionally, the severity of COVID-19 symptoms negatively correlated to some bacterial species, such as *Fecalibacterium prausnitzii*, *Bifidobacterium bifidum* and *Eubacterium rectale*, already known for their immunomodulatory potential. Similar results were obtained by Schult et al[37], who performed 16S rRNA gene sequencing on fecal and saliva samples from SARS-CoV-2 infected and post-COVID-19 patients and controls. Their data indicated an association between the gut and oral microbiota and the COVID-19-associated complications and disease severity, whereas a stable gut bacterial composition was found to be associated with a favorable outcome of the disease. Particularly, increasing complication rates associated with pathogenic taxa (e.g., *Parabacteroides* ssp.), concomitantly with decreased anti-inflammatory microbiota, and low-risk bacteria (e.g., *Faecalibacterium prausznitzii*) were found to be more prevalent in better clinical outcomes[37].

In line with previous results, Wu et al[38] reported an altered composition of the gut microbiome (e.g., elevated *Granulicatella and Rothia mucilaginosa*) in hospitalized COVID-19 patients as opposed to healthy controls, while Zhong et al[39] showed an increased Burkholderia cepacia complex, *Staphylococcus epidermidis*, *Mycoplasma hominis*, and *Mycoplasma* orale in severely *vs*. mildly affected ill COVID-19 patients[12]. Also, Chen et al[40] observed a decreased microbial diversity in patients with COVID-19 during a 6-month follow-up compared to controls. Post-COVID-19 recovery (after 30 days and at 3 months) has been associated with loss of anti-inflammatory bacteria (*Faecalibacterium prausnitzii, Eubacterium rectale*, and *Bifidobacterium adolescentis*) and with an increase in pathogens (*Rothia, Erysipelatoclostridium, Ruminococcus gnavus, Ruminococcus torques*, and *Bacteroides dorei*)[1,41]. Another recent study[42] provided evidence that SARS-CoV-2 infection promotes gut microbiome dysbiosis in COVID-19 patients, with increased barrier permeability and translocation of bacteria from the gut lumen into the systemic circulation. In support to these findings, fecal microbiota transplantation proved to be an effective measure in restoring gut microbiota and alleviated gastrointestinal symptoms in COVID-19 patients[16]. This result lends support to possible correlations between microbial species and immune dysregulation, ending in multisystem organ failure and critical illness[1]. However, it is difficult to establish whether the relative abundances of some microorganisms could play an active role in the pathogenesis of COVID-19 or are instead a consequence of mucosal inflammation, dietary changes during illness and bowel habit abnormalities (e.g., diarrhea).

Moreover, the composition of the gut microbiota could influence post-COVID symptoms, such as fatigue, dyspnea, and arthralgia, symptoms that can persist even more than 80 days after viral negativization. Yeoh et al[1] addressed this aspect by investigating symptom persistence after negativization of the nasopharyngeal swab assessed via RT-PCR. They analyzed fecal samples of subjects up to 30 days after hospital discharge and showed persistent dysbiosis in all patients with greater microbial abnormalities in those who received antibiotic therapy. Also, although antibiotics are known to cause prolonged dysbiosis, in this study they

did not influence the length of hospitalization or the outcome of these patients. Nonetheless, it is worthy to highlight that these results further support a cautionary approach when administering antibiotics[43,44]. Broad-spectrum antibiotics are a key element in the treatment of COVID-19, as they are highly used to avoid bacterial superinfection in hospitalized patients[45]. Notably, it has been estimated that more than half of COVID-19 inpatients have been treated with antibiotics, despite less than 7% had a demonstrable bacterial infection[46,47]. This approach promotes dysbiosis from resistant strains, which, added to the COVID-related dysbiosis, could lead to post-hospital clinical consequences. In this line, it has been reported that COVID-19 patients treated with broad-spectrum antibiotics at hospital admission are more prone to multi-drug resistant infections and almost double the mortality rate from septic shock[44,48,49]. In our study, about 91% of hospitalized patients were treated with antibiotics due to severe clinical conditions and for suspected bacterial over-infection. Although unavoidable, this aspect represents the main limitation of the present analysis. Indeed, SARS-CoV-2 infection promotes the occurrence of bacterial infections due to immune and cellular dysregulation[50], in addition to facilitating factors, i.e., the nosocomial environment, comorbidities, systemic steroid therapy, and the use of assisted ventilation device. Therefore, studies on the stratified microbial composition for the use of antibiotics (type, doses, duration of treatment) in COVID-19 patients are eagerly awaited.

Our study shows some shortcomings, e.g., small and very heterogeneous study cohort, the broad age range of patients (as the intestinal microbiota changes dynamically during the aging), the different number of males and females, and the variability of comorbidities, are all possible confounding factors. Moreover, specific conditions, including bed rest, cognitive impairment and neurodegenerative diseases, metabolic abnormalities, neoplasms, and the high number of drugs taken by patients, can be independent variables affecting the gut microbiota and thereby leading to a non-homogeneous cohort of patients. More solid evidence will be obtained by designing future case-control study including comparable cohorts for clinical severity, comorbidities, and treatment modalities. Finally, a further issue arises from some critically ill patients who were also hospitalized in ITU, preventing the analysis on two comparable independent groups. Another critical aspect that should be considered is the persistence of intestinal dysbiosis following the resolution of the underlying cause. In this regard, a prolonged follow-up would be essential to better assess the evolution of dysbiosis over time, even up to 1 year after viral clearance from the airways, especially if antibiotic treatment was started in the initial phase of the disease. Thus, the relationship between dysbiosis and post-COVID symptoms might be more accurately established.

The reconstruction of the SARS-CoV-2 genome from fecal specimens of our patients demonstrated the accuracy and sensibility of the metatranscriptomic approach to identify possible virus variants. Moreover, the simple collection and storage of the faecal samples and the isolation-free procedure render the metatranscriptomic analysis an excellent method to track and study SARS-CoV-2 evolution through the population. The need for follow-up after the virus clearance is also supported by evidence describing how SARS-CoV-2 is detectable in the feces even after complete clearance from the respiratory system regardless gastrointestinal symptoms[51,52].

Finally, unveiling the potential role of gut microbiota changes in COVID-19 may allow assessing the risk profiles of each patient by deciphering the presence of specific quantitative and qualitative abnormalities of single or cluster of microorganisms along with related networking with other members of the microbial community. This strategy is expected to enable the identification of subjects with a greater likelihood of complications and inflammatory sequelae following SARS-CoV-2 infection.

CONCLUSIONS

The gut microbiota plays a central role in the body homeostasis and health in addition to being involved in various mechanisms leading to disease state. Hence, gut dysbiosis occurs in pathological conditions, including SARS-CoV-2 infection, influencing not only the clinical phenotype, but also severity and outcome. Our study, although did not yield statistically significant results, expand and confirm that gut microbiota changes correlate with the severity

of the COVID-19 and that the depletion of some microorganisms with immunomodulatory effect may exert a critical pathogenetic role. Furthermore, the dysbiosis in COVID-19 patients could explain the inflammatory abnormalities, which persist after viral negativization and likely contribute to post-COVID-19 clinical consequences. In this perspective, the management of gut microbiota alterations should involve active disease as well as its resolution.

Conflict of Interest

The authors declared no conflict of interest.

Authors' Contributions

G.C., R.C., S.V., N.S., R.D.G. conceptualized and designed the present research; R.C., L.L., A.C., M.G., S.V. and F.M. collected and processed samples and clinical data; A.B.M., F.A., F.A., C.C., L.N., performed the experiments; A.B.M., F.A., F.A., C.C., L.N., A.C., M.G. and N.S. analyzed data; G.C., R.C., S.V., N.S., R.D.G interpreted the results; A.B.M, F.A., F.A., C.C., L.N., A.C., M.G. and N.S., prepared the figures and tables; G.C., L.L., A.C., F.M, N.S. and R.D.G. drafted the manuscript; N.S. and R.D.G. edited and revised the manuscript; all the authors approved the final version of the manuscript.

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Institutional Review Board Statement

The study was carried out in accordance with the guidelines provided by the Declaration of Helsinki (World Medical Association, www.wma.net) and received approval from the Local Ethic Committee of Arcispedale S. Anna, Ferrara, Italy (286/2020/Oss/AOUFe).

Informed Consent Statement

Signed informed consent, which was written in compliance with local and national ethical guidelines, was obtained from each patient before the inclusion into the study. All subjects gave their consent to participate and for the publication of the obtained data.

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