

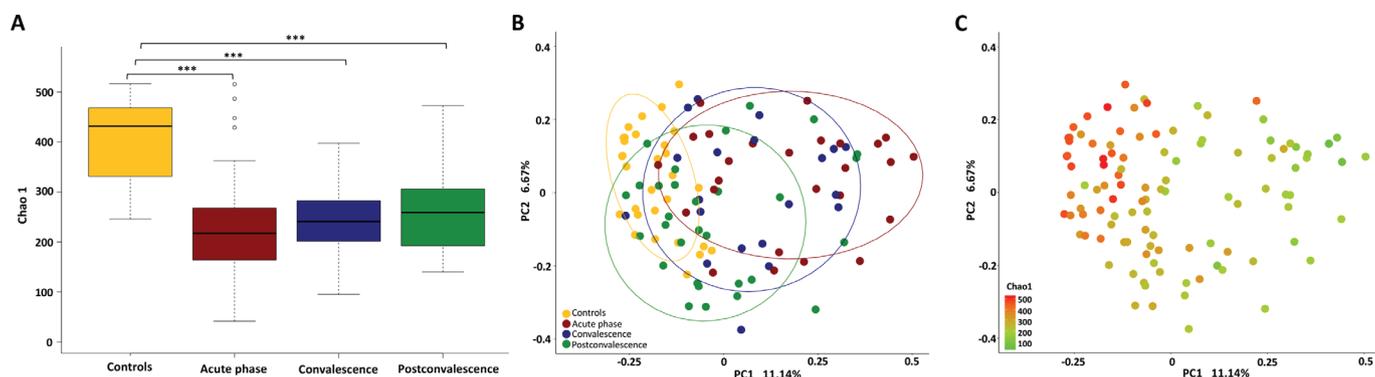
## Six-month follow-up of gut microbiota richness in patients with COVID-19

We read with great interest the recent article published in Gut in which Yeoh

*et al* demonstrated that gut microbiota composition of recovered patients with COVID-19 remained significantly distinct from uninfected controls.<sup>1</sup> Persisting symptoms, also known as ‘long COVID-19’, have been reported in a significant proportion of patients following hospital discharge.<sup>2 3</sup> Gut dysbiosis might link to long COVID-19 risks.<sup>1</sup> Few studies have focused on the recovery process of gut microbiota following SARS-CoV-2 infection.

Here, we conducted a prospective study to longitudinally monitor alterations of gut microbiota in patients with COVID-19 using 16S rDNA sequencing (detailed methods in online supplementary materials). Faecal microbiota was monitored at three timepoints, acute phase (from illness onset to viral clearance), convalescence (from viral clearance to 2 weeks after hospital discharge), postconvalescence (6 months after hospital discharge).

The gut microbiota richness, measured by Chao 1 index, was obviously lower ( $p < 0.01$ , Wilcoxon rank-sum test; figure 1A) in the acute phase of COVID-19 (median 217, IQR 164–266) as compared with uninfected controls (median 432, IQR 332–468). There was a non-significant increase of the Chao 1 index from the acute phase (median 217, IQR 164–266) to the convalescence (median 241, IQR 202–279) and postconvalescence (median 259, IQR 193–302). A Bray-Curtis based principal coordinated analysis revealed that the overall microbial composition of patients with COVID-19 deviated from the uninfected controls (analysis of similarities,  $R = -0.20$ ,  $p = 0.001$ , figure 1B). There was a tendency of the gut microbiota composition moving toward the controls from the acute phase to recovery phase along the first principal coordinate. Notably, the species richness as estimated by Chao



**Figure 1** Changes of faecal microbial communities in different stages (acute, convalescence, postconvalescence) of patients with COVID-19 (n=30), compared with uninfected controls (n=30). (A)  $\alpha$ -Diversity, illustrated by microbiota richness (Chao 1 index), was reduced in COVID-19 ( $p < 0.01$ , Wilcoxon rank-sum test). Boxes represent the 25th–75th percentile of the distribution; the median is shown as a thick line in the middle of the box; whiskers extend to values with 1.5 times the difference between the 25th and 75th percentiles. \*\*\* $P < 0.001$ . (B) Principal coordinate analysis (PCoA) of Bray-Curtis distance analysis demonstrated that the overall microbial composition of patients with COVID-19 deviated from the uninfected controls (analysis of similarities,  $R = -0.201$ ,  $p = 0.001$ ). (C) The same PCoA plot as (B), coloured by  $\alpha$ -diversity measured by Chao 1 index.

1 index, can explain the differences along the first principal coordinate (figure 1C).

The median Chao 1 index in postconvalescence was 259. Patients were further divided into two subgroups according to their Chao 1 index in postconvalescence: low ( $\leq 259$ ,  $n = 15$ ) and high ( $> 259$ ,  $n = 15$ ) (table 1). Patients with reduced postconvalescence richness had higher level of CRP ( $p = 0.01$ ), as well as higher occurrence of intensive care unit admission ( $p = 0.03$ ) and high flow nasal catheter oxygen therapy therapy ( $p = 0.03$ ) during the acute phase. In postconvalescence, low richness was associated with reduced pulmonary function of forced vital capacity ( $p = 0.03$ ), forced expiratory volume in the first 1 s of expiration ( $p = 0.02$ ), inspiratory vital capacity ( $p = 0.05$ ) and total lung capacity ( $p = 0.05$ ).

The present study found that microbiota richness was not restored to normal levels after 6-month recovery. Patients with lower postconvalescence richness showed higher level of CRP and illness severity during the acute phase, suggesting close correlations between inflammatory response and gut dysbiosis in COVID-19, as illustrated in previous studies.<sup>1–4</sup> Microbial diversity is a critical determinant of microbial ecosystem stability.<sup>5</sup> Stable ecosystems provide colonisation resistance to opportunistic pathogens.<sup>6</sup> Therefore, the persistent reduction of gut microbiota richness may have long-term biological influence during the COVID-19 pandemic.<sup>7</sup> Follow-up studies of 3 months and 6 months have shown pulmonary function impairment along with cardiac abnormalities in patients with COVID-19.<sup>2,8</sup> The results here indicated that postconvalescence patients with lower microbial richness had worse pulmonary functions. Gut microbiota is implicated in the pathogenesis of acute

lung injury via several potential mechanisms, including direct translocation of bacteria from gut to the lung and immune modulation effects of microbes related metabolites.<sup>9,10</sup> Our study corroborates the growing evidence that gut dysbiosis is associated with the recovery process of COVID-19. Due to the relatively small sample size, our results need to be confirmed in further studies with larger sample size and more techniques. Targeted manipulation to promote the microbial diversity could be an important strategy to treat long COVID-19 and speed up recovery.

Yanfei Chen,<sup>1</sup> Silan Gu,<sup>1</sup> Yunbo Chen,<sup>1</sup> Haifeng Lu,<sup>1</sup> Ding Shi,<sup>1</sup> Jing Guo,<sup>1</sup> Wen-Rui Wu,<sup>1</sup> Ya Yang,<sup>1</sup> Yongtao Li,<sup>1</sup> Kai-Jin Xu,<sup>1</sup> Cheng Ding,<sup>1</sup> Rui Luo,<sup>1</sup> Chenjie Huang,<sup>1</sup> Ling Yu,<sup>1</sup> Min Xu,<sup>1</sup> Ping Yi,<sup>2</sup> Jun Liu,<sup>3</sup> Jing-jing Tao,<sup>4</sup> Hua Zhang,<sup>1</sup> Longxian Lv,<sup>4</sup> Baohong Wang,<sup>1</sup> Jifang Sheng,<sup>1</sup> Lanjuan Li<sup>1</sup>

<sup>1</sup>State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, National Clinical Research Center for Infectious Diseases, Zhejiang University School of Medicine First Affiliated Hospital, Hangzhou, Zhejiang, China

<sup>2</sup>Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, School of Medicine First Affiliated Hospital, Zhejiang University, Hangzhou, Zhejiang, China

<sup>3</sup>National Clinical Research Center for Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Zhejiang University School of Medicine First Affiliated Hospital, Hangzhou, Zhejiang, China

<sup>4</sup>State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, Zhejiang University School of Medicine First Affiliated Hospital, Hangzhou, Zhejiang, China

**Correspondence to** Professor Lanjuan Li, National Clinical Research Center for Infectious Diseases Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Zhejiang University First Affiliated Hospital State Key Laboratory for Diagnosis and Treatment of

Infectious Diseases, Hangzhou, Zhejiang, China; ljli@zju.edu.cn

**Twitter** Baohong Wang @Baohong Wang

**Acknowledgements** We thank Ling Yu, Cheng Ren, and Ting Xu, who are staff in the infectious department of the First Affiliated Hospital, School of Medicine, Zhejiang University for their efforts in organising patients' follow-up visits.

**Contributors** Concept and design: LL. Acquisition and interpretation of data: all authors. Drafting of the manuscript: YC and SG. Critical revision of the manuscript: LL. Final approval: all authors.

**Funding** This study was funded by National Natural Science Foundation of China (U20A20343); Zhejiang Province key research and development plan emergency project (No. 2020C03123); National Science and Technology Major Project (No. 2017ZX10204401).

**Competing interests** None declared.

**Patient consent for publication** Not required.

**Ethics approval** The State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, National Clinical Research Centre for Infectious Diseases, Collaborative Innovation Centre for Diagnosis and Treatment of Infectious Diseases, Department of Infectious Diseases, The First Affiliated Hospital, College of Medicine, Zhejiang University. Ethics approval was obtained from the Institutional Review Board of the First Affiliated Hospital, Zhejiang University School of Medicine (IIT20200069A-R1).

**Provenance and peer review** Not commissioned; externally peer reviewed.

**Supplemental material** This content has been supplied by the author(s). It has not been vetted by BMJ Publishing Group Limited (BMJ) and may not have been peer-reviewed. Any opinions or recommendations discussed are solely those of the author(s) and are not endorsed by BMJ. BMJ disclaims all liability and responsibility arising from any reliance placed on the content. Where the content includes any translated material, BMJ does not warrant the accuracy and reliability of the translations (including but not limited to local regulations, clinical guidelines, terminology, drug names and drug dosages), and is not responsible for any error and/or omissions arising from translation and adaptation or otherwise.

**Table 1** Comparison of clinical characteristics between patients with high or low microbial richness in the recovery phase

	All (n=30)	Low (n=15)	High (n=15)	P value
Age, years	53.5 (39.75, 59)	53 (40, 57.5)	53 (37, 59.75)	0.72
Male, n (%)	19 (63.3%)	10 (66.7%)	9 (60%)	0.70
BMI of acute phase, kg/m <sup>2</sup>	24.2 (21.6, 25.2)	24.9 (22.3, 25.8)	23.8 (21.3, 25.0)	0.36
BMI of postconvalescence, kg/m <sup>2</sup>	24.1 (21.1, 26.6)	24.7 (22.2, 27.2)	23.1 (21.0, 25.3)	0.41
Severe illness during hospitalisation, n (%)	10 (33.3%)	7 (46.7%)	3 (20.0%)	0.12
White cell count, ×10 <sup>9</sup> /L *	5.7 (4.1, 8.9)	5.7 (4.3, 9.9)	6.5 (4.1, 8.0)	0.79
Haemoglobin, g/L *	138 (128, 149)	146 (128, 152)	137 (129, 141)	0.28
Platelet count, ×10 <sup>9</sup> /L *	187 (158, 231)	188 (168, 238)	182 (139, 225)	0.53
Neutrophil count, ×10 <sup>9</sup> /L *	3.9 (2.6, 7.1)	4.3 (2.9, 8.7)	3.9 (2.5, 6.7)	0.75
Lymphocyte count, ×10 <sup>9</sup> /L *	0.8 (0.6, 1.2)	0.7 (0.5, 1.2)	0.9 (0.6, 1.1)	0.59
D-dimer, mg/L *	236 (170, 467)	407 (175, 913)	199 (170, 320)	0.08
CRP, mg/L *	10.8 (5.9, 21.5)	15.4 (10.5, 45.7)	7.5 (2.2, 11.4)‡	<b>0.01</b>
HFNC during hospitalisation	7 (23.3%)	6 (40.0%)	1 (6.7%)‡	<b>0.03</b>
ICU admission	4 (13.3%)	4 (26.7%)	0 (0%)‡	<b>0.03</b>
Duration from illness onset to hospital admission, d	6 (4, 9.7)	6 (4.5, 10.5)	6 (1.75, 7.5)	0.55
Duration of viral shedding in respiratory tract, d	17.5 (14, 23.7)	18 (14, 22.5)	17.5 (14.5, 26.25)	0.98
Days of hospitalisation, d	17 (14.2, 23.7)	17 (15, 22)	19.5 (13.75, 25.75)	0.65
<i>PFTs</i> †				
FVC	95.5 (89, 105)	93 (84, 96)	101.5 (94.7, 107.2)‡	<b>0.03</b>
FEV1	95.5 (86.2, 107)	91 (82.5, 97)	103 (90.5, 112)‡	<b>0.02</b>
PEF	82 (71.2, 101)	77 (71.5, 90)	95.5 (75.2, 101)	0.41
FEV1/FVC ratio	80.25 (74.4, 88.0)	80.7 (76.4, 87.9)	80.25 (73.8, 88.0)	0.84
FEF25%–75%	87.5 (65, 120.2)	87 (69.5, 101)	87 (65, 135)	0.63
MEF 75%	86 (72.7, 111)	80 (74.5, 89.5)	104 (77, 111.7)	0.55
MEF 50%	80.5 (70, 102.7)	81 (70, 91)	79.5 (71.7, 118.7)	0.88
MEF 25%	74.5 (57.2, 114)	74 (50, 100)	73 (58.7, 127.2)	0.48
MVV	88 (67.2, 103.7)	75 (64, 95.5)	96 (78.7, 109.2)	0.08
DLCO	88.5 (78.5, 95)	86 (78, 92)	94.5 (79.5, 99)	0.07
DLCO/VA	73 (67, 79.7)	73 (67, 83.5)	73 (68.2, 79.2)	0.82
IVC	83.5 (77.2, 92)	82 (70.5, 86.5)	88.5 (82.5, 93.5)‡	<b>0.05</b>
TLC	96 (91.2, 105.7)	92 (84.5, 98.5)	98 (96, 106)‡	<b>0.05</b>
RV	120 (106.2, 130.7)	123 (102.5, 130.5)	120 (106.7, 129)	0.90
RV/TLC	124.5 (111.2, 142.7)	132 (113.5, 150)	120.5 (110.7, 133.5)	0.23
<i>Exercise capacity</i>				
Pre-6WMT heart rate	84 (75.7, 91)	85 (81.5, 96)	82 (74.5, 86.5)	0.15
Pre-6WMT systolic blood pressure	130.5 (116.2, 142.7)	133 (114, 156.5)	123 (115.5, 136.2)	0.40
Pre-6WMT diastolic blood pressure	77.5 (69, 90.7)	71 (66, 99)	77.5 (70.5, 86.2)	0.85
Pre-6WMT O <sub>2</sub> saturation, %	98 (97, 99)	98 (98, 99)	98 (97, 99)	0.88
6WMT distance, m	600 (540, 640)	620 (575, 640)	560 (515, 654)	0.77
Post-6WMT heart rate	103.5 (98.2, 113.7)	106 (100, 115)	101.5 (97.5, 106.75)	0.20
Post-6WMT systolic blood pressure	129 (122, 142.5)	140 (124, 158.5)	127.5 (121.2, 132.2)	0.06
Post-6WMT diastolic blood pressure	80 (70.7, 86)	81 (69, 92.5)	78.5 (73.7, 84)	0.71
Post-6WMT O <sub>2</sub> saturation, %	98 (97, 98)	98 (97, 98)	98 (97, 98)	0.71

Continued



## OPEN ACCESS

**Open access** This is an open access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited, appropriate credit is given, any changes made indicated, and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>.

© Author(s) (or their employer(s)) 2022. Re-use permitted under CC BY-NC. No commercial re-use. See rights and permissions. Published by BMJ.

YC and SG contributed equally.



**To cite** Chen Y, Gu S, Chen Y, *et al.* *Gut* 2022;**71**:222–225.

Received 10 January 2021

Revised 3 March 2021

Accepted 18 March 2021

Published Online First 8 April 2021

*Gut* 2022;**71**:222–225. doi:10.1136/gutjnl-2021-324090

## ORCID iD

Lanjuan Li <http://orcid.org/0000-0001-6945-0593>

## REFERENCES

- Yeoh YK, Zuo T, GC-Y L. Gut microbiota composition reflects disease severity and dysfunctional immune responses in patients with COVID-19. *Gut* 2021;**70**:698–706.
- Huang C, Huang L, Wang Y, *et al.* 6-Month consequences of COVID-19 in patients discharged from Hospital: a cohort study. *Lancet* 2021;**397**:220–32.
- Nabavi N. Long covid: how to define it and how to manage it. *BMJ* 2020;**370**:m3489.
- Gu S, Chen Y, Wu Z. Alterations of the gut microbiota in patients with COVID-19 or H1N1 influenza. *Clin Infect Dis* 2020.
- Lahti L, Salojärvi J, Salonen A, *et al.* Tipping elements in the human intestinal ecosystem. *Nat Commun* 2014;**5**:4344.
- Buffie CG, Pamer EG. Microbiota-mediated colonization resistance against intestinal pathogens. *Nat Rev Immunol* 2013;**13**:790–801.
- Finlay BB, Amato KR, Azad M, *et al.* The hygiene hypothesis, the COVID pandemic, and consequences for the human microbiome. *Proc Natl Acad Sci U S A* 2021;**118**. doi:10.1073/pnas.2010217118
- Sonnweber T, Sahanic S, Pizzini A, *et al.* Cardiopulmonary recovery after COVID-19 - an observational prospective multi-center trial. *Eur Respir J* 2020. doi:10.1183/13993003.03481-2020. [Epub ahead of print: 10 Dec 2020].
- Dickson RP, Singer BH, Newstead MW, *et al.* Enrichment of the lung microbiome with gut bacteria in sepsis and the acute respiratory distress syndrome. *Nat Microbiol* 2016;**1**:16113.
- Zhang Q, Ran X, He Y, *et al.* Acetate downregulates the activation of NLRP3 inflammasomes and attenuates lung injury in neonatal mice with bronchopulmonary dysplasia. *Front Pediatr* 2020;**8**:595157.

Table 1 Continued

	All (n=30)	Low (n=15)	High (n=15)	P value
The quantitative data are shown as median data and IQR data in brackets.				
The occurrence data are shown as no. (%). Values indicate no. of positive results/total no. of patients with available assay results.				
Between-group comparisons of continuous variable in patients with low and high richness were tested by Kruskal-Wallis test. For categorical variable, $\chi^2$ test was used for comparison between groups.				
Statistically significance with a p value $\leq 0.05$ was marked as bold.				
*The results of laboratory test in the acute phase were compared, usually the first day after hospital admission.				
†Pulmonary function tests were expressed as per cent of the predicted value.				
‡ A p value $\leq 0.05$ was denoted as statistically significant.				
MEF 25%, mean expiratory flow at 25%; MEF 50%, mean expiratory flow at 50%; MEF 75%, mean expiratory flow at 75%; BMI, body mass index; CRP, C reactive protein; DLCO, diffusing capacity of the lung for carbon monoxide; DLCO/VA, diffusing capacity divided by the alveolar volume; FEF25%–75%, forced expiratory flow at 25%–75%; FEV1, forced expiratory volume in the first 1 s of expiration; FVC, forced vital capacity; HFNC, high flow nasal catheter oxygen therapy; ICU, intensive care unit; IVC, inspiratory vital capacity; MVV, maximal voluntary ventilation; PEF, peak expiratory flow; PFTs, pulmonary function tests; RV, residual volume; RV/TLC, residual volume divided by the total lung capacity; TLC, total lung capacity; 6WMT, 6 min walk tests.				

## 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 <sup>2</sup>	24.1 (21.9, 25.1)	23.8 (21.7 - 25.2)	0.35
<i>Comorbidities</i>			
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 ( $\chi^2$ ) 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°C for 3 min, followed by 28 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 45 s, and single extension at 72°C for 10 min, and end at 4°C. 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 ddH<sub>2</sub>O 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](http://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](http://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.