

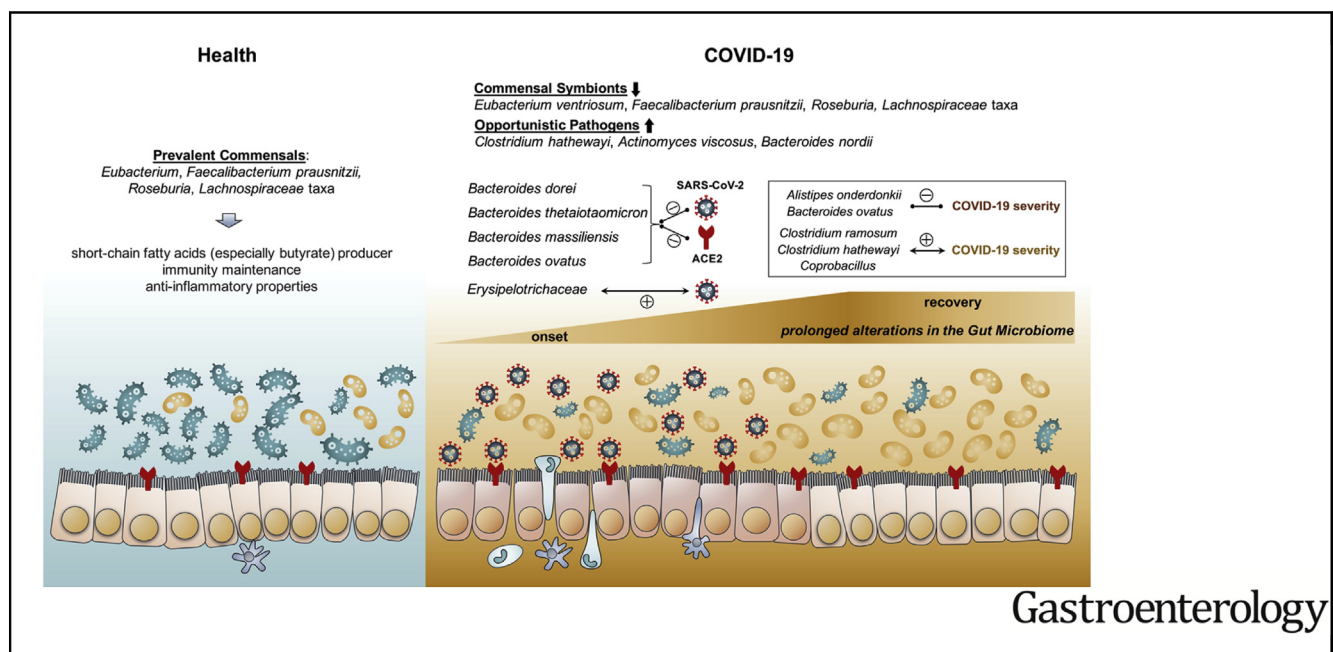
BASIC AND TRANSLATIONAL—ALIMENTARY TRACT

Alterations in Gut Microbiota of Patients With COVID-19 During Time of Hospitalization



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BACKGROUND & AIMS: Although severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infects gastrointestinal tissues, little is known about the roles of gut commensal microbes in susceptibility to and severity of infection. We investigated changes in fecal microbiomes of patients with SARS-CoV-2 infection during hospitalization and associations with severity and fecal shedding of virus. **METHODS:** We performed shotgun metagenomic sequencing analyses of fecal samples from 15 patients with Coronavirus Disease 2019 (COVID-19) in Hong Kong, from February 5 through March 17, 2020. Fecal samples were collected 2 or 3 times per week from time of hospitalization until discharge; disease was categorized as mild (no radiographic evidence of pneumonia), moderate (pneumonia was present), severe (respiratory rate ≥ 30 /min, or

oxygen saturation $\leq 93\%$ when breathing ambient air), or critical (respiratory failure requiring mechanical ventilation, shock, or organ failure requiring intensive care). We compared microbiome data with those from 6 subjects with community-acquired pneumonia and 15 healthy individuals (controls). We assessed gut microbiome profiles in association with disease severity and changes in fecal shedding of SARS-CoV-2. **RESULTS:** Patients with COVID-19 had significant alterations in fecal microbiomes compared with controls, characterized by enrichment of opportunistic pathogens and depletion of beneficial commensals, at time of hospitalization and at all time-points during hospitalization. Depleted symbionts and gut dysbiosis persisted even after clearance of SARS-CoV-2 (determined from throat swabs) and resolution of respiratory

symptoms. The baseline abundance of *Coprobacillus*, *Clostridium ramosum*, and *Clostridium hathewayi* correlated with COVID-19 severity; there was an inverse correlation between abundance of *Faecalibacterium prausnitzii* (an anti-inflammatory bacterium) and disease severity. Over the course of hospitalization, *Bacteroides dorei*, *Bacteroides thetaiotaomicron*, *Bacteroides massiliensis*, and *Bacteroides ovatus*, which downregulate expression of angiotensin-converting enzyme 2 (ACE2) in murine gut, correlated inversely with SARS-CoV-2 load in fecal samples from patients. **CONCLUSIONS:** In a pilot study of 15 patients with COVID-19, we found persistent alterations in the fecal microbiome during the time of hospitalization, compared with controls. Fecal microbiota alterations were associated with fecal levels of SARS-CoV-2 and COVID-19 severity. Strategies to alter the intestinal microbiota might reduce disease severity.

Keywords: Coronavirus; Bacteria; Gut Microbiome; Fecal Nucleic Acid.

Coronavirus Disease 2019 (COVID-19) is a respiratory illness caused by a novel coronavirus (severe acute respiratory syndrome coronavirus 2 [SARS-CoV-2]) and more than 3.3 million people worldwide have been infected as of May 1, 2020. Although most cases of COVID-19 are mild, disease can be severe, resulting in hospitalization, respiratory failure, or death.¹ Early reports from Wuhan showed that 2% to 10% of patients with COVID-19 had gastrointestinal (GI) symptoms, including diarrhea, but a recent meta-analysis reported that up to 20% had GI symptoms.²⁻⁵ Studies have detected SARS-CoV-2 virus in anal swabs and stool samples in almost 50% of patients with COVID-19, suggesting that the digestive tract might be an extrapulmonary site for virus replication and activity.^{6,7} Moreover, fecal calprotectin was found to be elevated in patients with COVID-19 with diarrhea,⁸ an indicator of inflammatory responses in the gut. SARS-CoV-2 uses the angiotensin-converting enzyme 2 (ACE2) receptor to enter the host, and this receptor is highly expressed in both the respiratory and GI tracts.⁹⁻¹¹ ACE2 is important in controlling intestinal inflammation and gut microbial ecology.¹² With trillions of diverse bacteria dwelling in our gut, the gut microbiome has a myriad of effects on gene regulation of immune response and metabolism. The commensal microbiota ecosystem in the gut is dynamic and can be regulated by invading viruses to facilitate a stimulatory or suppressive response.¹³ Studies have shown that respiratory viral infections may be associated with altered gut microbiome, which predispose patients to secondary bacterial infections.^{14,15} Recent meta-transcriptome sequencing of bronchoalveolar lavage fluid showed that the microbiota in SARS-CoV-2-infected patients was dominated by pathogens or oral and upper respiratory commensal bacteria.¹⁶ In addition, comorbidities commonly associated with severe COVID-19 are known to be associated with alterations in bacteria taxa from the phyla Bacteroidetes and Firmicutes,¹⁷⁻²⁰ which were reported to regulate ACE2 expression in rodents.²¹ There is an urgent need to understand host microbial perturbations that underlie SARS-CoV-

WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

SARS-CoV-2 infects gastrointestinal tissues. The authors investigated changes in fecal microbiomes of patients with SARS-CoV-2 infection during hospitalization and associations with severity and fecal shedding of virus.

NEW FINDINGS

Fecal microbiomes from patients with COVID-19 had depletion of symbionts and enrichment of opportunistic pathogens, which persisted after clearance of SARS-CoV-2. Baseline microbiome composition associated with COVID-19 severity. Multiple species from the Bacteroidetes phylum correlated inversely with fecal shedding of SARS-CoV-2.

LIMITATIONS

This was a pilot exploratory study of 15 patients with COVID-19; further studies are needed of alterations in intestinal microbiomes of these patients over time.

IMPACT

These findings indicate the prolonged effect of SARS-CoV-2 infection on the gut microbiomes of patients with COVID-19. Strategies to alter the gut microbiome might be developed to manage gastrointestinal effects of the virus in these patients.

2 infection, which may affect response to infection and efficacy of various future immune interventions, such as vaccines.²²

In this pilot study, we hypothesize that the intestinal microbiota is altered in SARS-CoV-2 infection and is associated with susceptibility to severe disease. We prospectively included 15 hospitalized patients with COVID-19 admitted between February 16, 2020, and March 2, 2020, in Hong Kong, China, followed from hospital admission until discharge. Through the application of deep shotgun metagenomics, we investigated longitudinal changes of the gut microbiome in COVID-19.


Methods

Study Subject and Design

This prospective study involved 15 patients with COVID-19 hospitalized with laboratory-confirmed SARS-CoV-2 infection, 6 patients hospitalized with community-acquired pneumonia (pneumonia controls), and 15 healthy individuals (healthy controls) (Table 1, Supplementary Table 1, Figure 1).

*Authors share co-first authorship.

Abbreviations used in this paper: ACE2, angiotensin-converting enzyme 2; COVID-19, coronavirus disease 2019; GI, gastrointestinal; RT-PCR, reverse-transcriptase polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

 Most current article

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Table 1. Subject Characteristics

Variables	COVID-19 cases	Pneumonia controls	Healthy controls
Number	15	6	15
Male	7 (47)	4 (67)	9 (60)
Median age, y (IQR)	55 (44, 67.5)	50 (44, 65)	48 (45, 48)
Comorbidities, n (%)	6 (40)	6 (100)	0 (0)
Recent exposure history, n (%)			
Travel to cities of Hubei province	1 (7)	0 (0)	0 (0)
Contact with person with COVID-19	5 (33)	0 (0)	0 (0)
Have family cluster outbreak	4 (27)	0 (0)	0 (0)
Symptoms at admission, n (%)			
Fever	9 (60)	4 (67)	
Gastrointestinal symptoms			
Diarrhea	1 (7)	2 (33)	
Respiratory symptoms			
Cough	11 (73)	4 (67)	
Sputum	5 (33)	3 (50)	
Rhinorrhea	3 (20)	1 (17)	
Shortness of breath	4 (27)	3 (50)	
Blood result			
Lymphocyte counts ($\times 10^9/L$, normal range 1.1–2.9)	0.9 (0.7, 1.1)	1.1 (0.9, 1.2)	
Antibiotic therapy at presentation, n (%)			
Amoxicillin Clavulanate	7 (47)	6 (100)	
Cephalosporin	4 (27)	3 (50)	
Tetracycline	5 (33)	6 (100)	
Tetracycline	4 (27)	0 (0)	
Antiviral therapy, n (%)			
Lopinavir-Ritonavir	13 (87)	0 (0)	
Lopinavir-Ritonavir	13 (87)	0 (0)	
Ribavirin	7 (47)	0 (0)	
Interferon beta-1b	1 (7)	0 (0)	
Death, n (%)	0 (0)	0 (0)	

NOTE. Values are expressed in number (percentage) and median (interquartile range).

SARS-CoV-2 infection was confirmed by 2 consecutive reverse-transcriptase polymerase chain reaction (RT-PCR) tests targeting different regions of the RdRp gene performed by the local hospital and Public Health Laboratory Service. Pneumonia controls were patients admitted with community-acquired pneumonia tested negative for SARS-CoV-2 PCR on 2 respiratory samples. Patients with COVID-19 and pneumonia controls were admitted to the Prince of Wales Hospital or the United Christian Hospital, Hong Kong. Healthy controls were individuals with no past medical history or history of antibiotic intake in the past 3 months recruited via advertisement from the general population and tested negative for SARS-CoV-2. All subjects were recruited between February 5 and March 17, 2020. Severity of COVID-19 infection was categorized as (1) mild, if there was no radiographic evidence of pneumonia; (2) moderate, if pneumonia was present along with fever and respiratory tract symptoms; (3) severe, if respiratory rate ≥ 30 /min, oxygen saturation $\leq 93\%$ when breathing ambient air, or $PaO_2 / FiO_2 \leq 300$ mm Hg (1 mm Hg = 0.133 kPa); or (4) critical, if there was respiratory failure requiring mechanical ventilation, shock, or organ failure requiring intensive care.²³ This study was approved by the Joint Chinese University of Hong Kong–New Territories East Cluster Clinical Research Ethics Committees (2020.076). All patients provided informed consent to participate in this study. Data including demographics, laboratory results, imaging results, and medical therapy were extracted from the electronic medical records in

the Hong Kong Hospital Authority clinical management system. Fecal samples from patients with COVID-19 were collected serially 2 to 3 times per week until discharge. This study was conducted in accordance with the Declaration of Helsinki.

Detection of Fecal SARS-CoV-2 Viral Load

SARS-CoV-2 viral loads in stool were measured using real-time RT-PCR assay. Viral RNA from stool samples was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany); 0.1g of stool was suspended in 1 mL of viral transport medium (in 1:10 dilution) and centrifuged for 20 minutes at 4000g. A 140- μ L aliquot of the filtrate was used as starting material following the manufacturer's protocol. SARS-CoV-2 RNA was quantified using real-time RT-PCR. The primer-probe set N1 (2019-nCoV_N1-F: 5'-GAC CCC AAA ATC AGC GAA AT-3', 2019-nCoV_N1-R: 5'-TCT GGT TAC TGC CAG TTG AAT CTG-3', and 2019-nCoV_N1-P: 5'-FAM-ACC CCG CAT TAC GTT TGG ACC-BHQ1-3') designed by US Centers for Disease Control and Prevention were purchased from Integrated DNA Technologies (Coralville, IA). The 1-step real-time RT-PCR reaction contained 10 μ L of the extracted preparation, 4 μ L TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems, Foster City, CA) in a final reaction volume of 20 μ L. The primer and probe concentration were 0.5 μ M and 0.125 μ M, respectively. The cycling conditions, 25°C for 2 minutes, 50°C for 15 minutes, 95°C for 2 minutes, followed by 45 cycles of 95°C for

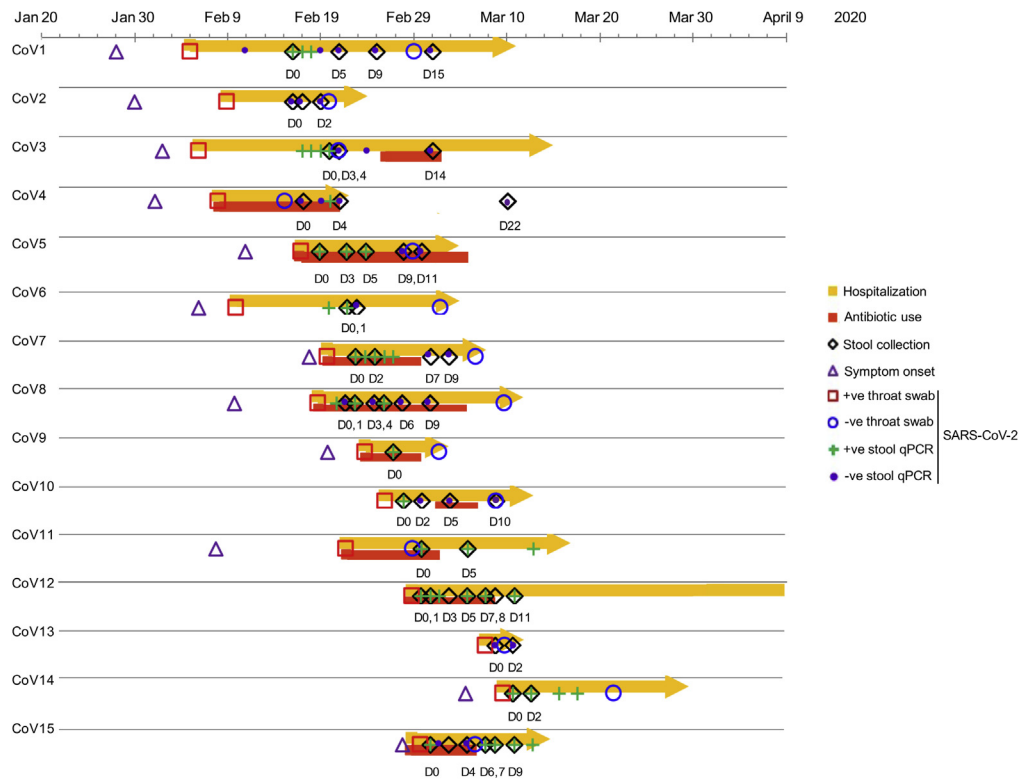


Figure 1. Schematic diagram of stool sample collection, SARS-CoV-2 PCR test results and hospitalization duration in patients with COVID-19 (n = 15). “CoV” denotes patient with COVID-19. Stool specimens were serially collected for shotgun metagenomics sequencing and quantitative RT-PCR test for SARS-CoV-2 virus; “D0” denotes baseline date when the first stool was collected after hospitalization; the following timepoints starting with “D” represent days since baseline stool collection. “+ve throat swab”: the first positive result for SARS-CoV-2 virus in nasopharyngeal/throat/pooled swabs; “-ve throat swab”: the first negative result for SARS-CoV-2 virus in 2 consecutive negative nasopharyngeal/throat/pooled swab tests, on which patient was then discharged.

15 seconds, and 55°C for 30 seconds, were performed with the StepOnePlus Real-Time PCR System (Applied Biosystems). The cycle threshold values of real-time RT-PCR were converted into viral RNA copies based on a standard curve prepared from 10-fold serial dilutions of known copies of plasmid containing the full N gene (2019-nCoV_N_Positive Control; Integrated DNA Technologies). Samples were considered as negative if the cycle threshold values exceeded 39.9 cycles. The detection limit of real-time RT-PCR was 347 copies/mL.

Microbial Profiling of Fecal Samples With Metagenomic Sequencing

An approximately 0.1 g fecal sample was prewashed with 1 mL double-distilled H₂O and pelleted by centrifugation at 13,000g for 1 minute. The fecal DNA was subsequently extracted from the pellet using Maxwell RSC PureFood GMO and Authentication Kit (Promega, Madison, WI) following the manufacturer’s instructions. Briefly, the fecal pellet was added to 1 mL of CTAB buffer and vortexed for 30 seconds, then the sample was heated at 95°C for 5 minutes. After that, the samples were vortexed thoroughly with beads at maximum speed for 15 minutes. Then, 40 μL of proteinase K and 20 μL of RNase A was added to the sample and the mixture was incubated at 70°C for 10 minutes. The supernatant was then obtained by centrifuging at 13,000g for 5 minutes and was added into the Maxwell RSC machine for DNA extraction. Extracted DNA was

subject to DNA libraries construction, completed through the processes of end repairing, adding A to tails, purification and PCR amplification, using Nextera DNA Flex Library Preparation kit (Illumina, San Diego, CA). Libraries were subsequently sequenced on our in-house sequencer Illumina NextSeq 550 (150 base pairs paired-end) at the Center for Microbiota Research, The Chinese University of Hong Kong. Raw sequence reads were filtered and quality-trimmed using Trimmomatic v0.36²⁴ as follows: (1) trimming low-quality base (quality score <20); (2) removing reads shorter than 50 base pairs; (3) removing sequencing adapters. Contaminating human reads were filtering using Kneaddata (Reference database: GRCh38 p12) with default parameters. Profiling of bacterial communities was performed using MetaPhlan2 (V2.9) by mapping reads to clade-specific markers.²⁵

Statistical Analysis

Relative abundance data from MetaPhlan2 were imported into R v3.5.1. Nonmetric multidimensional scaling analyses were performed on all baseline fecal microbiomes between groups, and serial fecal microbiomes in each COVID-19 case during the disease course, based on Bray-Curtis dissimilarities using vegan package (v2.5–3). Differential bacterial taxa between patients with COVID-19 (with or without antibiotics treatment at inclusion), patients with community-acquired pneumonia, and healthy controls were identified using

Table 2. Gut Microbiome Features in Patients With COVID-19

Gut microbiome feature	Taxon	Group	Coefficient	P value	Q value
COVID-19 (antibiotics naïve: abx-)					
Specifically enriched in COVID-19(abx-)	p_Actinobacteria c_Actinobacteria o_Actinomycetales f_Actinomycetaceae g_Actinomyces s_Actinomyces_viscosus	COVID-19 (Abx-)	0.243	8.2E-08	6.4E-05
	p_Firmicutes c_Clostridia o_Clostridiales f_Clostridiaceae g_Clostridium s_Clostridium_hathewayi	COVID-19 (Abx-)	1.130	4.8E-06	2.5E-03
	p_Bacteroidetes c_Bacteroidia o_Bacteroidales f_Bacteroidaceae g_Bacteroides s_Bacteroides_nordii	COVID-19 (Abx-)	0.164	2.3E-05	8.5E-03
Underrepresented in both COVID-19 and pneumonia	p_Firmicutes c_Clostridia o_Clostridiales f_Eubacteriaceae g_Eubacterium s_Eubacterium_ventriosum	COVID-19 (Abx-)	-0.280	1.2E-04	2.5E-02
COVID-19 (antibiotics exposed: abx+)					
Underrepresented in COVID-19(abx+)	p_Firmicutes c_Clostridia o_Clostridiales f_Lachnospiraceae g_Dorea s_Dorea_formicigenerans	COVID-19 (Abx+)	-0.812	2.6E-05	0.00853
	p_Firmicutes c_Clostridia o_Clostridiales f_Lachnospiraceae g_Blautia	COVID-19 (Abx+)	-0.441	6.1E-05	0.01491
	p_Firmicutes c_Clostridia o_Clostridiales f_Ruminococcaceae g_Faecalibacterium	COVID-19 (Abx+)	-0.537	3.2E-05	0.00924
	p_Firmicutes c_Clostridia o_Clostridiales f_Ruminococcaceae g_Faecalibacterium s_Faecalibacterium_prausnitzii	COVID-19 (Abx+)	-0.537	3.2E-05	0.00924
	p_Firmicutes c_Clostridia o_Clostridiales f_Eubacteriaceae	COVID-19 (Abx+)	-0.289	4.6E-05	0.01236
	p_Firmicutes c_Clostridia o_Clostridiales f_Eubacteriaceae g_Eubacterium	COVID-19 (Abx+)	-0.289	4.6E-05	0.01236
	p_Firmicutes c_Clostridia o_Clostridiales f_Eubacteriaceae g_Eubacterium s_Eubacterium_rectale	COVID-19 (Abx+)	-0.903	1.7E-04	0.03316
	p_Firmicutes c_Clostridia o_Clostridiales f_Ruminococcaceae	COVID-19 (Abx+)	-0.300	2.0E-04	0.03709
	p_Firmicutes c_Clostridia o_Clostridiales f_Lachnospiraceae g_Roseburia	COVID-19 (Abx+)	-0.598	2.3E-04	0.04018
	p_Firmicutes c_Clostridia o_Clostridiales f_Lachnospiraceae g_Coprococcus	COVID-19 (Abx+)	-0.447	1.6E-04	0.03239
	p_Firmicutes c_Clostridia o_Clostridiales f_Lachnospiraceae g_Blautia s_Ruminococcus_obeum	COVID-19 (Abx+)	-0.623	4.3E-06	0.00243
	p_Firmicutes c_Clostridia o_Clostridiales f_Lachnospiraceae g_Lachnospiraceae_noname s_Lachnospiraceae_bacterium_5_1_63FAA	COVID-19 (Abx+)	-0.341	2.5E-05	0.00853
Underrepresented in both COVID-19 and pneumonia	p_Firmicutes c_Clostridia o_Clostridiales f_Eubacteriaceae g_Eubacterium s_Eubacterium_ventriosum	COVID-19 (Abx+)	-0.307	8.6E-06	0.00376
Pneumonia patients					
Underrepresented in pneumonia	p_Firmicutes c_Bacilli o_Lactobacillales f_Enterococcaceae g_Enterococcus s_Enterococcus_faecium	Pneumonia	0.228	5.0E-05	0.01261
	p_Firmicutes c_Erysipelotrichia o_Erysipelotrichales f_Erysipelotrichaceae g_Erysipelotrichaceae_noname s_Clostridium_amosum	Pneumonia	0.195	3.1E-06	0.00190
	p_Firmicutes c_Erysipelotrichia o_Erysipelotrichales f_Erysipelotrichaceae g_Coprobacillus	Pneumonia	0.402	5.2E-06	0.00257
	p_Firmicutes c_Clostridia o_Clostridiales f_Lachnospiraceae g_Lachnospiraceae_noname s_Lachnospiraceae_bacterium_5_1_63FAA	Pneumonia	-0.348	7.6E-05	0.01752
Underrepresented in both COVID-19 and pneumonia	p_Firmicutes c_Clostridia o_Clostridiales f_Eubacteriaceae g_Eubacterium s_Eubacterium_ventriosum	Pneumonia	-0.256	3.5E-04	0.03539

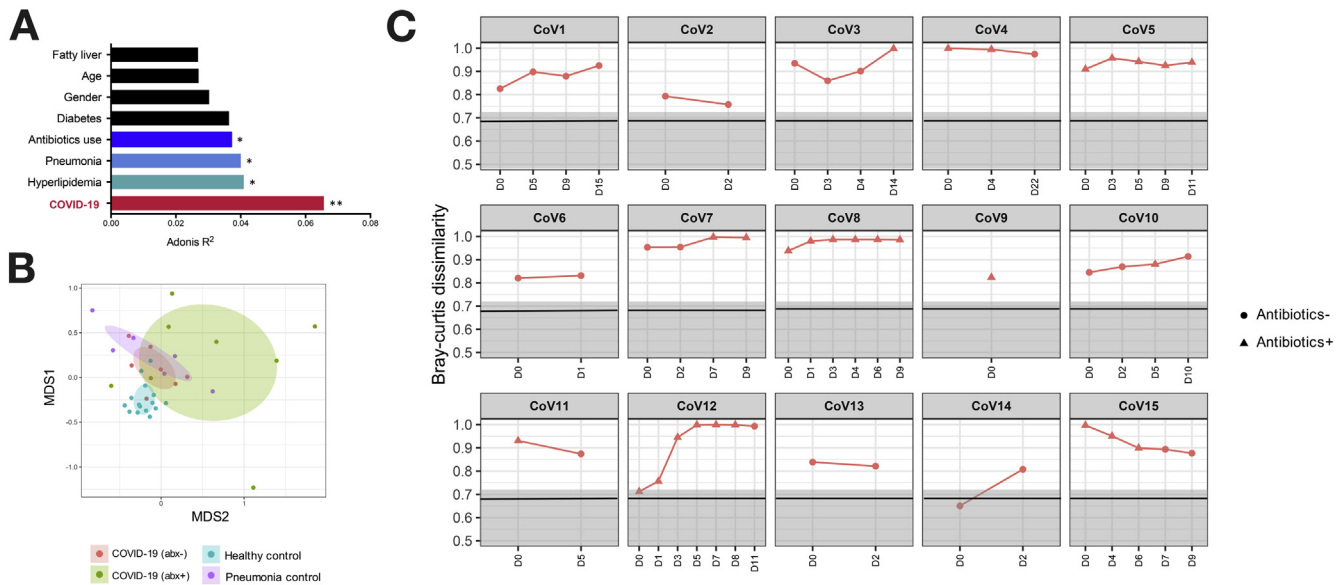


Figure 2. Gut microbiome alterations in patients with COVID-19 and longitudinal changes over the disease course. (A) The effect size of subject metadata in gut microbiome composition, as determined by PERMANOVA test. $**P < .01$; $*P < .05$. (B) Microbiome community alterations in COVID-19, viewed by NMDS (nonmetric multidimensional scaling) plot based upon Bray-Curtis dissimilarities. The microbiomes were compared among healthy controls ($n = 15$), COVID-19 (abx $-$, $n = 7$), COVID-19 (abx $+$, $n = 8$), and pneumonia controls ($n = 6$). (C) Dissimilarity of the gut microbiome of patients with COVID-19 to that of healthy controls during the disease course. The microbiome dissimilarity was calculated as Bray-Curtis dissimilarity. The gray area denotes the range of Bray-Curtis dissimilarities among gut microbiomes of healthy controls, and the solid black line indicates the median dissimilarity among healthy individuals. “CoV” denotes patient with COVID-19. “D0” denotes baseline date when the first stool was collected after hospitalization; the following timepoints starting with “D” represent days since baseline stool collection.

Multivariate Association with Linear Models (MaAsLin).²⁶ Spearman correlation analyses were conducted to associate baseline microbiome profiles of 7 antibiotics-naïve patients at baseline with COVID-19 severity, and to associate longitudinal fecal SARS-CoV-2 loads with timepoint-matched bacterial profiles across all 15 patients with COVID-19, while adjusting for confounding factors.

Data Availability

Metagenomics Sequencing dataset was deposited to the National Center for Biotechnology Information Sequence Read Archive under BioProject accession number PRJNA624223.

Results

Fecal Microbiome Alterations in COVID-19

Among the 15 patients with COVID-19, 7 were antibiotics-naïve (COVID-19[abx $-$]) and 8 received empirical antibiotics (COVID-19[abx $+$]) at baseline (defined as date of the first stool collection after hospitalization). The median ages of patients with COVID-19, pneumonia controls, and healthy controls were 55, 50, and 48 years, respectively; 40% and 100% of patients with COVID-19 and pneumonia controls, respectively, had underlying comorbidities (Table 1, Supplementary Table 1). All patients with COVID-19 presented with respiratory symptoms but only 1 had diarrhea at presentation. None of the patients developed GI symptoms during hospitalization. Median duration of hospitalization was 21 ± 2.4 days (mean \pm SE) in COVID-19 and pneumonia cases.

To understand alterations of the gut microbiome that underlie SARS-CoV-2 infection, we compared baseline fecal microbiome of patients with COVID-19 with healthy controls and pneumonia controls adjusting for age, gender, antibiotic use, and comorbidities. Antibiotic-naïve patients with COVID-19 were enriched in opportunistic pathogens known to cause bacteremia,^{27,28} including *Clostridium hathewayi*, *Actinomyces viscosus*, and *Bacteroides nordii* compared with controls (Table 2). COVID-19(abx $+$) patients demonstrated a further depletion of multiple bacterial species, which are symbionts beneficial to host immunity including *Faecalibacterium prausnitzii*, *Lachnospiraceae bacterium 5_1_63FAA*, *Eubacterium rectale*, *Ruminococcus obeum*, and *Dorea formicigenerans* compared with COVID-19(abx $-$) patients (Table 2). Regardless of antibiotic use, underrepresented bacterial species in patients with COVID-19 were consistently absent or present at very low abundance during the disease course, even when SARS-CoV-2 virus was cleared from the nasopharyngeal swab and stool, and respiratory symptoms had resolved (Supplementary Figures 1–6).

Among all host factors, COVID-19 infection showed the largest effect size in affecting the gut microbiome (PERMANOVA test, $R^2 = 0.066$, $P = .002$, Figure 2A), followed by hyperlipidemia, pneumonia, and antibiotics, whereas age and gender showed no significant effects on gut microbiome alterations (Figure 2A). At the whole microbiome community level, healthy subjects' fecal microbiome clustered together, whereas that of COVID-19(abx $-$) patients

Table 3. Correlation of Gut Bacteria With COVID-19 Severity

Correlation	Bacteria taxa	Correlation coefficient	
		Rho	P value
Positive correlation with COVID-19 severity	p__Firmicutes c__Erysipelotrichia o__Erysipelotrichales f__Erysipelotrichaceae g__Coprobacillus	0.92	.003
	p__Firmicutes c__Erysipelotrichia o__Erysipelotrichales f__Erysipelotrichaceae g__Erysipelotrichaceae_noname s__Clostridium_ramosum	0.92	.003
	p__Firmicutes c__Clostridia o__Clostridiales f__Clostridiaceae g__Clostridium s__Clostridium_hathewayi	0.90	.005
	p__Firmicutes c__Erysipelotrichia	0.90	.006
	p__Firmicutes c__Erysipelotrichia o__Erysipelotrichales	0.90	.006
	p__Firmicutes c__Erysipelotrichia o__Erysipelotrichales f__Erysipelotrichaceae	0.90	.006
	p__Firmicutes c__Erysipelotrichia o__Erysipelotrichales f__Erysipelotrichaceae g__Erysipelotrichaceae_noname	0.90	.006
	p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Actinomycetaceae g__Actinomyces s__Actinomyces_odontolyticus	0.87	.011
	p__Firmicutes c__Erysipelotrichia o__Erysipelotrichales f__Erysipelotrichaceae g__Erysipelotrichaceae_noname s__Erysipelotrichaceae_bacterium_6_1_45	0.87	.011
	p__Proteobacteria c__Gammaproteobacteria o__Enterobacteriales f__Enterobacteriaceae g__Enterobacter	0.87	.011
	p__Proteobacteria c__Gammaproteobacteria o__Enterobacteriales f__Enterobacteriaceae g__Enterobacter s__Enterobacter_cloacae	0.87	.011
	p__Bacteroidetes c__Bacteroidia o__Bacteroidales f__Porphyromonadaceae g__Parabacteroides s__Parabacteroides_unclassified	0.81	.029
	p__Bacteroidetes c__Bacteroidia o__Bacteroidales f__Rikenellaceae g__Alistipes s__Alistipes_indistinctus	0.81	.029
	p__Actinobacteria c__Actinobacteria o__Bifidobacteriales f__Bifidobacteriaceae g__Bifidobacterium s__Bifidobacterium_pseudocatenulatum	-0.81	.026
Negative correlation with COVID-19 severity	p__Firmicutes c__Clostridia o__Clostridiales f__Lachnospiraceae g__Dorea	-0.81	.026
	p__Firmicutes c__Clostridia o__Clostridiales f__Lachnospiraceae g__Dorea s__Dorea_longicatena	-0.81	.026
	p__Bacteroidetes c__Bacteroidia o__Bacteroidales f__Bacteroidaceae g__Bacteroides s__Bacteroides_ovatus	-0.84	.019
	p__Firmicutes c__Clostridia o__Clostridiales f__Lachnospiraceae g__Anaerostipes s__Anaerostipes_hadrus	-0.87	.011
	p__Firmicutes c__Clostridia o__Clostridiales f__Lachnospiraceae g__Lachnospiraceae_noname s__Lachnospiraceae_bacterium_5_1_63FAA	-0.87	.011
	p__Firmicutes c__Clostridia o__Clostridiales f__Lachnospiraceae g__Roseburia	-0.87	.011
	p__Firmicutes c__Clostridia o__Clostridiales f__Ruminococcaceae g__Faecalibacterium	-0.87	.011
	p__Firmicutes c__Clostridia o__Clostridiales f__Ruminococcaceae g__Faecalibacterium s__Faecalibacterium_prausnitzii	-0.87	.011
	p__Bacteroidetes c__Bacteroidia o__Bacteroidales f__Rikenellaceae g__Alistipes s__Alistipes_onderdonkii	-0.90	.005

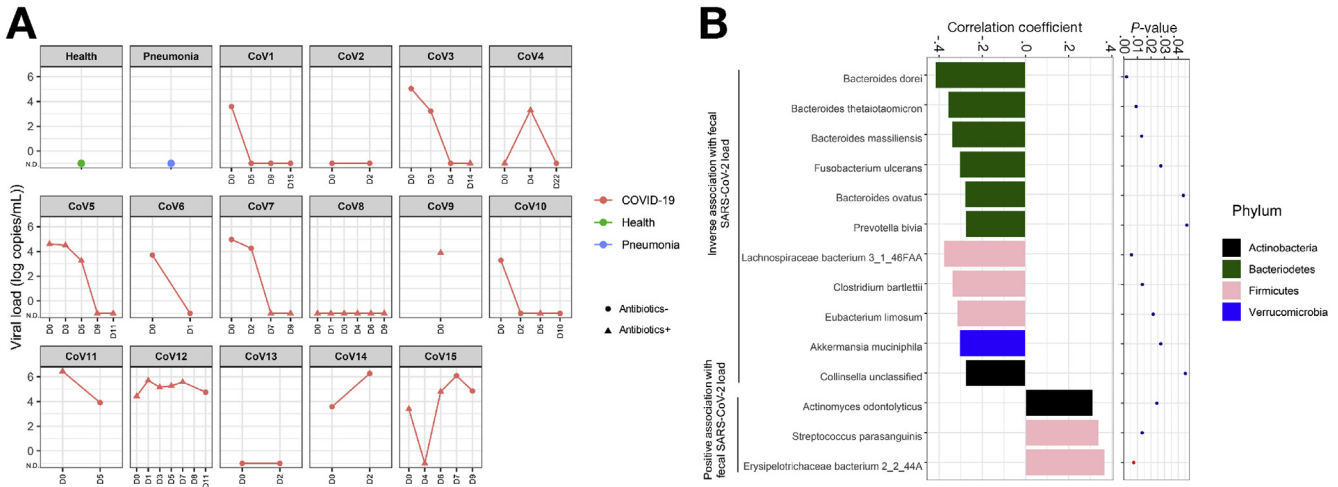


Figure 3. Correlation between gut bacteria and fecal SARS-CoV-2 shedding in patients with COVID-19 over the disease course. (A) Longitudinal changes in fecal viral loads of patients with COVID-19. (B) Bacteria significantly associated with fecal viral load during disease course, as determined by Spearman correlation test.

clustered separately (PERMANOVA test, $P = .001$) and were more heterogeneous (Figure 2B). Antibiotic treatment in patients with COVID-19 was associated with a more heterogeneous microbiome configuration and accompanied by further shift of the gut microbiome away from a healthy microbiome (Figure 2B).

We next explored whether recovery from SAR-CoV-2 infection was associated with restoration of gut microbiome to a community level similar to that of healthy individuals. Overall, the gut microbiome of all patients with COVID-19 remained stable but were markedly disparate from that of healthy controls, both during the disease course and after clearance of SARS-CoV-2 (Figure 2C). Although the microbiome of 5 patients with COVID-19 (CoV1, 4, 7, 11, 15) showed closer proximity to healthy microbiomes over time, patients CoV3, 5, 8, 10, and 12 became more disparate from healthy microbiomes over time (Supplementary Figure 7). At the last follow-up, the gut microbiome of these 10 patients remained substantially different from that of healthy controls, despite clearance of SARS-CoV-2 infection as defined by negative SARS-CoV-2 tests on nasopharyngeal swab or deep throat saliva (Figure 2C, Supplementary Figure 7). Of note, patient CoV4 was discharged on day 5 but his gut microbiome on day 22 was persistently different from that of healthy individuals.

Baseline Gut Microbiome and Disease Severity of COVID-19

To understand whether baseline gut microbiome affects the severity of COVID-19, we assessed association between baseline fecal microbiome and COVID-19 severity (mild, moderate, severe, or critical) in 7 antibiotic-naïve COVID-19 cases. A total of 23 bacterial taxa were found to be significantly associated with COVID-19 disease severity, most of which (15 of 23) were from the Firmicutes phylum (Table 3). Among them, 8 and 7 Firmicutes members, respectively, showed positive and negative correlation with

disease severity. These data are in line with a report showing that different Firmicutes bacteria have diverse roles in upregulating or downregulating ACE2 expression in the murine gut.²¹ Our finding of the association of gut Firmicutes bacteria with COVID-19 severity highlights the potential importance of bacterial membership in modulating human response to SARS-CoV-2 infection.

Three bacterial members from the Firmicutes phylum, the genus *Coprobacillus*, the species *Clostridium ramosum* and *C. hathewayi*, were the top bacteria positively associated with COVID-19 disease severity (Spearman correlation coefficient $Rho > 0.9$, $P < .01$, Table 3). Both *C. ramosum* and *C. hathewayi* have been associated with human infection and bacteremia.^{27,29} Importantly, *Coprobacillus* bacterium has been shown to strongly upregulate colonic expression of ACE2 in the murine gut.²¹ In contrast, 2 beneficial species, *Alistipes onderdonkii* and *Faecalibacterium prausnitzii*, were top bacterial species to show a negative correlation with COVID-19 severity (Table 3). *Alistipes* species are indole positive, involved in the serotonin precursor tryptophan metabolism and in maintaining gut immune homeostasis,^{30,31} whereas *F. prausnitzii* has anti-inflammatory properties.³² Although we cannot assign a causative or preventive role of these bacteria in disease pathogenesis or severity, our data underscore a potential role for bacteria in determining response to SARS-CoV-2 infection and intensity of the infection in the host.

Fecal SARS-CoV-2 Virus Load and Gut Bacterial Abundance

Eleven of the 15 patients had SARS-CoV-2 nucleic acid detected in feces at hospitalization (median 3.86×10^3 copies per mL inoculum, as determined by RT-PCR) and 5 of them cleared the SARS-CoV-2 virus over time (Figure 3A). We investigated whether gut bacteria were associated with fecal SARS-CoV-2 load over the course of hospitalization. A total of 14 bacterial species were identified to be

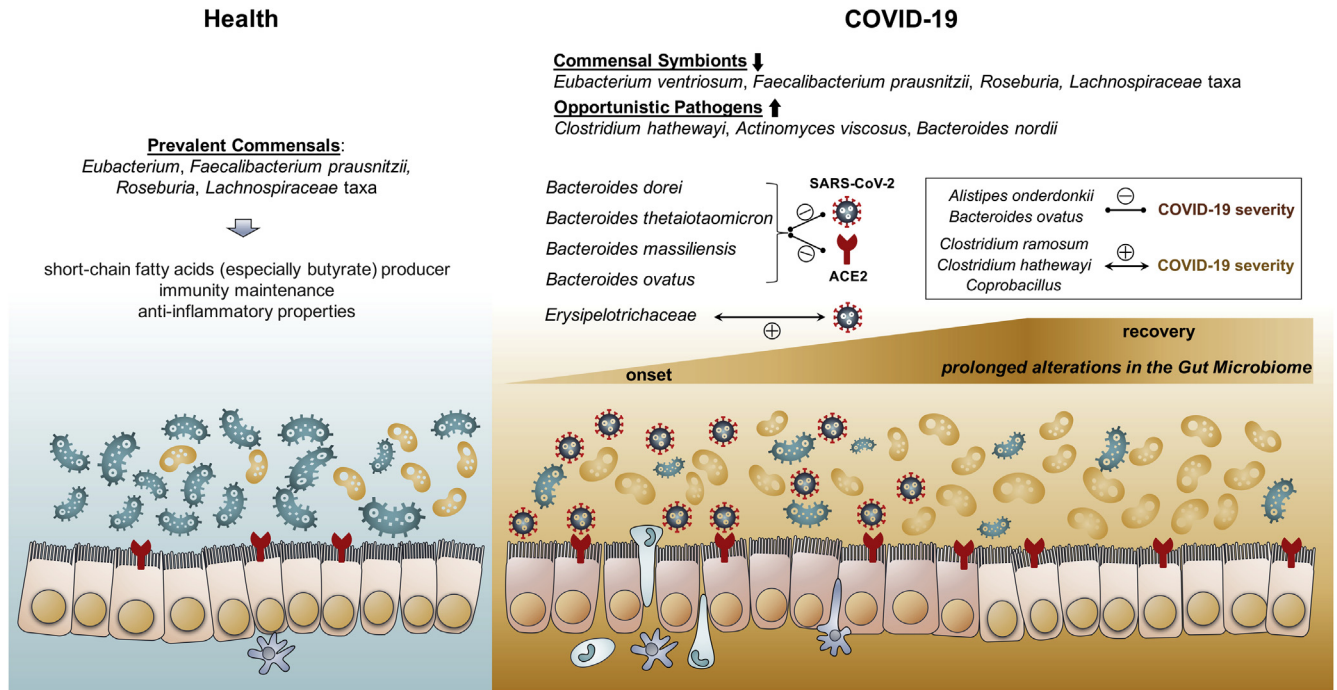


Figure 4. Schematic summary of the gut microbiome alterations in COVID-19. In healthy individuals, *Eubacterium*, *Faecalibacterium prausnitzii*, *Roseburia*, and *Lachnospiraceae* taxa are prevalent in their gut microbiome. However, the gut microbiome of patients with COVID-19 is characterized by enrichment of opportunistic pathogens and depletion of commensals in the gut. Such gut dysbiosis persists during the COVID-19 disease course, even after clearance/recovery of SARS-CoV-2 infection. Baseline fecal abundance of the bacteria *Coprobacillus*, *Clostridium ramosum*, and *Clostridium hathewayi* showed significant correlation with COVID-19 severity, whereas an anti-inflammatory bacterium *Faecalibacterium prausnitzii* showed an inverse correlation. Four Bacteroidetes members, including *Bacteroides dorei*, *Bacteroides thetaiotaomicron*, *Bacteroides massiliensis*, and *Bacteroides ovatus*, known to downregulate ACE2 expression in the murine gut, showed significant inverse correlation with fecal SARS-CoV-2 viral load in patients with COVID-19.

significantly associated with fecal viral load of SARS-CoV-2 across all fecal samples (Figure 3B). Among them, 6 species were from the Bacteroidetes phylum. Four *Bacteroides* species, including *Bacteroides dorei*, *Bacteroides thetaiotaomicron*, *Bacteroides massiliensis*, and *Bacteroides ovatus*, showed significant inverse correlation with fecal SARS-CoV-2 load (all Spearman correlation coefficient $Rho < -0.2$, $P < .05$, Figure 3B). Interestingly, all these 4 species were associated with downregulation of ACE2 expression in the murine colon.²¹ Taken together, these data suggest that *Bacteroides* species may have a potential protective role in combating SARS-CoV-2 infection by hampering host entry through ACE2. In contrast, *Erysipelotrichaceae* bacterium 2_2_44A, a Firmicutes species, showed the strongest positive correlation with fecal SARS-CoV-2 load (Spearman correlation coefficient $Rho = 0.89$, $P = .006$, Figure 3B). *Erysipelotrichaceae* has been implicated in inflammation-related disorders of the GI tract.³³ Considering the strong association of baseline abundance of *Erysipelotrichaceae* with COVID-19 severity (Spearman correlation $Rho = 0.89$, $P = .006$, Table 3), gut *Erysipelotrichaceae* may play a role in augmenting SARS-CoV-2 infection in the host gut.

Discussion

We showed for the first time that the gut microbiome was disturbed in patients with COVID-19. The alterations,

observed even in patients with COVID-19 naive to antibiotic therapy, were characterized by enrichment of opportunistic pathogens and depletion of beneficial commensals (Figure 4). Loss of salutary species in COVID-19 persisted in most patients despite clearance of SARS-CoV-2 virus, suggesting that exposure to SARS-CoV-2 infection and/or hospitalization may be associated with a more long-lasting detrimental effect to the gut microbiome.

Studies have shown that respiratory viral infections can alter the gut microbiome, such as pulmonary infections by influenza and respiratory syncytial virus.^{15,34–36} Viral infections predispose patients to secondary bacterial infections, which often have a more severe clinical course.^{14,37} We found that a number of pathogens and opportunistic pathogens were enriched in the gut microbiome of patients with COVID-19, including *C. hathewayi*, *B. nordii*, *A. viscosus*, and a higher baseline abundance of *C. hathewayi* correlated with more severe COVID-19. Most of these bacteria are bacteremia-associated bacteria, indicating susceptibility for severe disease course due to potential secondary bacterial infection. We also identified an opportunistic pathogen of the oral cavity and upper respiratory tract, *A. viscosus*, in the gut of patients with COVID-19.³⁸ Its presence suggests the passage or transmission of extra-intestinal microbes into the gut.

Recently, a study provided direct evidence that SARS-CoV-2 can bind to human ACE2 as host entry point.⁹ ACE2

is highly expressed in the intestine especially in colonocytes of healthy subjects and in patients with inflammatory bowel disease,¹⁰ and can regulate amino acid transport, microbial ecology, and inflammation in the gut.¹² Interestingly, Bacteroidetes species have been shown to downregulate ACE2 expression in the murine colon, whereas Firmicutes species showed variable effects in modulating ACE2 expression.²¹ We found that baseline abundance of Bacteroidetes species, *A. onderdonkii* and *B. ovatus*, negatively correlated with COVID-19 severity, and 4 species from the genus *Bacteroides* of the phylum Bacteroidetes (*B. dorei*, *B. thetaiotaomicron*, *B. massiliensis*, and *B. ovatus*) showed inverse correlation with fecal viral load of SARS-CoV-2 (Figure 4). Among them, *B. dorei* has been reported to suppress colonic ACE2 expression²¹ and to calibrate host immune response.^{39,40} The highest SARS-CoV-2 mortality and morbidity have been reported in older patients and in those with underlying chronic diseases that are associated with inflammation, such as hypertension, obesity, diabetes mellitus, and coronary artery disease.^{41–43} Interestingly, these subjects were also reported to have a lower abundance of *Bacteroides* species than healthy individuals.^{17–20} These findings altogether suggest that an individual's gut microbiome configuration may affect the subject's susceptibility and response to SARS-CoV-2 infection.

Cytokine profile associated with hyperinflammation state in severe COVID-19 has been characterized by increased interferon- γ inducible protein and other cytokines. Given limited proven treatment for COVID-19, understanding host cytokine pathways and microbiota interactions with cytokine responses in SARS-CoV-2 infection is essential in developing new treatment approaches.⁴⁴

One major limitation of this exploratory study is the modest sample size. Although assigning a causative relationship between COVID-19 and gut dysbiosis requires larger validation studies, this pilot study presents the first data to examine the influence of SARS-CoV2 infection on gut microbiome composition and dynamics. We attempted to adjust for factors, such as age, gender, therapy, and comorbidities, which may explain the observed variance in the data. As we included only hospitalized patients with moderate/severe disease, such findings may not be generalizable to all COVID-19 cases, including those with mild or asymptomatic COVID-19. Stool collected after hospitalization for microbiome analysis does not represent the bona fide baseline microbiome at COVID-19 onset, nor the baseline microbiome before disease onset. Further studies should prospectively include asymptomatic subjects, and if infected with SARS-CoV-2, followed up at disease onset, during disease course, and long term after discovery to delineate the role of microbiome changes in SARS-CoV-2 infection and postinfection recovery.

The use of empirical antibiotics (which was common in the initial outbreak of SARS-CoV-2 when secondary bacterial infection was a concern) led to further loss of salutary symbionts and exacerbation of gut dysbiosis in patients with COVID-19, and our data support avoidance of unnecessary antibiotics use in the treatment of viral pneumonitis, as antibiotics can eliminate beneficial bacteria and weaken the

gut barrier.⁴⁵ In addition, antibiotics-driven gut microbiome perturbation can alter immunity to vaccines in humans.⁴⁶ Improving efficacy of future immune interventions such as vaccines, through modulating the gut microbiome, in combating COVID-19 should be considered. One approach for promoting a healthy microbiome may include measures to enhance intestinal butyrate production through the promotion of microbial interactions by dietary changes, and reduction of proinflammatory states.

In conclusion, our study provides evidence of prolonged gut microbiome dysbiosis in COVID-19 and its association with fecal SARS-CoV-2 virus shedding and disease severity. These data highlight a new concept that novel and targeted approach of modulation of the gut microbiota may represent a therapeutic avenue for COVID-19 and its comorbidities.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at <https://doi.org/10.1053/j.gastro.2020.05.048>.

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CRedit Authorship Contributions

Tao Zuo, PhD (Formal analysis: Lead; Investigation: Equal; Methodology: Lead; Writing – original draft: Lead; Writing – review & editing: Lead). Fen Zhang, PhD

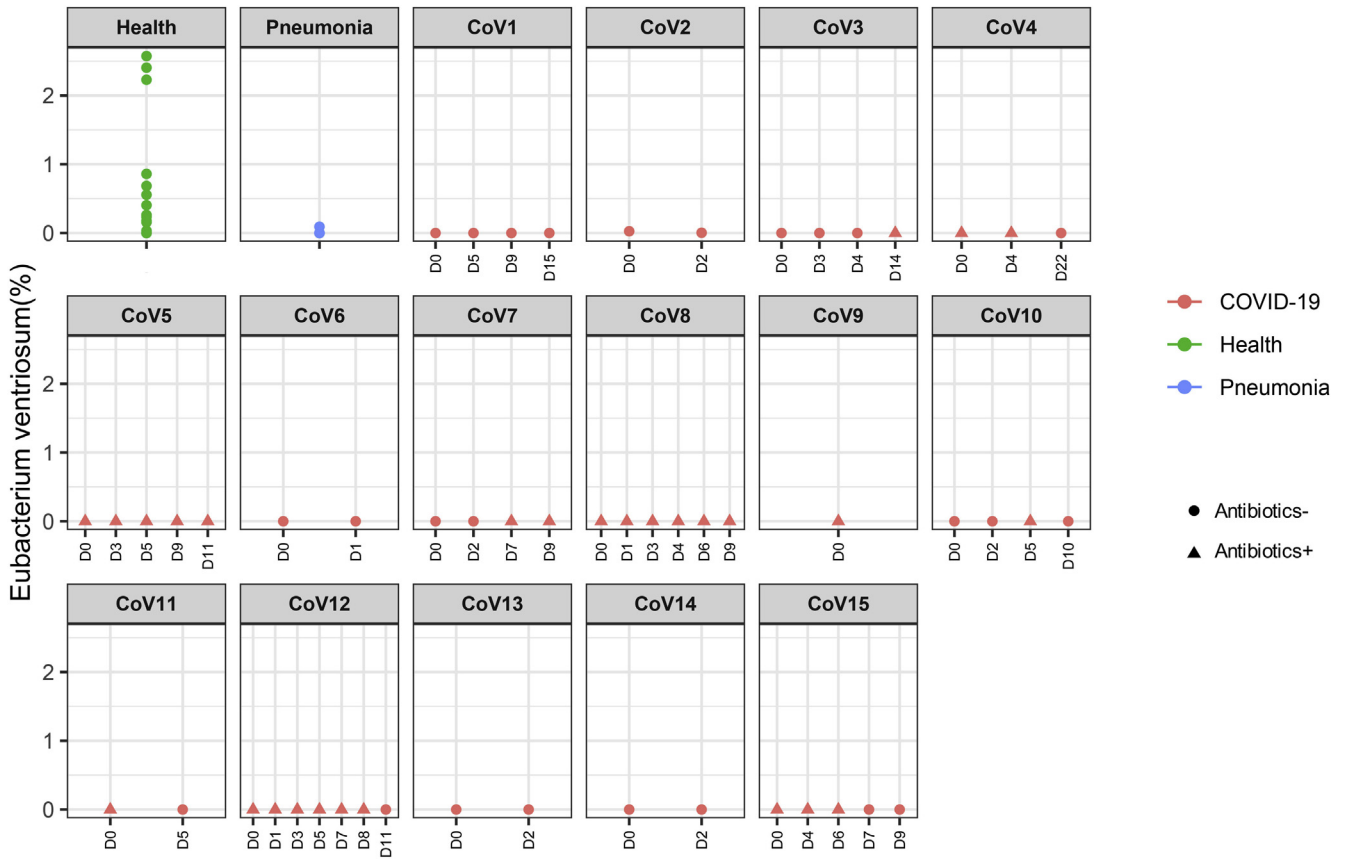
(Formal analysis: Equal; Methodology: Equal). Grace C.Y. Lui, Dr (Conceptualization: Equal; Resources: Lead; Writing – review & editing: Equal). Yun Kit Yeoh, PhD (Writing – review & editing: Lead). Amy Y.L. Li, Bachelor (Data curation: Lead). Hui Zhan, PhD (Investigation: Supporting). Yating Wan, PhD (Methodology: Supporting). Arthur Chung, PhD (Methodology: Supporting). Chun Peng Cheung, Bachelor (Project administration: Lead). Nan Chen, Master (Investigation: Supporting). Christopher K.C. Lai, PhD (Writing – review & editing: Supporting). Zigui Chen, PhD (Writing – review & editing: Equal). Eugene Y.K. Tso, MD (Resources: Equal). Kitty S.C. Fung, MD (Resources: Equal). Veronica Chan, MD (Resources: Equal). Lowell Ling, MD (Resources: Equal). Gavin Joynt, PhD (Supervision: Supporting). David S.C. Hui, MD (Supervision: Supporting). Francis K.L. Chan, MD (Supervision: Lead). Paul K.S. Chan, PhD (Project administration: Lead). Siew C. Ng, PhD (Conceptualization: Lead; Funding acquisition: Lead; Resources: Lead; Supervision: Lead; Writing – review & editing: Lead).

Conflict of interest

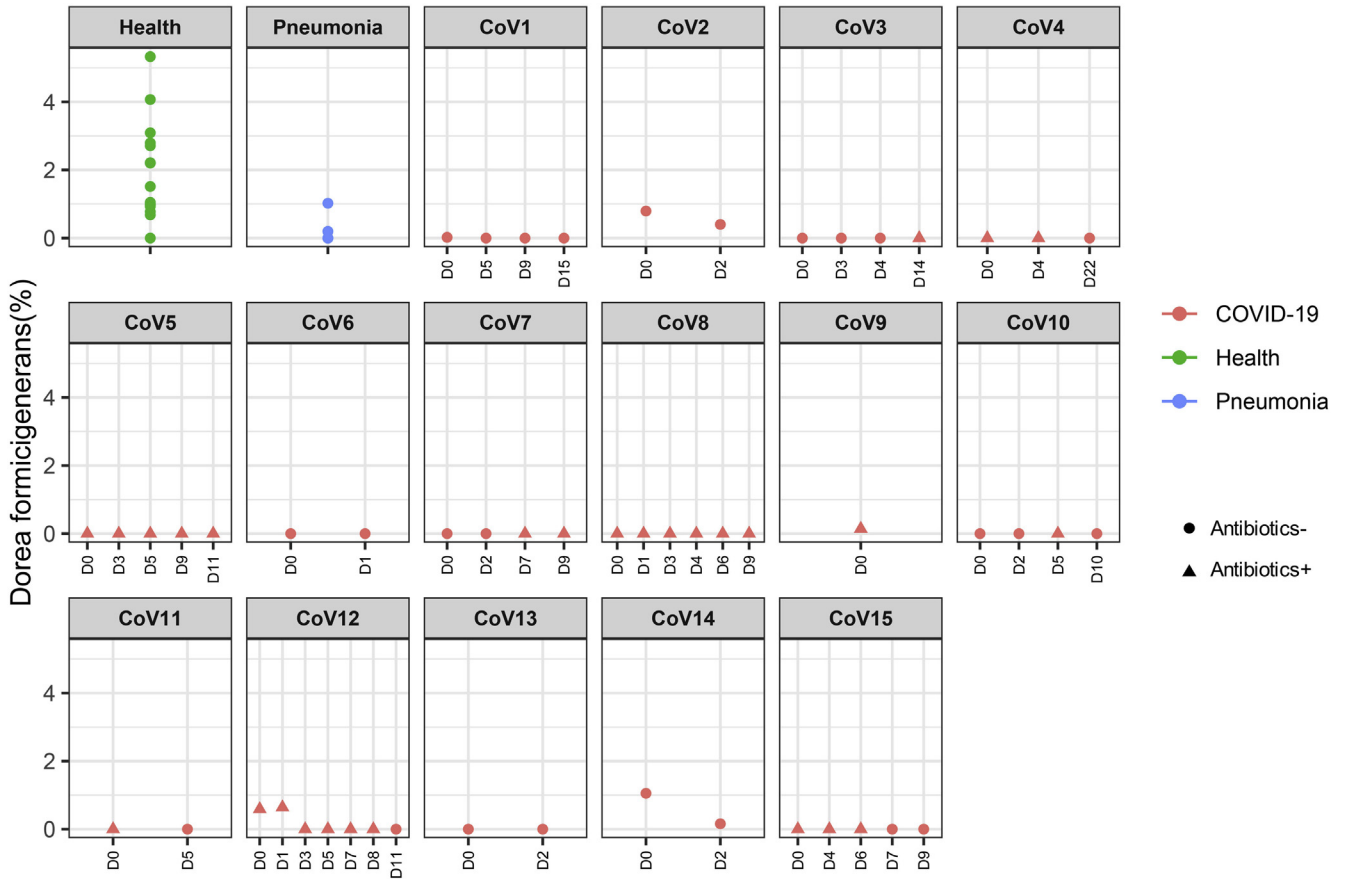
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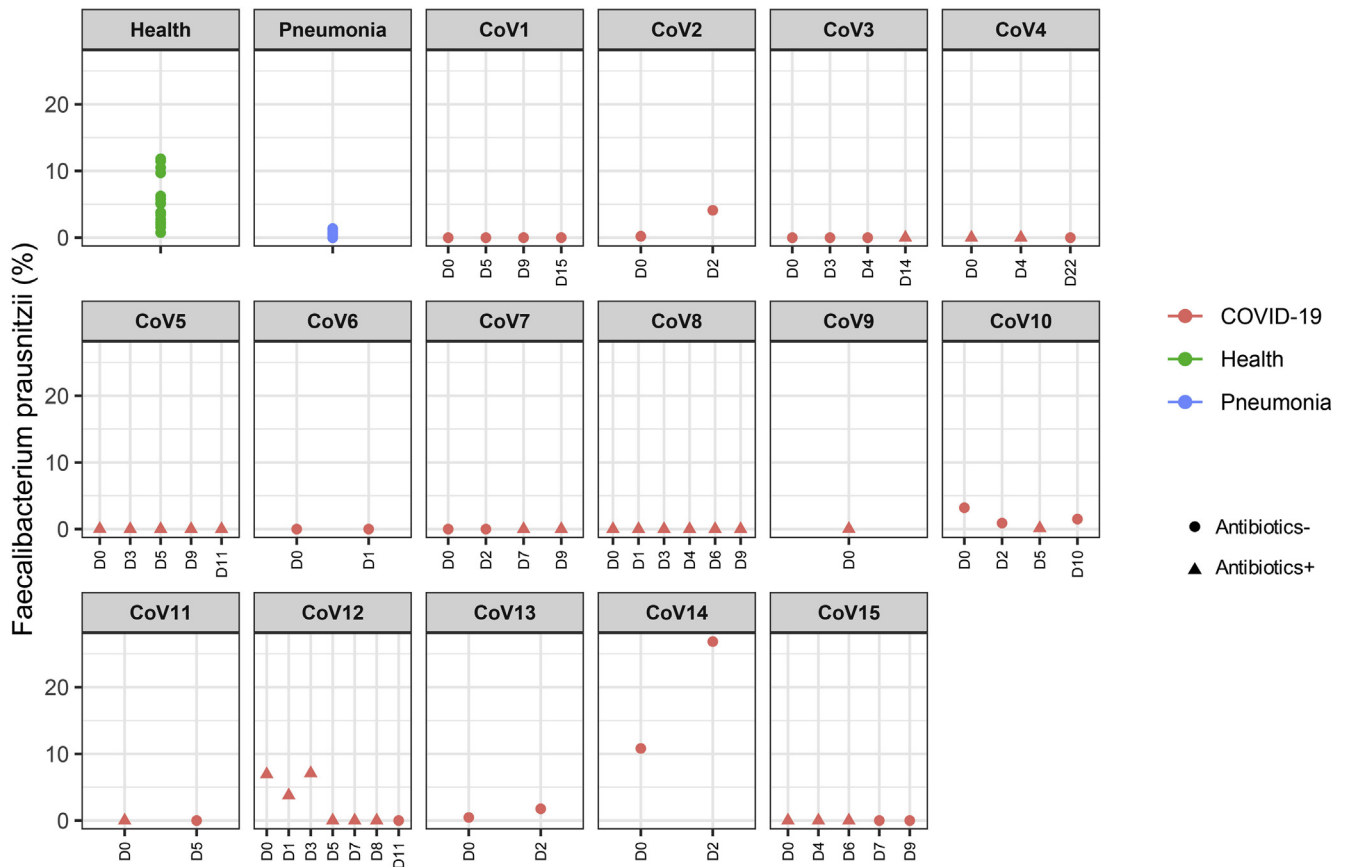
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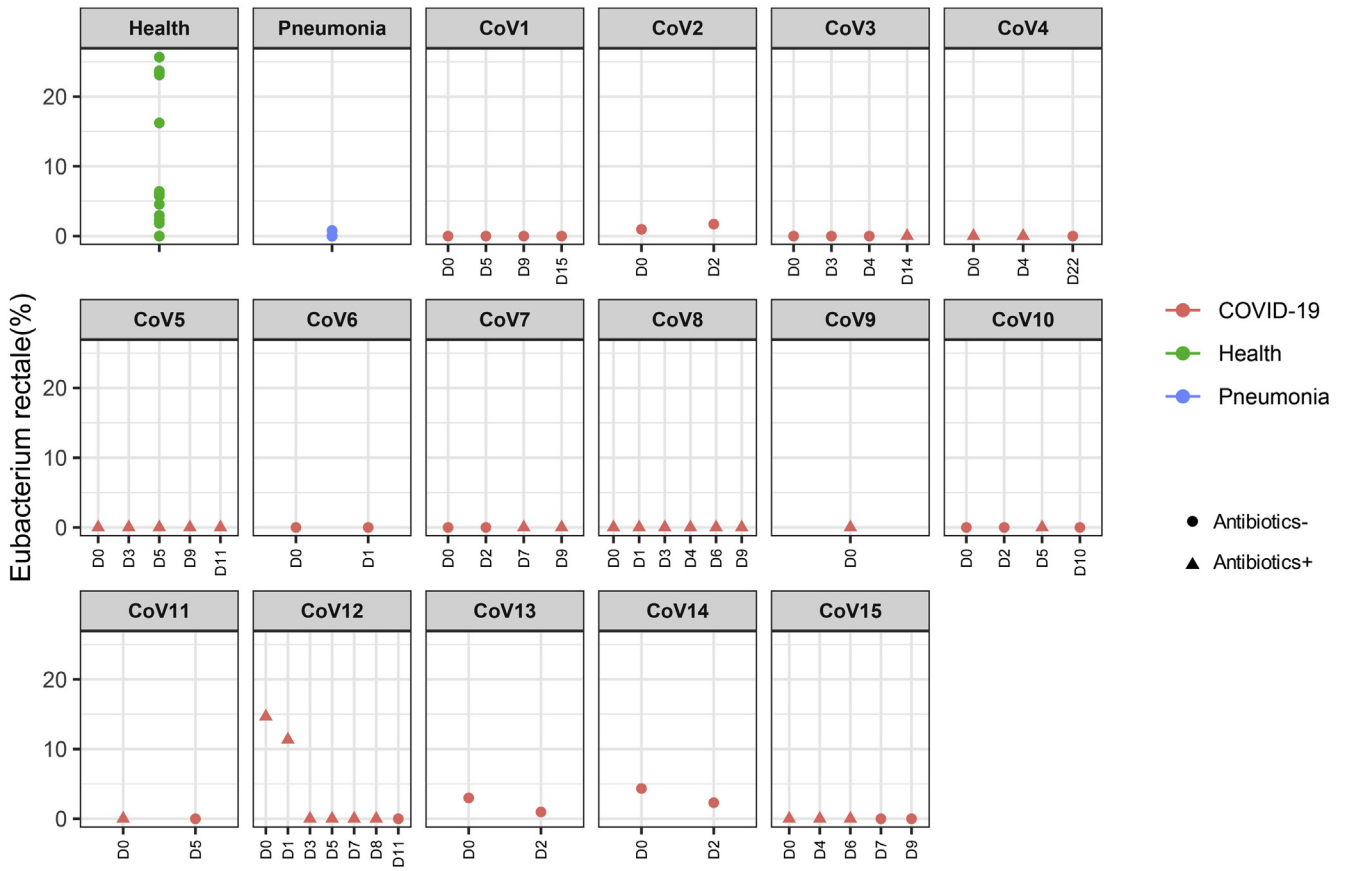
Supplementary Figure 1. Longitudinal changes of fecal abundance of *Eubacterium ventriosum* in patients with COVID-19 over the disease course. Bacterial species abundance is expressed as fractional abundance (%). “D0” denotes baseline date when the first stool was collected after hospitalization; the following timepoints starting with “D” represent days since baseline stool collection.



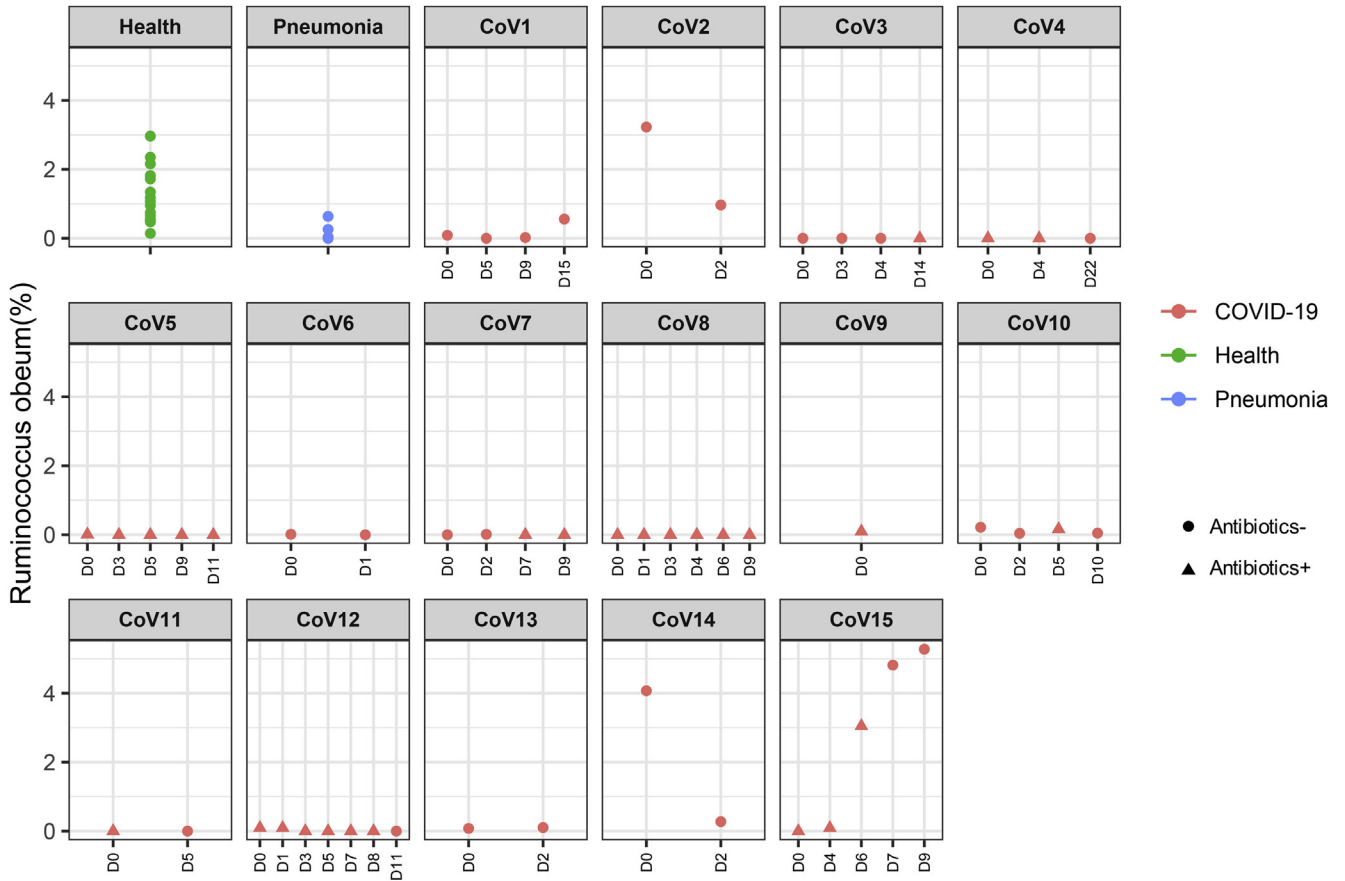
Supplementary Figure 2. Longitudinal changes of fecal abundance of *Dorea formicigenerans* in patients with COVID-19 over the disease course. Bacterial species abundance is expressed as fractional abundance (%). “D0” denotes baseline date when the first stool was collected after hospitalization; the following timepoints starting with “D” represent days since baseline stool collection.



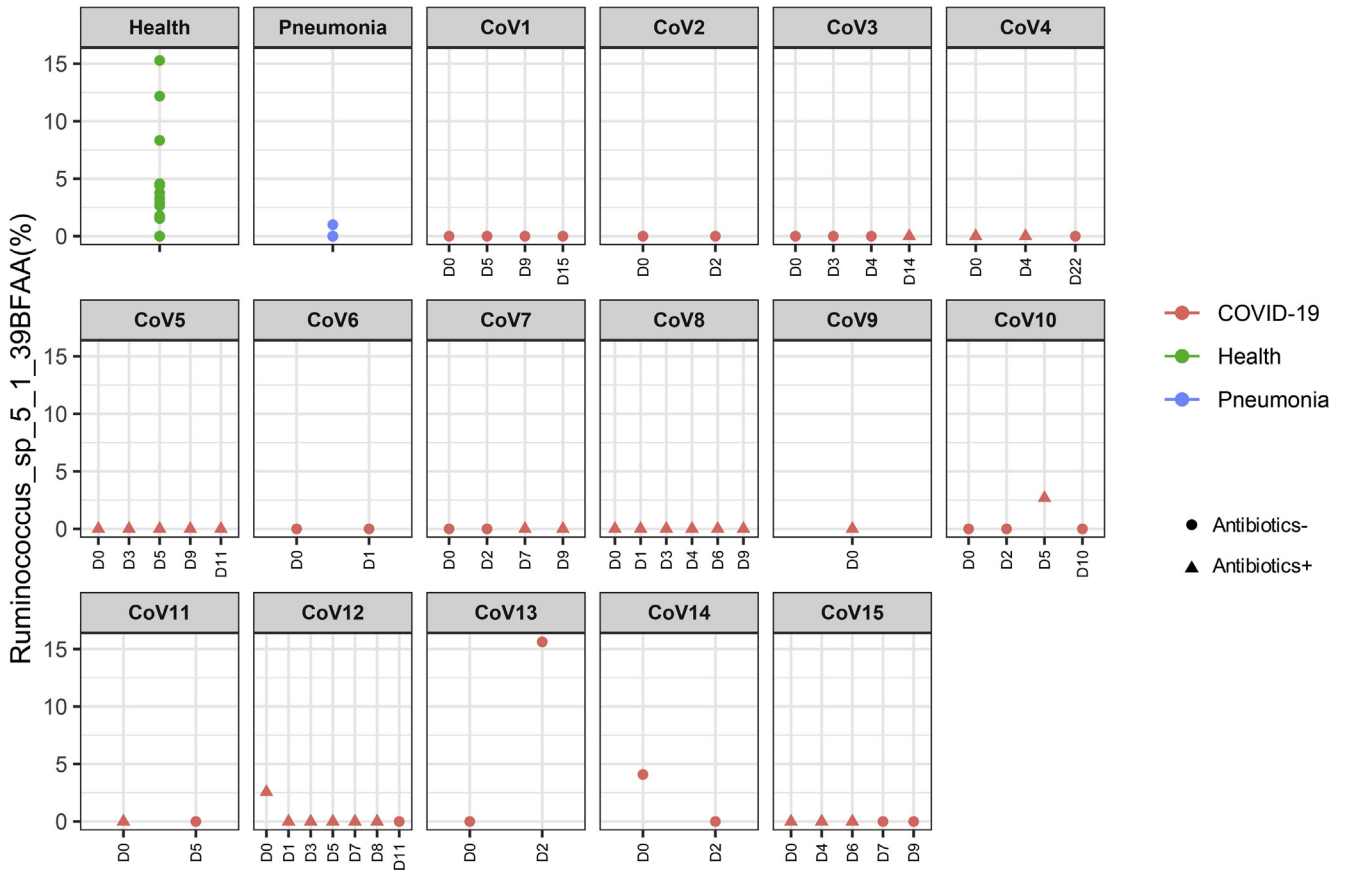
Supplementary Figure 3. Longitudinal changes of fecal abundance of *Faecalibacterium prausnitzii* in patients with COVID-19 over the disease course. Bacterial species abundance is expressed as fractional abundance (%). “D0” denotes baseline date when the first stool was collected after hospitalization; the following timepoints starting with “D” represent days since baseline stool collection.



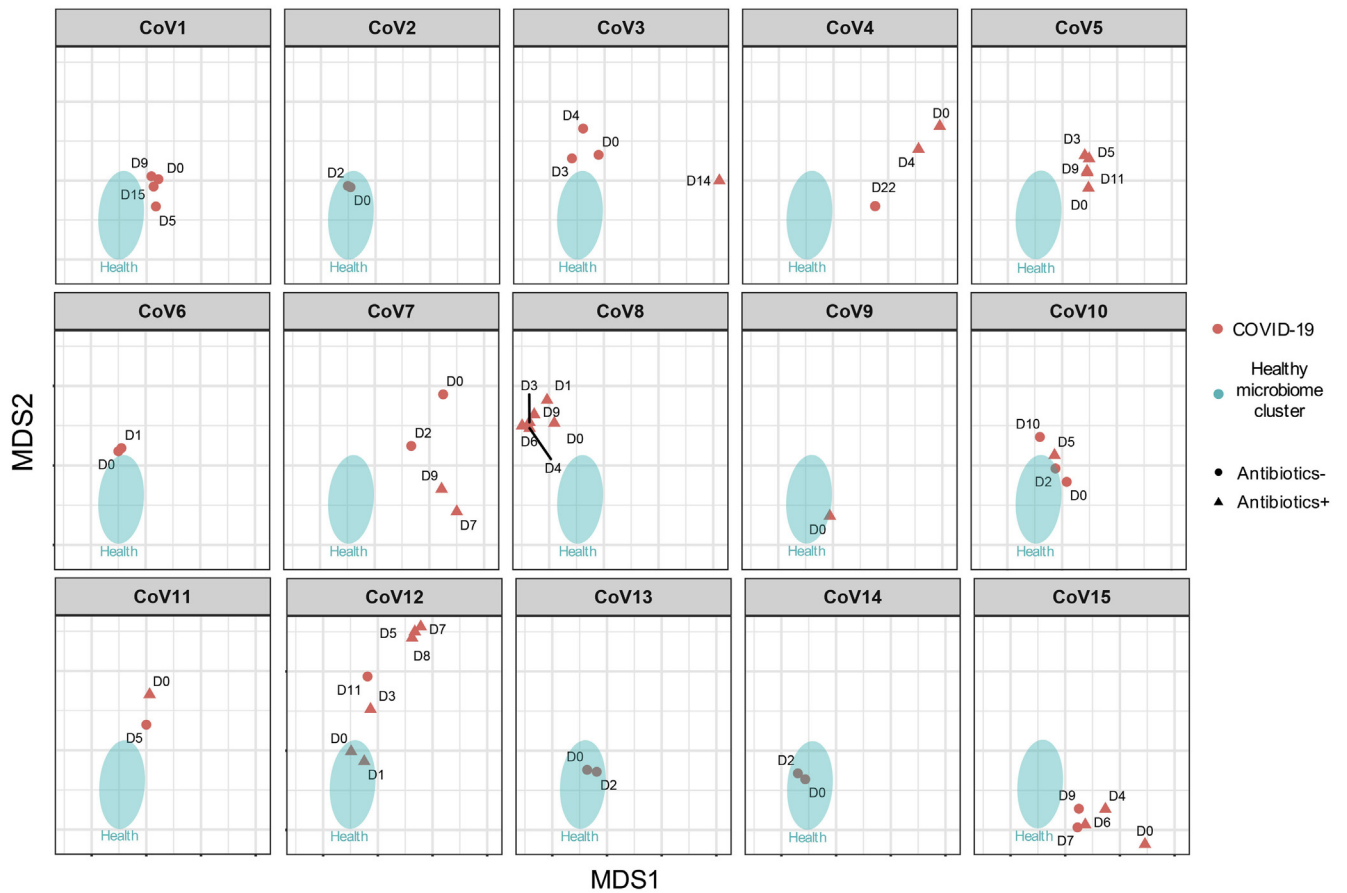
Supplementary Figure 4. Longitudinal changes of fecal abundance of *Eubacterium rectale* in patients with COVID-19 over the disease course. Bacterial species abundance is expressed as fractional abundance (%). “D0” denotes baseline date when the first stool was collected after hospitalization; the following timepoints starting with “D” represent days since baseline stool collection.



Supplementary Figure 5. Longitudinal changes of fecal abundance of *Ruminococcus obeum* in patients with COVID-19 over the disease course. Bacterial species abundance is expressed as fractional abundance (%). “D0” denotes baseline date when the first stool was collected after hospitalization; the following timepoints starting with “D” represent days since baseline stool collection.



Supplementary Figure 6. Longitudinal changes of fecal abundance of *Lachnospiraceae bacterium_5_1_63FAA* in patients with COVID-19 over the disease course. Bacterial species abundance is expressed as fractional abundance (%). “D0” denotes baseline date when the first stool was collected after hospitalization; the following timepoints starting with “D” represent days since baseline stool collection.



Supplementary Figure 7. Longitudinal changes of the fecal microbiome in patients with COVID-19, at the community level, during the disease course. “CoV” denotes patient with COVID-19. “D0” denotes baseline date when the first stool was collected after hospitalization; the following timepoints starting with “D” represent days since baseline stool collection.

Supplementary Table 1. Detailed Clinical Characteristics of Patients With COVID-19 and Patients With Pneumonia

Case	Sex	Age	Comorbidities	Recent exposure history	Symptoms at admission		Admitted to ICU	Chest radiograph findings	COVID-19 severity
					Fever and respiratory	GI			
CoV1	F	65	Hypertension, Chronic hepatitis B carrier	No	Fever, cough, sputum	Nil	Yes	Bilateral LZ haziness	Critical
CoV2	F	55	None	Contact with person with COVID-19	Fever, runny nose	Nil	No	Bilateral LZ haziness	Moderate
CoV3	M	42	None	Travel to Hubei province	Fever, cough	Nil	Yes	RLZ haziness, RLL collapse	Critical
CoV4	M	70	Hyperlipidemia, duodenal ulcer	No	Cough, shortness of breath	Nil	No	Bilateral lung haziness	Severe
CoV5	M	58	None	No	Fever, cough	Diarrhea	No	Slight RLZ haziness	Moderate
CoV6	M	71	None	No	Fever, cough, shortness of breath	Nil	No	Bilateral lung infiltration	Severe
CoV7	M	48	Diabetes mellitus, hypertension, hyperlipidemia	No	Fever, cough	Nil	No	LLZ haziness	Moderate
CoV8	F	38	None	No	Fever, cough, sputum, runny nose	Nil	No	Bilateral LZ infiltrates	Moderate
CoV9	M	33	None	Contact with person with COVID-19	Fever, cough	Nil	No	Bilateral LZ haziness	Moderate
CoV10	F	70	Obesity, hypertension	No	Cough	Nil	No	Bilateral LZ haziness	Moderate
CoV11	M	62	Diabetes, hyperlipidemia, left subclavian artery occlusion	No	Fever, cough, sputum, shortness of breath	Nil	No	Bilateral lung infiltrates	Severe
CoV12	F	71	Hypertension, renal impairment, hyperlipidemia	Contact with person with COVID-19	Cough	Nil	No	Bilateral lung infiltrates	Moderate
CoV13	F	47	None	Contact with person with COVID-19	Cough	Nil	No	Bilateral lung infiltrates	Moderate
CoV14	F	22	None	Contact with person with COVID-19	Fever, runny nose	Nil	No	Bilateral lung infiltrates	Moderate
CoV15	F	46	None	Contact with person with COVID-19	Cough, shortness of breath	Nil	No	Clear	Mild
P1	F	69	Hypertension, diabetes mellitus, Tricuspid regurgitation	No	Fever	Nil	No	LMZ pneumonia	N/A
P2	M	43	Nonalcoholic Fatty liver disease	No	Cough	Nil	No	Right sided infiltrates	N/A
P3	F	92	Diabetes, Hypertension, pulmonary fibrosis, Paroxysmal atrial fibrillation, Acute coronary syndrome	No	Cough, sputum, shortness of breath	Nil	No	Bilateral lung infiltrate	N/A
P4	M	47	Diabetes mellitus	No	Fever, sputum	Nil	No	Left effusion, LMZ infiltrates	N/A
P5	M	36	Ischemic priapism	No	Fever, cough, sputum, shortness of breath	Diarrhea	No	N/A	N/A
P6	M	52	Epilepsy, Hepatitis	No	Fever, cough, runny nose, shortness of breath	Diarrhea	No	N/A	N/A

LLZ, left lower zone; LMZ, left middle zone; N/A, not applicable.