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Gut bacteria after recovery from COVID-19: a pilot study

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Abstract. – OBJECTIVE: COVID-19 has been a major infectious disease lately in humans. 10% of people experience persistent symptoms twelve weeks after having COVID-19. The gut microbiota is essential for host immunity. Thus, gut microbiota composition may contribute to the recovery of COVID-19 patients. The impact of COVID-19 on the gut microbiota of patients during recovery is less explored. We investigated the potential alterations of bacterial gut microbiota of immediately recovered COVID-19 patients, and six months after their recovery.

MATERIALS AND METHODS: Stool samples were collected from 8 patients with COVID-19 immediately after their recovery, and six months after SARS-CoV-2 clearance, as well as from 8 healthy donors as a control group. 16S rRNA gene sequencing was performed to analyze the correlation between disease recovery and microbiota using the immediately recovered and control group. Specific primers were designed for the most significantly altered bacteria and used to analyze the changes in intestinal microbiota composition of patients using qPCR. qPCR comparisons were performed on three groups: newly recovered from COVID-19, after six months of COVID-19 recovery, and healthy controls.

RESULTS: Compared with the healthy control group, patients who immediately recovered from COVID-19 had significantly less presence of 15 bacterial groups. The immediately recovered patients had a very significantly higher relative abundance of the opportunistic pathogen *Mycolicibacterium*. No differences were found between the immediately recovered patients, and after six months of recovery using the qPCR analyses.

CONCLUSIONS: Our results contribute novel insights regarding the alteration of human gut microbiota and the emergence of opportunistic pathogens in recovered patients of COVID-19. Further studies with a larger experimental size are needed to reveal balance or dysbiosis in patients after COVID-19 recovery.

Key Words:
Long COVID, Dysbiosis, Intestinal microbiota, Infectious disease, Opportunistic pathogen, *Mycolicibacterium*.

Introduction

The novel coronavirus called SARS-CoV-2 causes the respiratory coronavirus disease COVID-19. In most patients, the disease presents moderate symptoms that include headache, cough, fever, lethargy, shortness of breath, diarrhea, vomiting, altered smell, altered taste, and pain in the abdomen, chest, joints, and muscles[1-3]. In fewer cases, the disease can be severe, resulting in vascular complications, acute kidney and lung injury, multi-organ failure, and death[4-6]. SARS-CoV-2 causes systemic infection and viral genetic material can be found for months in the tissues of the intestines, lymph nodes, and other areas of the body[7]. One in ten people still experience persistent symptoms up to twelve weeks after having COVID-19, termed "long COVID" or post-COVID-19 conditions[8]. The nature of long COVID is still not fully understood, and the true prevalence is currently unknown[9,10]. Known manifestations of long COVID conditions include fatigue, back pain, post-traumatic stress syndrome, depression, cognitive impairment "brain fog", memory loss, somatoform pain disorder, panic disorder, impaired immune system, increased risk of damage to the heart, lungs, and brain, chronic inflammation, hypercoagulability, and gut dysbiosis[9,11-17]. The gut-lung axis is an intersecting mucosal immune system that connects the gastrointestinal and respiratory systems[18]. Because some symptoms of COVID-19 and long COVID are linked to the digestive tract, there is a suspected link between the lung and intestinal microbiota that influences the results of clinical

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manifestations[12]. Therefore, research into the effects of COVID-19 on the human gut microbiota, particularly the long-term effects are needed to understand the implications of this disease on health as well as on potential remediation or minimization of symptoms.

The human gut microbiota, by definition, is the community of trillions of microorganisms resident in the gastrointestinal tract, which mostly consist of non-pathogenic archaea, bacteria, fungi, and viruses[19]. The intestinal microbiota is unique to each individual and has a high variability since its composition depends on genetic factors, lifestyle, feeding, and birth route, among others[20-24]. In the intestinal mucosa, some bacteria are a functional continuum with mutualistic, commensalistic, opportunistic, and pathogenic microbes. The vast majority are mutualistic or commensalistic and few are pathogenetic, that is those that can cause disease. This community together is at times referred to as a 'metabolic organ' due to its effects on human well-being, metabolism, physiology, nutrition, and immune system[25].

The intestinal microbiota is essential for regulating homeostasis in the human immune and metabolic systems [26-28]. The disturbance of intestinal microbiota has been linked to colorectal cancer, autism, chronic diseases such as heart disease, respiratory diseases, inflammatory bowel disease, obesity, and some metabolic diseases such as diabetes, liver cirrhosis, and non-alcoholic fatty liver disease[29-37]. Moreover, the microbiota can have an impact on the outcomes of viral diseases[38]. Therefore, having a balanced gut microbiota is vital for health. Whether a change in the microbiota causes the disease to develop or the disease causes a change in the microbiota, the causal relationship between disease and microbiota composition remains an unanswered question in most diseases.

In the case of COVID-19, dysbiosis affects the integrity of the intestinal barrier causing the translocation of the virus from the lung to the intestinal lumen through the circulatory and lymphatic systems[39]. In patients with COVID-19, there are primary inflammatory stimuli that trigger the release of intestinal cytokines into the circulatory system, which can cause microbial dysbiosis[40,41].

The microbiota of patients with COVID-19 during hospitalization are characterized by enrichment of opportunistic pathogens and depletion of commensals in the gut[42,43]. Due to the gut microbiota's crucial importance, dysbiosis could increase COVID-19 severity and prolong patient recovery. The majority of COVID-19's most serious clinical illnesses and deaths occur in people who have certain risk factors, such as old age and the existence of one or more comorbidities, all of which are associated with an unbalanced microbiota[44]. Dysbiosis in COVID-19 patients enhances inflammation and long-term symptoms[13,45]. Altogether, the available data suggests a potential role of gut microbiota in COVID-19 progression, severity, and perhaps susceptibility.

In this pilot study, we hypothesize that the intestinal microbiota is altered during the recovery process following SARS-CoV-2 infection. The participants of our study included a total of 16 individuals: 8 participants had been diagnosed and had recovered from COVID-19 in December 2020 with mild symptoms and no need for hospitalization, versus 8 participants that had never contracted COVID-19. Through the application of bacterial high-throughput sequencing and analyses, we investigated the differences in the gut microbiota of these participants. In addition, after identification of the most significantly varying bacteria from the sequencing data, we used quantitative PCR (qPCR) to study the levels of twelve bacterial groups among the COVID-19 patients in samples collected in December 2020 and after six months of their infection in July 2021.

Materials and Methods

Sample Collection

The COVID-19 positive participants were laboratory-confirmed SARS-CoV-2 positive by quantitative reverse transcription PCR (RT-qPCR) performed on nasopharyngeal swabs collected by hospital staff. Eight recovered from COVID-19 at the time with clearance of SARS-CoV-2, and 8 that had never contracted COVID-19 at the time of the sampling participated in this study. The stool samples were collected in December 2020 in Istanbul (Turkey). Although the SARS-CoV-2 variants were not identified in the patients, from the dates of the infections, we assume the variants were either Alpha or Beta[46]. None of the participants had been vaccinated against COVID-19 at the time of the stool sampling. Biometric, lifestyle, and medical history information was recorded using a standard form self-filled by the participants. The non-COVID-19 subjects or control group were recruited among the person-

nel of Beykoz Institute of Life Sciences and Biotechnology (Istanbul, Turkey) as healthy donors. Stool samples were collected in a collection tube (100 × 16 mm and 13 ml, Marienfeld Superior, Germany). Participants provided stools on the day of follow-up on self-sampling at home and stored them at -80°C until processing.

Stool DNA Extraction

For DNA extraction from stool in preparation for next-generation sequencing, we used DNA Tissue Kit STM QuickGene (Kurabo, Japan). First, a 25 mg stool sample was transferred to the homogenization tube with a 250 µl MDT (Tissue Lysis) solution. To homogenize, 15 mg 0.1 mm ø glass beads were added to the tube. The samples were treated twice for 120 seconds each at 3,075 g in the homogenizer. After the sample was homogenized, 25 µl of EDT (Proteinase K) solution was added and incubated at 56°C for 60 minutes. Then, the samples were centrifuged at 15,000 g for 10 minutes at room temperature. After centrifugation, 200 µl of supernatant was transferred to a 1.5 ml tube. After adding 180 µl of LDT (cell lysis) solution and vortexing for 15 seconds, the micro-tube was incubated at 70°C for 10 minutes. In the next step, 240 µl of 99% ice-cold ethanol was added and the sample was vortexed for 15 seconds. All the contents of the micro-tube were transferred to the QuickGene filtered cassette and the washing and elution process was performed following the manufacturer's suggested protocol. After the extraction process, the samples were diluted with 200 µl CDT (elution buffer) of obtained genomic DNA to a final concentration of 50-60 ng per µl. DNA quality and purity were measured using Colibri Titertek Berthold and Qubit fluorometer 2.0 device. For qPCR analyses, the QIAamp DNA Stool Mini Kit (Qiagen, Hiden, Germany) was used following the manufacturer's instructions.

Next-Generation Sequencing

16S ribosomal DNA V3-V4 preparation phase

PCR was carried out with a 30 µl reaction solution containing 10 ng template DNA, 6 µM primers, 15 µL 2× Phusion Master Mix (New England Biolabs Inc., Ipswich, MA, USA), and 2 µL of water. The PCR condition included an initial denaturation at 98°C for 1 minute, 30 cycles of denaturation at 98°C for 10 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 30 seconds, and final extension at 72°C for 5 minutes. A mix of the same volume of 1X loading buffer (containing SYBR green) and run electrophoresis on a 2% agarose gel for detection was used. PCR products from samples with bands between 400 bp-450 bp were selected and mixed at equal concentrations. Mixed PCR products were purified with the Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were produced using the NEBNext® Ultra™ DNA Library Prep Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommendations and index codes were added. Library quality was evaluated on the Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Finally, the library was sequenced on an Illumina NovaSeq6000 platform and 250 bp double-ended reads were generated. The forward and reverse primers used are shown in **Supplementary Table I**.

De Novo Assembly and Data Filtering

The unique molecular barcodes, linker primer sequences from each sample (**Supplementary Table II**), and read pairs were separated and pair-end reads were combined using Geneious software. Afterward, reads were clustered into OTUs using the de novo assembler, and a taxonomy database was created. The batch BLAST OTUs and any potential chimeras were excluded using the taxonomy database. In addition, OTUs with very low total counts were excluded (20 reads in at least one of the samples were the minimum).

Quantitative PCR

To evaluate the relative abundance of specific genera or species of bacteria in the patients immediately and after six months of recovery from COVID-19, qPCR was performed to assess the presence and the relative quantity of specific bacterial DNA. Specific bacterial primers were designed (Table I). Primer specificity was evaluated by comparing the chosen sequence fragments with all the sequences obtained from 16S Ribosomal DNA analyses. We calculated the relative abundance (RA) of a specific bacterial genus or species by comparing the ΔCT using samples of water as no template calibrator control where abundance was calculated using the formula ($RA=2^{-\Delta Ct}$)[47].

For each sample, SYBR real-time qPCR was conducted in duplicate in a 36-well rotor using a Rotor-Gene Q-QIAGEN. The 20 µL PCR reaction

Table I. Details of specific primers designed for qPCR analyses.

Name	Sequence (5′- 3′)	Amplicon length (bp)	T annealing/ T melting (°C)
Acetivibrio-F1	TTCGGCAAGTCTGATGTGAAAG	144	62/67
Acetivibrio-R1	TGTCAGTTACTGTCCAGTAAGCC		
Aeromicrobium-F1	CCTGCAGAAGAAGGACCGGCC	110	64/69
Aeromicrobium-R1	GACAAACCGCCTACGAGCC		
Anaerotignum-F1	ATAAGAAGCCCCGGCTAACTA	220	56/61
Anaerotignum-R1	TTCCTAATATCTACGCATTTCA		
Bacilli-F1	TAGGGAATCTTCCGCAATGGA	200	62/67
Bacilli-R2	AACGCTTGCCACCTACGTATT		
Chloroflexi-F1	CAAGGAATTTTCCCCAATGG	160	58/63
Chloroflexi-R1	GTAGTTGGCCGCACCTTCTTCTG		
Desulfomicrobiae-F1	TGTGAGGGATGAAGGCCTTC	174	51/56
Desulfomicrobiae-R1	ATTCCGAATAACGCT		
Mycolicibacterium-F2	CGTAGGTGGTTTGTCGCGTTGTTC	95	66/71
Mycolicibacterium-R3	TTCCAGTCTCCCCTGCAGTACTC		
Paenarthrobacter-F1	CGCAAGCGTTATCCGGAATTAT	110	62/67
Paenarthrobacter-R1	TGCACTCTAGTCTGCCCGTAC		
Paenibacillaceae-F1	GAGAAGAAAGCCCCGGCTAAC	190	64/69
Paenibacillaceae-R1	TCACCGCTACACGTGGAATTCC		
Sphaerobacter thermophilus-F1	GACGTAGGGTGCGAGCGTTGT	140	67/72
Sphaerobacter thermophilus-R1	CACCAGCCTCTCCCTGCCTCTA		
Stenotrophobacter-F1	TGTGCTAGAGTGCAGAAGGGGC	103	67/72
Stenotrophobacter-R1	TGTCAGCCCAGCAACCCGTCTTCA		
Xanthobacter-F1	AAGGGGGCTAGCGTTGCTCGGAATC	117	62/67
Xanthobacter-R1	GTTCCACCAACCTCTCTCGAACTC		

was prepared as follows: 9 µL of 2× SeniFAST SYBR No-ROX kit (Bioline, UK), 1.8 µL of 4 µM of appropriate forward and reverse primers, 4.7 µL RNase-free water, and 2.7 µL DNA template of 20 ng/µl. "No template controls", in which RNase-free water was duplicated as no DNA template, were also performed for each master mix prepared. The real-time qPCR program was performed with hold 1 of 95°C/4 min, followed by 45 cycles of cycling annealing of step 1: hold of 95°C/10 s, step 2: hold of annealing temperature depending on each pair of primers characteristics used/15 s (Table I), step 3: 72°C/10 s acquiring to cycling A (Green), 95°C/1 s, with melting ramp depending on each pair of primers characteristics used (Table I) to 95°C, and hold of 90 s on the 1st step, hold for 4 s on next steps, for melting curve.

Statistical Analysis

Two-tailed significance test and response screening test were used to perform hypothesis tests on inter-group taxonomic abundance data, the occurrence rate >10% to obtain a *p*-value based on the taxonomic abundance table of different levels and the *p*-value. The relationships between the microbial data, clinical and no clinical parameters were assessed using a response screening correlation test. All statistical data analyses were performed using JMP software.

Two-tailed significance tests and box-and-whisker plots for descriptive statistics were performed with the results obtained from qPCR analyses.

Results

Sample Collection

The studied groups included 8 patients with confirmed SARS-CoV-2 infection and clearance and 8 participants without any experienced infection of SARS-CoV-2. The details of the participants of this study are described in Table II. The median age of COVID-19 patients was 41 years, and the control group was 32 years. 75% were women in each group. None of them were hospitalized or vaccinated against COVID-19.

Next-Generation Sequencing

More than 1,100 bacterial and 10 archaeal OTUs were amplified from the analysed stool samples. Of the ~1,100 different bacterial OTUs amplified, 65% belong to phylum Firmicutes, 21% to Bacteroidetes, 7% to the Actinobacteria,

Table II. Clinical characteristics of the studied groups.

Clinical characteristics		COVID-19 recovered	Control	Total
Gender:	Male	4	2	2
	Female	4	6	10
Blood type:	A+	3	5	8
	A-	0	1	1
	B+	2	2	4
	O+	1	-	1
	O-	1	-	1
	Unknown	1	-	1
High blood pressure		3	0	3
Thyroid condition		2	1	3
Antibiotic intake		2	7	9
Body mass index:	Obese	1	0	1
	Overweight	2	0	2
	Normal	5	8	13
Sport (at least two times per week)		0	8	8
Other medical conditions:	Asthma	1	0	1
	Autism spectrum	0	1	1
	Diabetes	1	0	1
	Gluten intolerance	0	1	0
	Irritable bowel disease	0	1	1

5% Proteobacteria, 1% Acidobacteria, and 1% others (including unidentified sequences). Of the Firmicutes phylum, 87% belong to the Clostridiales class (94% to Veillonellales order). Of the Bacteroidetes phylum, 97% belong to the Bacteroidia class. In the case of the Archaea, 91% belong to Euryarchaeota and 9% to Thaumarchaeota. No significant differences between groups were detected between the COVID-19 and uninfected controls group although COVID-19 group presented fewer Firmicutes and Bacteroidetes in proportion than the control group (Figure 1). These results roughly conform to what is considered a healthy human adult gut microbiota, normally dominated by two phyla: Firmicutes (which includes mainly *Clostridium, Enterococcus, Lactobacillus*, and *Faecalibacterium* genera) and *Bacteroidetes* (which includes notably *Bacteroides* and *Prevotella* genera). Other phyla like Actinobacteria, Proteobacteria, Verrucomicrobia, and Euryarchaeota, are represented in lower concentrations[48,49]. The relative abundance of bacteria found from the high throughput sequencing analyses in the COVID-19 recovered and control group are shown more in detail in **Supplementary Figure 1**.

The bacteria that are statistically significant in the response screen test between participants who recovered from COVID-19 and the control group are shown in Table III and Table IV. Among them, the most significantly altered genus was *Mycolicibacterium*. In addition, some bacteria were significantly different between gender, blood type, age, weight, high blood pressure, thyroid condition, and sport (Table IV). Our data showed a high correlation between individuals who recovered from COVID-19 and age, weight, and high blood pressure. These and other correlations are also shown in **Supplementary Figure 2**.

Quantitative PCR

From the sequences obtained in the next generation sequencing, twelve specific primer pairs had been designed for bacteria identified at phylum level (Chroroflexi), at class level (Bacilli), at family level (Desulfomicrobium and Paenibacil-

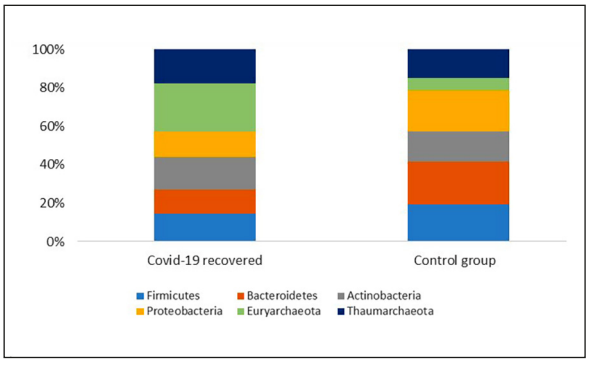
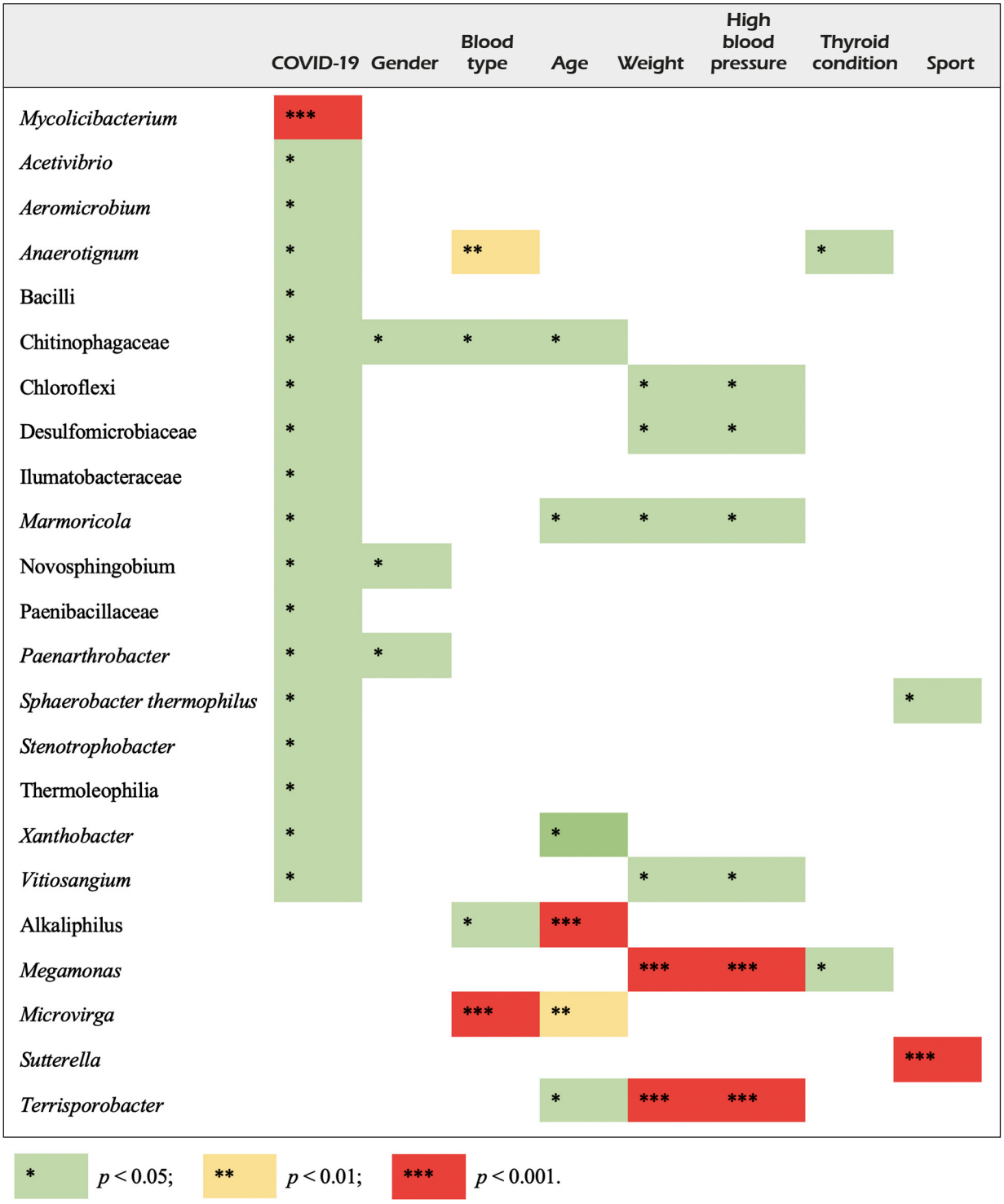


Figure 1. Average relative abundances of phyla of bacteria (Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and others) and archaea (Euryarchaeota and Thaumachaeota) detected in stool samples from patients newly recovered from COVID-19, and the control group.

Table III. Bacteria significantly different from the screen response analyses between newly recovered from COVID-19 and the control group.

Phylum	Class	Order	Family	Genus	Species	*p*-value
Acidobacteria	Blastocatellales	Blastocatellia	Blastocatellaceae	*Stenotrophobacter*	-	0.02
Actinobacteria	Acidimicrobiales	Acidimicrobiia	Ilumatobacteraceae	-	-	0.04
Actinobacteria	Actinomycetia	Micrococcales	Micrococcaceae	*Paenarthrobacter*	-	0.02
Actinobacteria	Actinomycetia	Corynebacteriales	Mycobacteriaceae	*Mycolicibacterium*	-	0.0002
Actinobacteria	Actinomycetia	Propionibacteriales	Nocardioidaceae	*Aeromicrobium*	-	0.04
Actinobacteria	Actinomycetia	Propionibacteriales	Nocardioidaceae	*Marmoricola*	-	0.02
Bacteroidetes	Chitinophagales	Chitinophagia	Chitinophagaceae	-	-	0.03
Chloroflexi	-	-	-	-	-	0.02
Chloroflexi	Thermomicrobia	Sphaerobacterales	Sphaerobacteraceae	*Sphaerobacter*	*Sphaerobacter thermophilus*	0.02
Firmicutes	Bacilli	-	-	-	-	0.04
Firmicutes	Bacilli	Bacillales	Paenibacillaceae	-	-	0.01
Firmicutes	Clostridiales	Eubacteriales	Lachnospiraceae	*Anaerotignum*	-	0.04
Firmicutes	Clostridiales	Eubacteriales	Ruminococcaceae	*Acetivibrio*	-	0.02
Proteobacteria	Rhizobiales	Alphaproteobacteria	Xanthobacteraceae	*Xanthobacter*	-	0.03
Proteobacteria	Desulfovibrionales	Deltaproteobacteria	Desulfomicrobiaceae		-	0.02
Proteobacteria	Myxococcales	Deltaproteobacteria	Cystobacterineae	*Vitiosangium*	-	0.03

Table IV. Significant differences found in screen response analyses between groups in terms of load of specific bacterial group and different factors.



laceae), genus level (*Acetivibrio*, *Anaerotignum*, *Aeromicrobium*, *Mycolicibacterium*, *Paenarthobacter*, *Sphaerobacter*, *Stenotrophobacter*, and *Xanthobacter*) and species level (*Sphaerobacter thermophilus*) (Table I). Specific primers for the sequences obtained of the families Chitinophagaceae and Ilumatobacteraceae, and the genera *Marmoricola* and *Vitiosangium*, were not

possible to design due to similarities to other sequences. Variability in microbial DNA abundance was found immediately post COVID-19, after six months of COVID-19, and in the control group although significant differences were not observed (Figure 2).

Discussion

COVID-19 is a serious global health problem. On 12 June 2022, over 533 million confirmed cases and over 6.3 million deaths have been reported globally[50]. In a survey of 3,762 participants, more than 91% had a time-to-recovery of longer than 35 weeks[51]. Long-term COVID patients experience multisystem involvement and significant disability. By seven months, many patients have not yet recovered (mainly from systemic and neurological/cognitive symptoms), have not returned to previous levels of work, and continue to experience a significant symptom burden[51]. Symptoms like fatigue, sleep disturbance, joint pain, anxiety, depression, headache, and diarrhea that appear with long COVID have also been correlated with dysbiosis of the gut microbiota[52-60]. Symptoms are very similar and given the fact that dysbiosis has consequences for SARS-CoV-2 infection, a strong connection between long COVID and microbiota dysbiosis should be considered, even though there is no direct evidence for this link yet.

In this study, we identified the bacterial gut microbiota from 16 people, 8 diagnosed with and recovered from COVID-19 at the time of sampling with 'clearance of SARS-CoV-2', and 8 participants that had never contracted COVID-19 at the time of sampling. We characterized each of these individuals' gut microbiota via Illumina sequencing of the V3-V4 region of bacterial 16S rRNA. We found significant differences between the control group and the patients immediately after recovery from COVID-19 in a total of 16 groups of bacteria from sequences identified at phylum level belonging to Chloroflexi, at class level belonging to Bacilli, at family level belonging to Chitinophagaceae, Desulfomicrobiaceae, Ilumatobacteraceae, and Paenibacillaceae, at genus level belonging to *Acetivibrio*, *Aeromicrobi-*

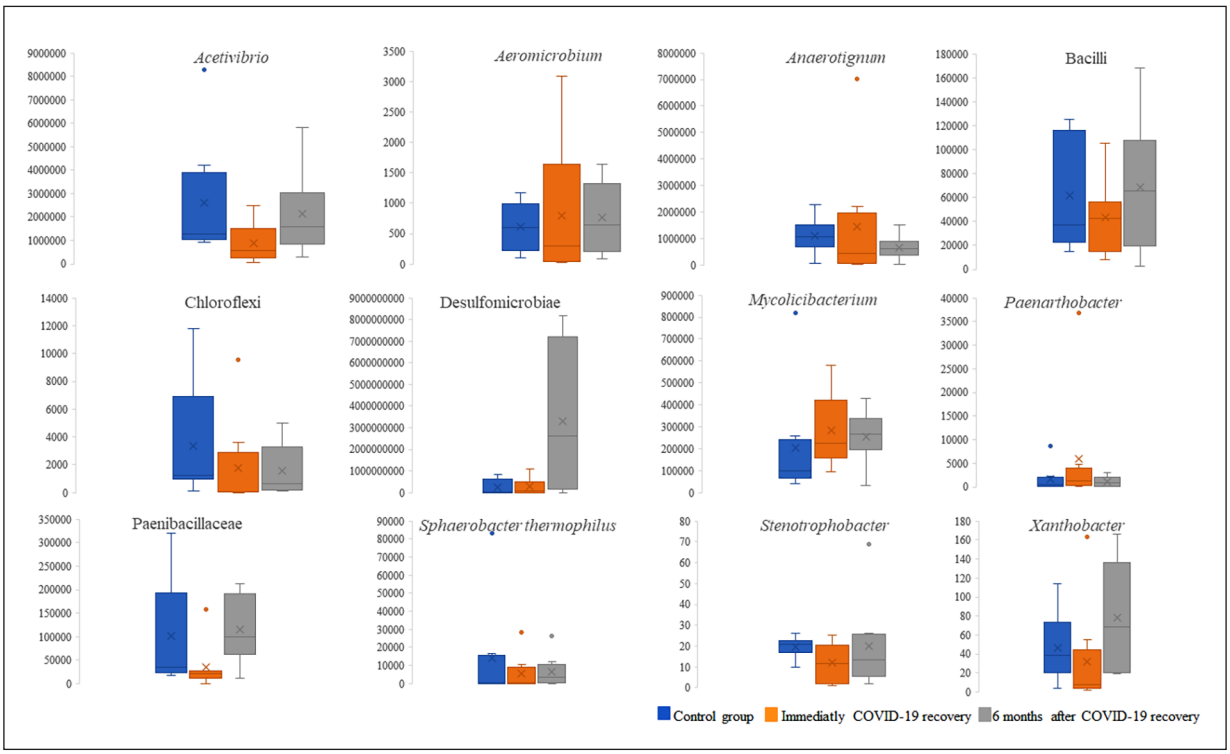


Figure 2. Box plot analyses of qPCR results. Box plot analyses display the Cq values for qPCR using specific primers for different bacterial groups. The samples analyzed belong to three groups: newly recovered from COVID-19, after six months of COVID-19 recovery, and healthy controls. Cq values for each group are represented by a box divided by the median line. Whiskers indicate the 5 to 95 percentile; individual outliers are indicated with dots.

um, *Anaerotignum*, *Marmoricola*, *Mycolicibacterium*, *Paenarthrobacter*, *Stenotrophobacter*, *Xanthobacter*, and *Vitiosangium* and at species level belonging to *Sphaerobacter thermophilus*. All of them were significantly higher in the control group, except for *Mycolicibacterium*, being significantly higher in the group of participants who immediately recovered from COVID-19. *Mycolicibacterium* is a genus of Gram-positive rod-shaped bacteria of the family Mycobacteriaceae from the order Mycobacteriales. The family *Mycobacteriaceae* also includes the genus Mycobacterium, which consists of a wide spectrum of environmental and pathogenic bacteria that frequently appear in clinics affecting human and animal health[61]. Some Mycobacteria are known to cause serious health problems in humans like leprosy and pulmonary diseases such as tuberculosis[62,63]. Members of *Mycolicibacterium* were demarcated from the larger genus *Mycobacterium* in 2018 by Gupta and co-workers[61] being part of the group named nontuberculous mycobacteria (NTM), which are mycobacteria other than *Mycobacterium tuberculosis* and *Mycobacterium leprae*. NTM species are spread among five genera: *Mycobacterium*, *Mycobacteroides*, *Mycolicibacillus*, *Mycolicibacter*, and *Mycolicibacterium*[61]. The incidence and the disease burden of NTM infections have increased significantly worldwide as a result of changes in demographics and advances in radiological diagnosis of pulmonary abnormalities[64]. NTMs can cause infections commonly in the lungs, with the presence of symptoms such as cough, shortness of breath, fever, weight loss, night sweats, decreased appetite, and fatigue[65]. Many of these symptoms are also present in COVID-19 infections. Therefore, COVID-19 patients look like good targets for the opportunistic pathogen *Mycolicibacterium*, which would explain its significantly higher presence in this group compared to the control group. So far, studies of *Mycolicibacterium* in humans are mainly represented by case reports[66-68]. The other 15 groups of bacteria were significantly higher in the control group compared with the immediate COVID-19 recovery. Like in this study, other pilot studies with small numbers of COVID-19 patients have also found an altered microbiota compared to healthy controls showing enrichment of opportunistic pathogens and, at the same time, depletion of salutary bacteria[13,42,43,45,69,70].

In our pilot study, we found a high correlation between altered microbiota in individuals who recovered from COVID-19 and parameters of age, weight, and high blood pressure. Although, we are aware that this study has several shortcomings, including heterogeneous patient clinical history, and gut microbial plasticity in response to factors such as diet, age, gender, and sport[22,71,72] (Table IV). Because a homogenous case-controlled cohort with higher representation in each disease group is desirable for a proper study, we consider that studies like ours need longitudinal sampling and large data sets to derive reliable patterns.

Among the statistically different bacteria between the patients and the healthy control group, we designed 12 specific qPCR primers for long-term monitoring of the quantity of these specific bacteria in patients post-recovery from COVID-19. For this pilot study, we were able to perform this immediately post-disease and after six months in comparison with the control group. This study has shown that although there are some variations in the quantity of these bacteria over time, the frequency has not varied significantly among the groups. This could be due to persistent gut dysbiosis, which has been observed even six months after recovery from COVID-19[73]. Although we think the follow-up period of six months permits a good extrapolation of gut microbiota composition to long-term persistent symptoms, this can be a prolonged time to address questions related to the duration of gut microbiota dysbiosis post-recovery, the link between microbiota dysbiosis and long-term persistent symptoms, and whether the dysbiosis or enrichment/depletion of specific gut microorganisms predisposes recovered individuals to future health problems. It is possible that these results are due to the small sample size, therefore these results should be interpreted carefully.

A biomarker is defined as a characteristic that can be objectively measured and evaluated as an indicator of normal biological and pathological processes or pharmacological responses to a therapeutic intervention[74]. This study provides a new set of specific primers for quantifying certain bacteria in the context of gut microbiota in COVID-19 recovered patients. Especially, *Mycolicibacterium* could be considered a good example of a potential biomarker for evaluating the level of this opportunistic pathogen although larger cohort studies are needed to corroborate the results of this pilot study.

Evaluating the composition of gut microbiota in COVID-19 patients compared with healthy in-

dividuals may have potential value as a diagnostic and/or prognostic biomarker for the disease. Stool samples can be acquired easily and non-invasively. Analysis of these samples periodically in conjunction with patient records could help us understand the human microbiota as a critical component and regulator of host immunity to individual and population-level recovery. Even at the end of the outbreak, molecular tools and new therapies for recovery diagnosis and treatment are still needed for patients experiencing long-term symptoms and to ensure efficient and effective responses to future health challenges. In this regard, qPCR using specific primers for specific bacteria could be a very useful, cheap, and fast method.

Conclusions

Because the symptoms in the long COVID are so vague, it is critical to manage and diagnose patients earlier and more accurately. Nowadays studies that follow patient recoveries from COVID-19 using a variety of diagnostic methods to understand the progression and regression of symptoms are crucial. Despite the fact that most of the long COVID cases are medically classified as mild, symptoms like fatigue, joint pain, headache, anxiety, depression, and dyspnea result in diminished quality of life.

Since gut microbiota is malleable and is modulated by several factors, it is imperative that personalized strategies may be implemented as a supplement to current routine therapies. This can be done by profiling the gut microbiota of the individual patients and checking the levels of some specific bacteria. Opportunistic pathogens could be used as potential biomarkers after COVID-19 recovery and/or long COVID to follow the progression of the patients. There is a need for more studies in humans to ascertain a causal and potential triggering role in inflammation for specific bacteria like *Mycobacterium* or others and more in-depth metagenomics studies are needed to reveal the consistency of the results of this study. Further studies are needed to find clinical evidence that intestinal microbiota modulation has a therapeutic role in the treatment of long COVID, being potentially a new therapeutic option or at least an adjuvant treatment of choice. Insights from such studies will add new dimensions to understanding infectious diseases and can help in taking decisive actions in the future.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding

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Authors' Contribution

AMR, PGP, and HS conceived the idea. PGP and BC conducted the experimental work. AMR, PGP, BC, and HS participated in writing, discussion, and data analysis.

Ethics Approval

This study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines and has been approved by the Bezmialem Vakif University Ethics Committee (reference 19/216).

Informed Consent

Written informed consent was obtained from all participants prior to collecting stool samples.

Data Availability

All relevant data are included in the article. Raw sequence data that support the findings of this study have been deposited in NCBI Sequence Read Archive with the Bioproject ID PRJNA865083.

References

1) Burke RM, Killerby ME, Newton S, Ashworth CE, Berns AL, Brennan S, Bressler JM, Bye E, Crawford R, Morano LH, Lewis NM, Markus TM, Read JS, Rissman T, Taylor J, Tate JE, Midgley CM. Symptom Profiles of a Convenience Sample of Patients with COVID-19-United States, January-April 2020. Morb Mortal Wkly Rep 2020; 69: 904-908.
2) Zhang H, Liao YS, Gong J, Liu J, Xia X, Zhang H. Clinical characteristics of coronavirus disease (COVID-19) patients with gastrointestinal symptoms: A report of 164 cases. Digest Liver Dis 2020; 52: 1076-1079.
3) Bolay H, Gul A, Baykan B. COVID-19 is a Real Headache! Headache 2020; 60: 1415-1421.
4) Wu T, Zuo Z, Kang S, Jiang L, Luo X, Xia Z, Liu J, Xiao X, Ye M, Deng M. Multi-organ dysfunction in patients with COVID-19: a systematic review and meta-analysis. Aging Dis 2020; 11: 874-894.

5) Mitchell WB. Thromboinflammation in COVID-19 acute lung injury. Paediatr Respir Rev 2020; 35: 20-24.
6) Mokhtari T, Hassani F, Ghaffari N, Ebrahimi B, Yarahmadi A, Hassanzadeh G. COVID-19 and multiorgan failure: A narrative review on potential mechanisms. J Mol Histol 2020; 51: 613-628.
7) Chertow D, Stein S, Ramelli S, Grazioli A, Chung J-Y, Singh M, Yinda CK, Winkler C, Dickey J, Ylaya K, Ko SH, Platt A, Burbelo P, Quezado M, Pittaluga S, Purcell M, Munster V, Belinky F, Ramos-Benitez M, Boritz E, Herr D, Rabin J, Saharia K, Madathil R, Tabatabai A, Mccurdy M, Cohen J, Wit ED, Vannella K, Hewitt S, Kleiner D. SARS-CoV-2 infection and persistence throughout the human body and brain. Res Sq 2021; https://doi.org/10.21203/rs.3.rs-1139035/v1.
8) Raveendran AV, Jayadevan R, Sashidharan S. Long COVID: An overview. Diabetes Metab Syndr 2021; 15: 869-875.
9) Huang CL, Huang LX, Wang YM, Li X, Ren LL, Gu XY, Kang L, Guo L, Liu M, Zhou X, Luo JF, Huang ZH, Tu SJ, Zhao Y, Chen L, Xu DC, Li YP, Li CH, Peng L, Li Y, Xie WX, Cui D, Shang LH, Fan GH, Xu JY, Wang G, Wang Y, Zhong JC, Wang C, Wang JW, Zhang DY, Cao B. 6-month consequences of COVID-19 in patients discharged from hospital: a cohort study. Lancet 2021; 397: 220-232.
10) Greenhalgh T KM, A'court C, Buxton M, Husain L. Management of post-acute covid-19 in primary care. BMJ 2020; 370.
11) Aiyegbusi OL, Hughes SE, Turner G, Rivera SC, Mcmullan C, Chandan JS, Haroon S, Price G, Davies EH, Nirantharakumar K, Sapey E, Calvert MJ. Symptoms, complications and management of long COVID: a review. J R Soc Med 2021; 114: 428-442.
12) Dhar D, Mohanty A. Gut microbiota and Covid-19-possible link and implications. Virus Res 2020; 285.
13) Yeoh YK, Zuo T, Lui GC, Zhang F, Liu Q, Li AY, Chung AC, Cheung CP, Tso EY, Fung KS, Chan V, Ling L, Joynt G, Hui DS, Chow KM, Ng SSS, Li TC, Ng RW, Yip TC, Wong GL, Chan FK, Wong CK, Chan PK, Ng SC. Gut microbiota composition reflects disease severity and dysfunctional immune responses in patients with COVID-19. Gut 2021; 70: 698-706.
14) Lopez-Leon S, Wegman-Ostrosky T, Perelman C, Sepulveda R, Rebolledo PA, Cuapio A, Villapol S. More than 50 Long-term effects of COVID-19: a systematic review and meta-analysis. Sci Rep 2021; 11: 16144.
15) Phetsouphanh C, Darley DR, Wilson DB, Howe A, Munier CML, Patel SK, Juno JA, Burrell LM, Kent SJ, Dore GJ, Kelleher AD, Matthews GV. Immunological dysfunction persists for 8 months following initial mild-to-moderate SARS-CoV-2 infection. Nat Immunol 2022; 23: 210-216.
16) Novak P, Mukerji SS, Alabsi HS, Systrom D, Marciano SP, Felsenstein D, Mullally WJ, Pilgrim DM. Multisystem Involvement in Post-Acute Sequelae of Coronavirus Disease 19. Ann Neurol 2022; 91: 367-379.
17) Pretorius E, Vlok M, Venter C, Bezuidenhout JA, Laubscher GJ, Steenkamp J, Kell DB. Persistent clotting protein pathology in Long COVID/Post-Acute Sequelae of COVID-19 (PASC) is accompanied by increased levels of antiplasmin. Cardiovasc Diabetol 2021; 20: 172.
18) Enaud R, Prevel R, Ciarlo E, Beaufils F, Wieërs G, Guery B, Delhaes L. The Gut-Lung Axis in Health and Respiratory Diseases: A Place for Inter-Organ and Inter-Kingdom Crosstalks. Front Cell Infect Microbiol 2020; 10.
19) Sender R, Fuchs S, Milo R. Revised estimates for the number of human and bacteria cells in the body. PLOS Biol 2016; 14: e1002533.
20) Hasan N YH. Factors affecting the composition of the gut microbiota, and its modulation. PeerJ 2019; 7: e7502.
21) Goodrich JK, Waters JL, Poole AC, Sutter JL, Koren O, Blekhman R, Beaumont M, Van Treuren W, Knight R, Bell JT, Spector TD, Clark AG, Ley RE. Human genetics shape the gut microbiome. Cell 2014; 159: 789-799.
22) Aleman FDD, Valenzano DR. Microbiome evolution during host aging. PLoS Pathog 2019; 15.
23) Reyman M, Van Houten MA, Van Baarle D, Bosch AaTM, Man WH, Chu MLJN, Arp K, Watson RL, Sanders EaM, Fuentes S, Bogaert D. Impact of delivery mode-associated gut microbiota dynamics on health in the first year of life. Nat Commun 2019; 10.
24) Odamaki T, Kato K, Sugahara H, Hashikura N, Takahashi S, Xiao JZ, Abe F, Osawa R. Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. BMC Microbiol 2016; 16: 90.
25) Guinane CM, Cotter PD. Role of the gut microbiota in health and chronic gastrointestinal disease: understanding a hidden metabolic organ. Therap Adv Gastroenterol 2013; 6: 295-308.
26) Maynard CL, Elson CO, Hatton RD, Weaver CT. Reciprocal interactions of the intestinal microbiota and immune system. Nature 2012; 489: 231-241.
27) Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. Cell 2014; 157: 121-141.
28) Carding S, Verbeke K, Vipond DT, Corfe BM, Owen LJ. Dysbiosis of the gut microbiota in disease. Microb Ecol Health Dis 2015; 26: 26191.
29) Hills RD, Jr., Pontefract BA, Mishcon HR, Black CA, Sutton SC, Theberge CR. Gut microbiome: profound implications for diet and disease. Nutrients 2019; 11.
30) Grabherr F, Grander C, Effenberger M, Adolph TE, Tilg H. Gut dysfunction and non-alcoholic fatty liver disease. Front Endocrinol 2019; 10.
31) Davis CD. The gut microbiome and its role in obesity. Nutr Today 2016; 51: 167-174.

32) Backhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF, Gordon JI. The gut microbiota as an environmental factor that regulates fat storage. PNAS 2004; 101: 15718-15723.

33) Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nature 2006; 444: 1022-1023.

34) Meng C, Bai C, Brown TD, Hood LE, Tian Q. Human gut microbiota and gastrointestinal cancer. Genom Proteom Bioinform 2018; 16: 33-49.

35) Minemura M, Shimizu Y. Gut microbiota and liver diseases. World J Gastroentero 2015; 21: 1691-1702.

36) Tai NW, Wong FS, Wen L. The role of gut microbiota in the development of type 1, type 2 diabetes mellitus and obesity. Rev Endocr Metab Dis 2015; 16: 55-65.

37) Arab JP, Arrese M, Shah VH. Gut microbiota in non-alcoholic fatty liver disease and alcohol-related liver disease: Current concepts and perspectives. Hepatol Res 2020; 50: 407-418.

38) Harper A, Vijayakumar V, Ouwehand AC, Ter Haar J, Obis D, Espadaler J, Binda S, Desiraju S, Day R. Viral infections, the microbiome, and probiotics. Front Cell Infect Microbiol 2020; 10: 596166.

39) Aguirre Garcia MM, Mancilla-Galindo J, Paredes-Paredes M, Tiburcio AZ, Avila-Vanzzini N. Mechanisms of infection by SARS-CoV-2, inflammation and potential links with the microbiome. Future Virol 2021; 10.2217/fvl-2020-0310.

40) Sharma L, Riva A. Intestinal barrier function in health and disease-any role of SARS-CoV-2? Microorganisms 2020; 8: 1744.

41) Villapol S. Gastrointestinal symptoms associated with COVID-19: impact on the gut microbiome. Transl Res 2020; 226: 57-69.

42) Zuo T, Zhang F, Lui GCY, Yeoh YK, Li AYL, Zhan H, Wan Y, Chung ACK, Cheung CP, Chen N, Lai CKC, Chen Z, Tso EYK, Fung KSC, Chan V, Ling L, Joynt G, Hui DSC, Chan FKL, Chan PKS, Ng SC. Alterations in gut microbiota of patients with COVID-19 during time of hospitalization. Gastroenterology 2020; 159: 944-955.

43) Farsi Y, Tahvildari A, Arbabi M, Vazife F, Sechi LA, Shahidi Bonjar AH, Jamshidi P, Nasiri MJ, Mirsaeidi M. Diagnostic, prognostic, and therapeutic roles of gut microbiota in COVID-19: a comprehensive systematic review. Front Cell Infect Microbiol 2022; 12: 804644.

44) Donati Zeppa S, Agostini D, Piccoli G, Stocchi V, Sestili P. Gut microbiota status in COVID-19: an unrecognized player? Front Cell Infect Microbiol 2020; 10: 576551.

45) Hilpert K, Mikut R. Is there a connection between gut microbiome dysbiosis occurring in COVID-19 patients and post-COVID-19 symptoms? Front Microbiol 2021; 12: 732838-732838.

46) Who. World Health Organization. www.who.int/en/activities/tracking-SARS-CoV-2-variants/.2022.

47) Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nat Protoc 2008; 3: 1101-1108.

48) Villanueva-Millan MJ, Perez-Matute P, Oteo JA. Gut microbiota: a key player in health and disease. A review focused on obesity. J Physiol Biochem 2015; 71: 509-525.

49) Reiss A., Jacobi M., Rusch K., A. S. Association of dietary type with fecal microbiota and short chain fatty acids in vegans and omnivores. J Int Soc Micr 2016; 1: 1-9.

50) Who. World Health Organization. https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports 2022.

51) Davis HE, Assaf GS, Mccorkell L, Wei H, Low RJ, Re'em Y, Redfield S, Austin JP, Akrami A. Characterizing long COVID in an international cohort: 7 months of symptoms and their impact. EClinicalMedicine 2021; 38: 101019.

52) Galland L. The gut microbiome and the brain. J Med Food 2014; 17: 1261-1272.

53) Poroyko VA, Carreras A, Khalyfa A, Khalyfa AA, Leone V, Peris E, Almendros I, Gileles-Hillel A, Qiao Z, Hubert N, Farré R, Chang EB, Gozal D. Chronic sleep disruption alters gut microbiota, induces systemic and adipose tissue inflammation and insulin resistance in mice. Sci Rep 2016; 6: 35405.

54) Molina-Torres G, Rodriguez-Arrastia M, Roman P, Sanchez-Labraca N, Cardona D. Stress and the gut microbiota-brain axis. Behav Pharmacol 2019; 30: 187-200.

55) Peirce JM, Alviña K. The role of inflammation and the gut microbiome in depression and anxiety. J Neurosci Res 2019; 97: 1223-1241.

56) Smith RP, Easson C, Lyle SM, Kapoor R, Donnelly CP, Davidson EJ, Parikh E, Lopez JV, Tartar JL. Gut microbiome diversity is associated with sleep physiology in humans. PLOS One 2019; 14: e0222394-e0222394.

57) Arzani M, Jahromi SR, Ghorbani Z, Vahabizad F, Martelletti P, Ghaemi A, Sacco S, Togha M. Gut-brain Axis and migraine headache: a comprehensive review. J Headache Pain 2020; 21: 15.

58) Matenchuk BA, Mandhane PJ, Kozyrskyj AL. Sleep, circadian rhythm, and gut microbiota. Sleep Med Rev 2020; 53: 101340.

59) Favazzo LJ, Hendesi H, Villani DA, Soniwala S, Dar Q-A, Schott EM, Gill SR, Zuscik MJ. The gut microbiome-joint connection: implications in osteoarthritis. Curr Opin Rheumatol 2020; 32: 92-101.

60) Li Y, Xia S, Jiang X, Feng C, Gong S, Ma J, Fang Z, Yin J, Yin Y. Gut microbiota and diarrhea: an updated review. Front Cell Infect Microbiol 2021; 11: 625210.

61) Gupta RS, Lo B, Son J. Phylogenomics and comparative genomic studies robustly support division of the genus *Mycobacterium* into an emended genus *Mycobacterium* and Four Novel Genera. Front Microbiol 2018; 9: 67.

62) Delogu G, Sali M, Fadda G. The biology of *Mycobacterium tuberculosis* infection. Mediterr J Hematol 2013; 5: e2013070.

63) Smith I. *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. Clin Microbiol Rev 2003; 16: 463-496.

64) Zhou L, Xu D, Liu H, Wan K, Wang R, Yang Z. Trends in the Prevalence and Antibiotic Resistance of Non-tuberculous Mycobacteria in Mainland China, 2000–2019: Systematic Review and Meta-Analysis. Front Public Health 2020; 8: 295.

65) Pennington KM, Vu A, Challener D, Rivera CG, Shweta FNU, Zeuli JD, Temesgen Z. Approach to the diagnosis and treatment of non-tuberculous mycobacterial disease. J Clin Tuberc Other Mycobact Dis 2021; 24: 100244-100244.

66) Tanaka S, Hoshino Y, Sakagami T, Fukano H, Matsui Y, Hiranuma O. Pathogenicity of *Mycolicibacterium phlei*, a non-pathogenic nontuberculous mycobacterium in an immunocompetent host carrying anti-interferon gamma autoantibodies: a case report. BMC Infect Dis 2019; 19: 454.

67) Niitsu T, Kuge T, Fukushima K, Matsumoto Y, Abe Y, Okamoto M, Haduki K, Saito H, Nitta T, Kawano A, Matsuki T, Motooka D, Tsujino K, Miki K, Nakamura S, Kida H, Kumanogoh A. Pleural effusion caused by *Mycolicibacterium mageritense* in an immunocompetent host: a case report. Front Med 2021; 8: 797171.

68) Kuge T, Fukushima K, Matsumoto Y, Abe Y, Akiba E, Haduki K, Saito H, Nitta T, Kawano A, Kawasaki T, Matsuki T, Kagawa H, Motooka D, Tsujino K, Miki M, Miki K, Kitada S, Nakamura S, Iida T, Kida H. Pulmonary disease caused by a newly identified mycobacterium: *Mycolicibacterium toneyamachuris*: a case report. BMC Infect Dis 2020; 20: 888.

69) Gu S, Chen Y, Wu Z, Chen Y, Gao H, Lv L, Guo F, Zhang X, Luo R, Huang C, Lu H, Zheng B, Zhang J, Yan R, Zhang H, Jiang H, Xu Q, Guo J, Gong Y, Tang L, Li L. Alterations of the Gut Microbiota in Patients With Coronavirus Disease 2019 or H1N1 Influenza. Clin Infect Dis 2020; 71: 2669-2678.

70) Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet 2020; 395: 507-513.

71) Shin JH, Sim M, Lee JY, Shin DM. Lifestyle and geographic insights into the distinct gut microbiota in elderly women from two different geographic locations. J Physiol Anthropol 2016; 35: 31-31.

72) Santos-Marcos JA, Haro C, Vega-Rojas A, Alcala-Diaz JF, Molina-Abril H, Leon-Acuña A, Lopez-Moreno J, Landa BB, Tena-Sempere M, Perez-Martinez P, Lopez-Miranda J, Perez-Jimenez F, Camargo A. Sex differences in the gut microbiota as potential determinants of gender predisposition to disease. Mol Nutr Food Res 2019; 63: e1800870.

73) Liu Q, Mak JWY, Su Q, Yeoh YK, Lui GC-Y, Ng SSS, Zhang F, Li AYL, Lu W, Hui DS-C, Chan PK, Chan FKL, Ng SC. Gut microbiota dynamics in a prospective cohort of patients with post-acute COVID-19 syndrome. Gut 2022; 71: 544-552.

74) Coravos A, Khozin S, Mandl KD. Developing and adopting safe and effective digital biomarkers to improve patient outcomes. NPJ Digit Med 2019; 2: 14.