Supplementary materials

Study design and Participants

This is a prospective longitudinal follow-up study of COVID-19 survivors. These patients were discharged from the First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China. In total, thirty patients with COVID-19 were included in the study and followed up to 6 months after hospital discharge. A control cohort with 30 uninfected subjects (age, gender and BMI matched) served as the baseline because stool specimens were not collected from patients before SARS-CoV-2 infection (**Table S1**). Exclusion criteria of the patients and controls include: history of gastrointestinal disease; antibiotic or prebiotic use in the previous months. Ethics approval was obtained from the Institutional Review Board of the First Affiliated Hospital, Zhejiang University School of Medicine (IIT20200069A-R1). Informed written consent was obtained from each of the participants before enrollment.

Table S1. Clinical characteristics of patients and uninfected controls in study

	COVID-19 Patients (n=30)	Controls (n=30)	p-value
Age, years	53.5 (39.75, 59)	53.5 (45.25, 58)	0.88
Male, n (%)	19 (63.3%)	19 (63.3%)	1
Body mass index, kg/m^2	24.1 (21.9, 25.1)	23.8 (21.7 - 25.2)	0.35
Comorbidities			
Hypertension, n (%)	9 (30%)	10 (33.3%)	0.78
Type 2 diabetes, n (%)	4 (13.3%)	5 (16.7%)	0.71
Coronary artery heart disease, n (%)	2 (6.7%)	4 (13.3%)	0.39

The quantitative data are shown as median data and inter quartile range data in brackets.

The occurrence data are shown as no. (%). Values indicate no. of positive results/total no. of patients with available assay results.

Kruskal-Wallis test and Chi-square (χ^2) test was used when applicable.

DNA extraction and 16S rDNA sequencing

Fecal bacterial DNA extraction were performed using the PowerSoil DNA Extraction kit (MoBio Inc., Carlsbad, CA). Given the potential presence of live virus in feces, all fecal samples were inactivated at 56°C for 30 min before DNA extraction. Samples collected in the

recovery phase and controls were treated under the same experimental conditions to reduce methodological bias. Bacterial genomic DNA extraction, PCR amplification of the 16S rDNA V3-4 region, Illumina MiSeq sequencing, and bioinformatic analysis were performed as described earlier [1].

Briefly, after extraction, bacterial DNA was quantified using a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA). The hypervariable region V3-V4 of the bacterial 16S rRNA gene were amplified with primer pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R(5'-GGACTACHVGGGTWTCTAAT-3') by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). The PCR amplification of 16S rRNA gene was performed as follows: initial denaturation at 95℃ for 3 min, followed by 28 cycles of denaturing at 95℃ for 30 s, annealing at 55℃ for 30 s and extension at 72℃for 45 s, and single extension at 72℃ for 10 min, and end at 4℃. The PCR mixtures contain 5 × TransStart FastPfu buffer 4 μL, 2.5 mM dNTPs 2 μL , forward primer (5 μM) 0.8 μL, reverse primer (5 μM) 0.8 μL, TransStart FastPfu DNA Polymerase 0.4 μL, template DNA 10 ng, and finally ddH2O up to 20 μL. PCR reactions were performed in triplicate.bacterial 16S rDNA V3-4 region was amplified using the 338F/806R primer set (338F 5'-ACTCCTACGGGAGGCAGCAG-3', 806R 5'-GGACTACHVGGGTWTCTAAT-3').

Then, the PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to manufacturer's instructions and quantified using Quantus™ Fluorometer (Promega, USA). Attachment of sequencing adapters to PCR products, amplification and library preparation were performed using the NEXTFLEX Rapid DNA-Seq Kit (Illumina, San Diego, CA, United States), as suggested by the manufacturer. Purified amplicons were pooled in

equimolar and paired-end sequenced (2×300) on an Illumina MiSeq platform (Illumina, San Diego, USA) by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). Genome sequences were processed and analyzed on the Majorbio Cloud Platform (www.majorbio.com). The raw sequences have been deposited into the NCBI Sequence Read Archive database (PRJNA703303).

Bioinformatic methods

The raw sequencing reads were quality-filtered and merged by Trimmomatic and FLASH. Operational taxonomic units (OTUs) with a similarity cut-off of 97% were clustered using UPARSE version 7.1 (http://drive5.com/uparse), and chimeric sequences were identified and removed. A total of 1,598 OTUs were defined at 3% distance. After filtering, an average of 44,503 reads per sample was obtained (minimum, 25,480; maximum, 66,612). The taxonomy of each OTU representative sequence was analyzed by RDP Classifier (http://rdp.cme.msu.edu/) against the 16S rRNA database (Silva SSU132). Because the Chao 1 index is dependent on the size of the sequence libraries, the sample sizes from different subjects were equalized by random subtraction to 25,480. Beta diversity was estimated by the Bray-Curtis distance and was visualized by principal coordinate analysis (PCoA).

Six-minute walk distance test (6MWT)

At 6 months after hospital discharge, 6MWT was performed [2]. Pre-walk and post-walk vital signs were determined. Pre-walk heart rate, systolic and diastolic blood pressure and pulse-oximetry were performed in sitting position at least five minutes and breathing room air.

Pulmonary function tests (PFTs)

PFTs was performed at the time of 6 months after discharge. Parameters of forced vital capacity (FVC), forced expiratory volume in the first 1 second of expiration (FEV1), peak expiratory flow (PEF), FEV1/FVC ratio, forced expiratory flow at 25-75% (FEF25-75%), mean expiratory flow at 75% (MEF 75%), mean expiratory flow at 50% (MEF 50%), mean expiratory flow at 25% (MEF 25%), maximal voluntary ventilation (MVV), diffusing capacity of the lung for carbon monoxide (DLCO), diffusing capacity divided by the alveolar volume (DLCO/VA), inspiratory vital capacity (IVC), total lung capacity (TLC), residual volume (RV), residual volume divided by the total lung capacity (RV/TLC) were measured using the SensorMedic Vmax System, USA. The spirometry and DLCO parameters were expressed as a percentage of predicted normal values [3].

Statistical analysis

Demographic characteristics were expressed as median data and inter quartile range (IQR) and as absolute values along with percentages for categorical variables. Severe illness of COVID-19 Patients were diagnosed based on the WHO criteria (https://www.who.int/publications/i/item/clinical-management-of-covid-19). Severe and critical cases during hospitalization were grouped as severe illness. Between-group comparisons for continuous variables were tested by Kruskal-Wallis test. Categorical variables were tested with a Chi-square test. Statistical analyses were performed using the SAS 9.4 software (SAS Institute Inc., Cary, NC, USA). The significance level of the hypothesis tests was set at 0.05 (two-sided). Data of 16S rDNA sequences were analyzed on the Majorbio Cloud Platform using the pipeline provided (www.majorbio.com).

Reference

1. Gu S, Chen Y, Wu Z, et al. Alterations of the Gut Microbiota in Patients with COVID-19 or H1N1 Influenza. Clin Infect Dis Published Online First: 4 June 2020. doi:10.1093/cid/ciaa709.
2. Agarwala P, Salzman SH. Six-Minute Walk Test: Clinical Role, Technique, Coding, and Reimbursement. Chest. 2020 Mar;157(3):603–11.
3. Pakhale S, Bshouty Z, Marras TK. Comparison of per cent predicted and percentile values for pulmonary function test interpretation. Can Respir J. 2009 Dec;16(6):189–93.