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Gut microbiota dynamics in a prospective cohort of patients with post-acute COVID-19 syndrome

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ABSTRACT

Background Long-term complications after COVID-19 are common, but the potential cause for persistent symptoms after viral clearance remains unclear.

Objective To investigate whether gut microbiome composition is linked to post-acute COVID-19 syndrome (PACS), defined as at least one persistent symptom 4 weeks after clearance of the SARS-CoV-2 virus.

Methods We conducted a prospective study of 106 patients with a spectrum of COVID-19 severity followed up from admission to 6 months and 68 non-COVID-19 controls. We analysed serial faecal microbiome of 258 samples using shotgun metagenomic sequencing, and correlated the results with persistent symptoms at 6 months.

Results At 6 months, 76% of patients had PACS and the most common symptoms were fatigue, poor memory and hair loss. Gut microbiota composition at admission was associated with occurrence of PACS. Patients without PACS showed recovered gut microbiome profile at 6 months comparable to that of non-COVID-19 controls. Gut microbiome of patients with PACS were characterised by higher levels of *Ruminococcus gnavus*, *Bacteroides vulgatus* and lower levels of *Faecalibacterium prausnitzii*. Persistent respiratory symptoms were correlated with opportunistic gut pathogens, and neuropsychiatric symptoms and fatigue were correlated with nosocomial gut pathogens, including *Clostridium innocuum* and *Actinomyces naeslundii* (all p<0.05). Butyrate-producing bacteria, including *Bifidobacterium pseudocatenulatum* and *Faecalibacterium prausnitzii* showed the largest inverse correlations with PACS at 6 months.

Conclusion These findings provided observational evidence of compositional alterations of gut microbiome in patients with long-term complications of COVID-19. Further studies should investigate whether microbiota modulation can facilitate timely recovery from post-acute COVID-19 syndrome.

Significance of this study

What is already known on this subject?
► Persistent symptoms after the convalescence period in patients with COVID-19 have been reported.
► Emerging evidence supports the potential role of gut dysbiosis in the severity of COVID-19 infection.
► The long-term impact of SARS-CoV-2 on the gut microbiome after the acute infection and whether gut microbiota composition affects risk of persistent symptoms in recovered patients with different severity of COVID-19 infection is unknown.

What are the new findings?
► Our results show that an individual's gut microbiome profile may affect their susceptibility to long-term complications of COVID-19.
► Gut microbiome pattern reflects different symptoms in patients with post-acute COVID-19 syndrome (PACS).
► Distinct gut microbiota composition at admission is associated with occurrence of PACS at 6 months.

How might it impact on clinical practice in the foreseeable future?
► These findings provide new insights into the intricate association between the gut microbiome and the long-term sequelae after COVID-19 infection.
► Microbiome-based profiling might be used as a tool in early risk stratification for occurrence of post-acute COVID-19 syndrome.

recognised.[1–3] Up to three-quarters of patients describe at least one symptom at 6 months after recovery, and multisystem symptoms, including fatigue, muscle weakness and sleep difficulties, are commonly reported.[4]

Reason underlying the development of PACS is largely unclear. Perturbations of immune and inflammatory responses, cellular damage by acute viral infection or sequelae of post critical illness may contribute to long-term symptoms after

INTRODUCTION

Clinical characteristics of COVID-19 during the acute infection are well described, but little is known of long-term complications of COVID-19. Post-acute COVID-19 syndrome (PACS), characterised by long-term complications and/or persistent symptoms after the onset of COVID-19, is increasingly

COVID-19 infection.[5] Increasing evidence has shown that gut dysbiosis is linked to the severity of COVID-19 infection and persists after disease resolution.[6] Several studies have shown substantial involvement of the GI tract in COVID-19, including enhanced expression of ACE2 in the GI tract[7] and gut microbiota perturbations in subjects infected with SARS-CoV-2.[8] Patients with COVID-19 had significant alterations in faecal microbiomes compared with non-COVID-19 controls, characterised by enrichment of opportunistic pathogens and depletion of beneficial commensals.[8 9] Several gut commensals with known immunomodulatory potential such as *Faecalibacterium prausnitzii*, *Eubacterium rectale* and bifidobacteria were under-represented in patients, and these bacteria remained low in samples collected up to 1 month after disease resolution.[10]

As the GI tract is the largest immunological organ in the body, aberrant immune response to COVID-19 infection induced by resident microorganisms may affect the recovery process. Emerging evidence supports the potential role of gut dysbiosis in the severity of COVID-19 infection.[6] We herein investigated alterations of gut microbiota composition and its association with persistent symptoms following acute COVID-19.

MATERIALS AND METHODS
Study population
This prospective cohort study was performed at three regional hospitals (Prince of Wales Hospital, United Christian Hospital and Yan Chai Hospital) in Hong Kong, China. All patients with a confirmed diagnosis of COVID-19, as shown by a positive reverse transcriptase-polymerase chain reaction (RT-PCR) test for SARS-CoV-2 in nasopharyngeal swab, nasal swab, deep throat saliva, sputum or tracheal aspirate, were recruited between 1 February and 31 August 2020. All patients with confirmed COVID-19 were required to be admitted to hospital according to local government policy. Patients could be discharged if they fitted one of the following criteria: two clinical specimens of the same type (ie, respiratory or stool) tested negative for nucleic acid of SARS-CoV-2 by RT-PCR taken at least 24 hours apart or tested positive for SARS-CoV-2 antibody. Patients received the same antibiotics (amoxicillin clavulanate) during hospitalisation. Patients were excluded if they were unable to be contacted, declined to participate in study or died before the follow-up visit. Demographics, clinical and laboratory data were extracted from electronic medical records in the clinical management system of the Hong Kong Hospital Authority. Severity of COVID-19 infection was categorised 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 ≤93% when breathing ambient air, or arterial oxygen pressure/fractional inspired oxygen ≤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.[11]

PACS was defined as at least one persistent symptom which could not be explained by an alternative diagnosis 4 weeks after clearance of SARS-CoV-2. We assessed the presence of the 30 most commonly reported symptoms post-COVID[12 13] at 3 and 6 months after illness onset (online supplemental table 1). Six-minute walk distance test, which is a simple functional assessment of aerobic capacity and endurance, was performed 6 months after discharge in a subset of patients who had recovered from COVID-19. The data were correlated with the gut microbiota analysis.

Given that diet is known to affect the gut microbiome, we documented dietary records for all patients with COVID-19 during the time of hospitalisation. Standardised meals were provided by the hospital catering service of each hospital, and the dietary component and pattern were consistent with the habitual diet commonly consumed by Hong Kong Chinese (online supplemental table 2). After discharge, patients with COVID-19 were advised to continue a diverse and standard Chinese diet that was consistent with habitual daily diets consumed by Hong Kong Chinese.

Controls were recruited before the COVID-19 pandemic (between September 2019 and November 2019) from the community through advertisement and from the endoscopy centre at the Prince of Wales Hospital in subjects who had a normal colonoscopy (stools collected before bowel preparation) with the same collection protocol. We selected controls matched for age and sex with similar comorbidities and standard dietary patterns for comparison of gut microbiota composition between subjects with and without COVID-19 infection. Demographics and comorbidities of controls are listed in online supplemental table 3. The exclusion criteria for non-COVID-19 controls were (1) the use of antibiotics in the past 6 months; (2) the use of laxatives or antidiarrhoeal drugs in the past 3 months; (3) recent dietary changes (eg, becoming vegetarian/vegan); (4) known complex infections or sepsis; (5) known history of severe organ failure (including decompensated cirrhosis, malignant disease, kidney failure, epilepsy, active serious infection, AIDS); (6) bowel surgery in the past 6 months (excluding colonoscopy/procedure related to perianal disease); (7) presence of an ileostomy/stoma; and (8) current pregnancy.

In total, 258 stool samples were collected and sequenced. All samples from patients with COVID-19 and controls were processed and analysed simultaneously.

The study was conducted in accordance with the declaration of Helsinki. All patients provided written informed consent. The study was approved by the Joint Chinese University of Hong Kong – New Territories East Cluster Clinical Research Ethics Committee (CREC Reference no.: 2020.076).

Stool samples
Stool samples were collected serially at admission, at 1 month and 6 months after discharge from hospital. Stool samples from in-hospital patients were collected by hospital staff while discharged patients provided stools on the day of follow-up at 1 month and 6 months after discharge or self-sampled at home and had samples couriered to the hospital within 24 hours of collection. Baseline (stools collected at admission) samples were collected before antibiotic treatment. All samples were collected in tubes containing preservative media (cat. 63700, Norgen Biotek Corp, Ontario Canada) and stored immediately at −80°C until processing. We have previously shown that data of gut microbiota composition generated from stools collected using this preservative medium is comparable to data obtained from samples that are immediately stored at −80°C.[14]

Stool DNA extraction and sequencing
Detailed methods are described in Zuo *et al*.[8] The faecal pellet was added to 1 mL of cetyltrimethylammonium bromide buffer and vortexed for 30 s, then the sample was heated at 95°C for 5 min. After that, the samples were vortexed thoroughly with beads at maximum speed for 15 min. 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 min. The supernatant was then

obtained by centrifuging at $13\,000g$ for $5\,\text{min}$ and was put 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, California, USA). Libraries were subsequently sequenced on our in-house sequencer Illumina NextSeq 550 (150 base pairs paired-end) at the Centre for Microbiota Research, the Chinese University of Hong Kong. Raw sequence data generated for this study are available in the Sequence Read Archive under BioProject accession: PRJNA714459.

Bioinformatics

Raw sequence data were quality filtered using Trimmomatic V.39 to remove adaptor, low-quality sequences (quality score <20), reads shorter than 50 base pairs. Contaminating human reads were filtered using Kneaddata (V.0.7.2 https://bitbucket.org/biobakery/kneaddata/wiki/Home, Reference database: GRCh38 p12) with default parameters. Following this, microbiota composition profiles were inferred from quality-filtered forward reads using MetaPhlAn3 version 3.0.5. GNU parallel[15] was used for parallel analysis jobs to accelerate data processing. Alpha diversity metrics (Shannon diversity, Chao1 richness) were calculated using the phyloseq package, version 1.26.0. Species whose average abundance and prevalence was less than 0.1% and 3%, respectively, were filtered out.

Statistical analysis and inferring gut microbiota composition

Continuous variables were expressed as median (IQR), whereas categorical variables were presented as number (percentage). Qualitative and quantitative differences between subgroups were analysed using X^2 or Fisher's exact tests for categorical parameters and Mann-Whitney test for continuous parameters, as appropriate. Odds ratio and adjusted odds ratio (aOR) with 95% CI were estimated using logistic regression to examine clinical parameters associated with development of PACS. The site by species counts and relative abundance tables were input into R V.3.5.1 for statistical analysis. Here we applied micropower,[16] a simulation-based method for permutational multivariate analysis of variance (PERMANOVA)-based β-diversity comparisons, to assess the effect size and statistical power of this study with 68 patients with or without PACS. This sample size provided 80% power to detect an effect size (corrected coefficient of determination ω2) of 0.015% and 90% power to detect a difference at ω2=0.006. Principal coordinates analysis (PCoA) was used to visualise the clustering of samples based on their species-level compositional profiles. Associations between gut community composition and patients' parameters were assessed using PERMANOVA. Associations of specific microbial species with patient parameters were identified using the linear discriminant analysis effect size and the multivariate analysis by linear models (MaAsLin2) statistical frameworks implemented in the Huttenhower Lab Galaxy instance (http://huttenhower.sph.harvard.edu/galaxy/). PCoA, PERMANOVA and Procrustes analyses are implemented in the vegan R package V.2.5–7.

RESULTS

Post-acute COVID-19 syndrome

Between 1 February and 31 August 2020, we recruited 106 patients from three regional hospitals in Hong Kong and followed them up for 6 months. Median age was 48.3 years (IQR 33–62 years) and 56 (52.9%) were female (table 1). Among the patients, hypertension (17%) was the most common comorbidity followed

Table 1 Clinical characteristics of the 106 patients who recovered from COVID-19

Female, n (%)	56 (52.8)
Age, years (IQR)	48.3 (33–62)
Non-smokers, n (%)	61 (57.5)
Presence of any comorbidities, n (%)	45 (42.5)
Types of comorbidities	
Hypertension	18 (17.0)
Diabetes mellitus	16 (15.1)
Hyperlipidaemia	12 (11.3)
Length of stay in hospital, days (IQR)	17 (10–25)
Severity of COVID-19, n (%)	
Asymptomatic	4 (3.8)
Mild	31 (29.2)
Moderate	55 (51.9)
Severe	10 (9.4)
Critical	6 (5.7)
Treatments for COVID-19, n (%)	
Lopinavir/ritonavir	56 (52.8)
Ribavirin	30 (28.3)
Interferon	39 (36.8)
Remdesivir	6 (5.7)
Antibiotics	25 (23.6)
Number of persistent symptoms by 3 months, n (%)	86 (81.1)
1	21 (19.8)
2–3	26 (24.5)
>3	39 (36.8)
Number of persistent symptoms by 6 months, n (%)	81 (76.4)
1	17 (16.0)
2–3	29 (27.4)
>3	35 (33.0)

by type 2 diabetes mellitus (15.1%). Most patients had mild to moderate severity of COVID-19 (81.1%) during hospitalisation and 25 patients (23.6%) received antibiotics during hospitalisation. Overall, PACS was reported in 86 (81.1%) and 81 (76.4%) patients with COVID-19 at 3 months and 6 months, respectively. The most common symptoms at 6 months were fatigue (31.3%), poor memory (28.3%), hair loss (21.7%), anxiety (20.8%) and difficulty in sleeping (20.8%; figure 1A). There were no significant differences in age, gender, comorbidities, use of antibiotics, use of anti-viral drugs and severity of COVID-19 in patients with or without PACS at 6 months (figure 1B, online supplemental tables 4 and 5, online supplemental figure 1).

Altered gut microbiota composition in patients with COVID-19 persisted for 6 months after disease resolution

Among 106 patients with COVID-19, 68 had stool samples collected at admission (n=47), 1 month (n=64) and 6 months' (n=68) follow-up for gut microbiota analysis. Stool samples were also collected from 11 patients at 9 months' follow-up (online supplemental table 6). We first examined gut microbiome composition of patients with COVID-19 at follow-up without (figure 2) or with (online supplemental figure 2) antibiotic use during hospitalisation. Gut microbiota composition of antibiotic-naive patients at 6 months remained distinct (figure 2A), and bacteria diversity (Shannon Index) and Chao1 richness at 6 months were significantly lower than in non-COVID-19 controls (figure 2B,C). In antibiotic-naive patients, gut microbiome composition at 1 month and 6 months showed
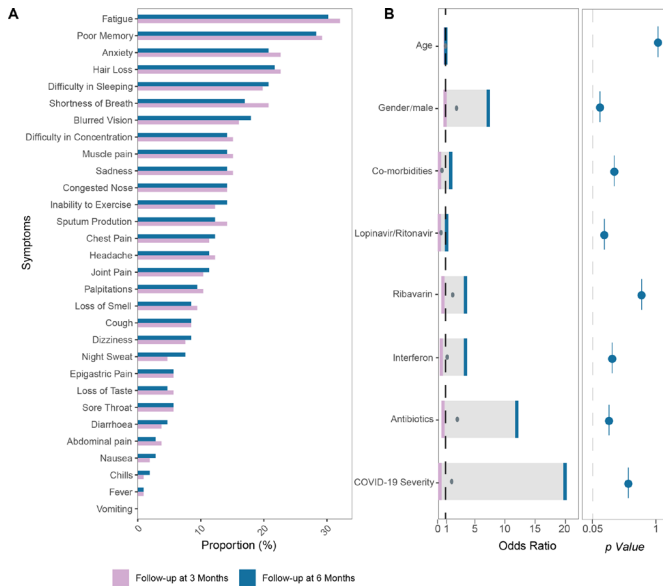
Figure 1 Post-acute COVID-19 syndrome (PACS) after virus clearance. (A) The proportion of 30 symptoms in 106 patients at 3 months and 6 months after acute COVID-19; (B) Multivariable analysis on factors associated with development of PACS. The centre dot denotes the mean value, the boxes denote the upper and lower interquartile ranges.

almost identical cluster centroids (p=0.555, figure 2A) but was distinct from samples of non-COVID-19 controls (figure 2A, online supplemental table 7). In addition, gut microbiota
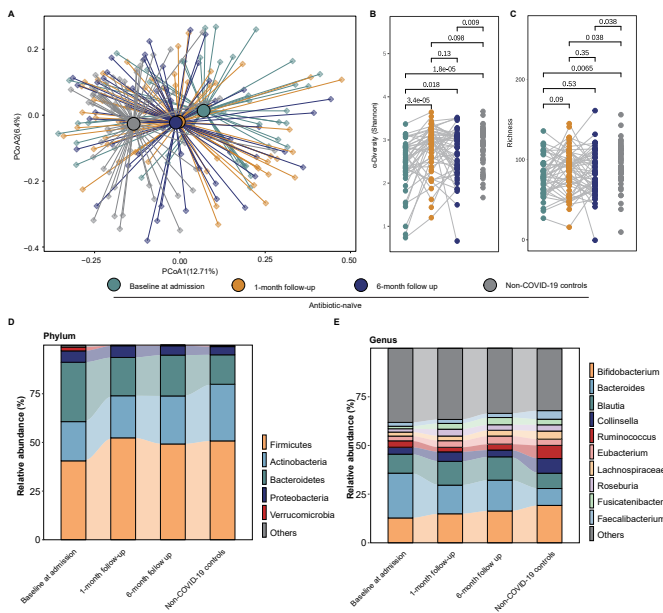
Figure 2 Compositional differences in gut microbiota of in-hospital patients (antibiotic-naïve) and their follow-up stools after negative SARS-CoV-2, and non-COVID-19 individuals. (A) Principal coordinates analysis (PCoA) of gut microbiota composition of patients with COVID-19 before and after negative SARS-CoV-2 compared with non-COVID-19 subjects. (B) Diversity and richness (C) Analysis of gut microbiota in patients with COVID-19 at 1 month and 6 months after virus clearance. (D) Average relative abundance of top five phyla and top 10 microbial genera (E) detected in stools from in-hospital patient and their follow-up within 1 month and longer than 6 months after negative SARS-CoV-2.

composition was stable in samples analysed at 1 month, 6 months and 9 months after disease resolution (online supplemental figure 3, online supplemental table 8). We observed dysbiosis and heterogeneity in gut microbiota profile defined by the top five phyla (figure 2D) and top 10 most prevalent genera in antibiotic-naive patients with COVID-19 at 1 and 6 months compared with non-COVID-19 controls (figure 2E). The relative abundance of members of *Ruminococcus* and *Bifidobacterium* were significantly lower in patients with COVID-19 than in non-COVID-19 controls (mean 3.02% vs 6.75%, and mean 16.36% vs 19.22%, respectively, p<0.001, Mann-Whitney test; figure 2E).

When the effect of antibiotics was examined at 6 months' follow-up, overall gut microbiota composition was similar between antibiotic-naïve and antibiotic-treated patients (p=0.351, (online supplemental figure 2D). At baseline, there were no significant differences in microbiome composition between antibiotic-naïve and antibiotic-treated patients (online supplemental figure 2B), whereas the overall gut microbiota composition was distinct at 1 month follow-up (online supplemental figure 2C). Patients were treated with antibiotics for 4 to 12 days, with a mean of 7.89 (IQR 6–11) days based on the discretion of the physician. We found no significant correlation between gut microbiota composition and duration of antibiotic treatment (online supplemental figure 2E,F). These data suggest that the effect of antibiotics on the gut microbiome in patients with COVID-19 did not persist beyond 6 months.

Patients with PACS have distinct gut microbiome dysbiosis
Among 68 patients with COVID-19 who had stool samples analysed at 6 months, 50 (73.5%) had PACS (online supplemental figure 4). We tested viral load and found no significant correlations between viral load (both in stool and respiratory samples) and PACS development (online supplemental figure 5). We found two distinct clusters of gut microbiome in patients with and without PACS at 6 months' follow-up (figures 3A and 4A, p<0.05). Patients without PACS showed recovered gut microbiome comparable to that of non-COVID-19 controls (figures 3A and 4A, p=0.470), whereas gut microbiome composition of patients with PACS remained distinct from that of
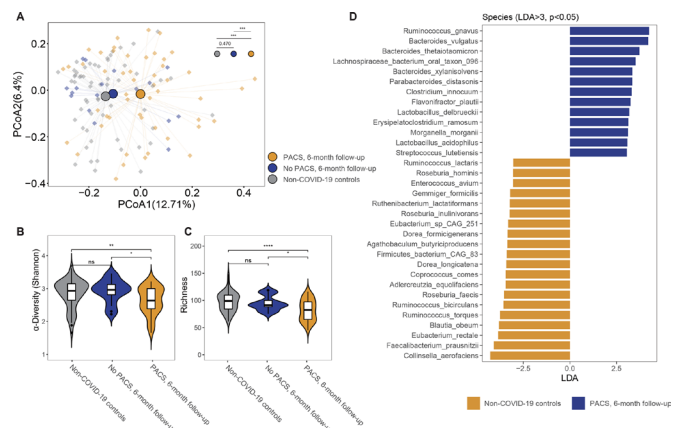

Figure 3 Gut microbiota composition in patients with COVID-19 with and without post-acute COVID-19 syndrome (PACS) at 6 months; (A) Principal coordinates analysis (PCoA) of gut microbiota composition of patients with COVID-19 with and without PACS at 6 months. (B) Bacteria diversity and richness. (C) Analysis of gut microbiota composition of patients with and without PACS. (C) Linear discriminant analysis effect size analysis of discriminant taxa in gut microbiome of patients with PACS at 6 months. LDA, linear discriminant analysis.
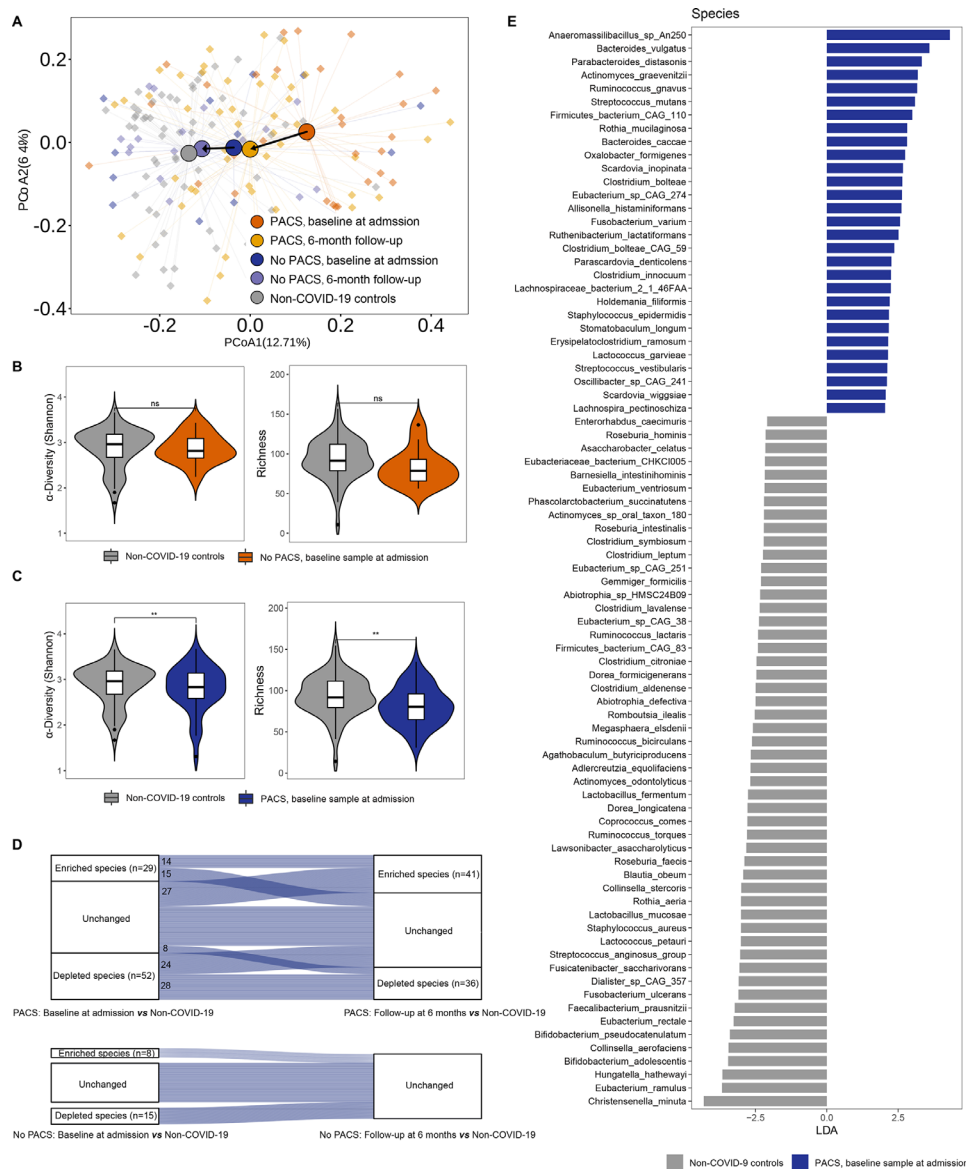
Figure 4 Compositional differences in gut microbiota of baseline and follow-up samples at different time points after virus clearance. (A) Principal coordinates analysis (PCoA) of gut microbiota composition of patients with COVID-19 with or without post-acute COVID-19 syndrome (PACS) before and after negative reverse transcriptase-quantitative polymerase chain reaction for SARS-CoV-2 compared with non-COVID-19 subjects. (B) Diversity and richness analysis (as measured in Simpson diversity and Chao1 richness index, respectively) of gut microbiota in patients with COVID-19 at baseline compared with non-COVID-19 subjects. (C) Diversity and richness analysis of gut microbiota in patients with COVID-19 at 6 months' follow-up compared with non-COVID-19 subjects. (D) Change of gut microbial composition from baseline to 6 months' follow-up after virus clearance in patients with COVID-19 with or without PACS. (E) Linear discriminant analysis effect size in gut microbiome of recovered patients with PACS at baseline.

non-COVID-19 controls at 6 months (figure 3A, p<0.001). At 6 months' follow-up, bacteria diversity and richness (as measured by Shannon index diversity and Chao1 richness index, respectively) in patients with PACS were significantly lower than in those without PACS and controls (figure 3B,C). In addition, bacteria Shannon diversity and richness of gut microbiome were significantly lower at admission in patients who developed PACS than non-COVID-19 controls (figure 4C), but there was no difference in Shannon diversity and richness between patients without PACS and controls (figure 4B). Among gut bacteria species detected in patients with PACS, 28 bacteria species were diminished and 14 were enriched at both baseline and follow-up samples (figure 4D,E, online supplemental tables 9 and 10). At 6 months, patients with PACS showed a significantly lower level

of *Collinsella aerofaciens*, *F. prausnitzii*, *Blautia obeum* and a higher level of *Ruminococcus gnavus* and *Bacteroides vulgatus* than non-COVID-19 controls (p<0.05, LefSe >2; figure 3D, online supplemental table 10). Subjects without PACS showed only 25 alterations of bacteria species at admission, which recovered completely by 6 months (figure 4D, online supplemental table 11). Patients with PACS are reported to display a spectrum of multiple persistent biochemical pathophysiology. We investigated functionality alterations of the gut bacteriome using HUMAnN3. We found that the relative abundance of 32 MetaCyc pathways were significantly different between patients with PACS and non-COVID-19 controls through MaAslin2 analysis (p<0.05; online supplemental table 12). The gut microbiome in patients with PACS exhibited increased abundance of
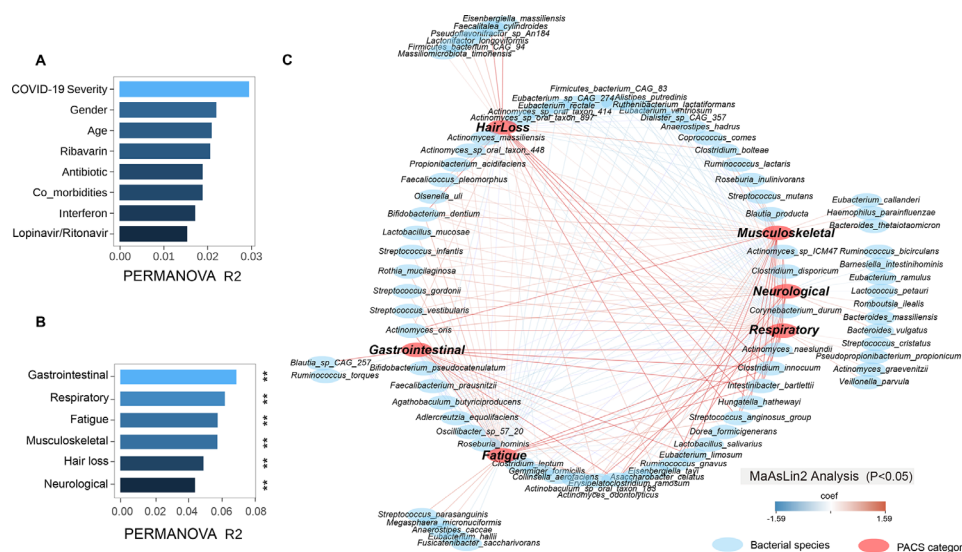
Figure 5 Factors affecting the gut microbiome in follow-up stools from patients after clearing the virus. (A) Effect size of subject metadata on gut microbiome composition determined by permutational multivariate analysis of variance (PERMANOVA) test. (B) Overall associations between gut microbiome composition with different subgroups of post-acute COVID-19 syndrome (PACS) determined by PERMANOVA test. (C) Associations of bacteria species with different categories of PACS at 6 months. MaAsLin, multivariate association with linear model.

urea cycle pathway, L-citrulline biosynthesis pathway and L-ornithine biosynthesis II pathway (online supplemental figure 6, online supplemental table 12).

Gut microbiome composition reflects different symptoms in patients with PACS

We next examined the composition of the gut microbiome and its association with different symptoms at 6 months. Based on PERMANOVA, gut composition was not associated with medical treatment during hospitalisation or disease severity (figure 5A, online supplemental table 13). We categorised symptoms of PACS into respiratory (cough, sputum, nasal congestion/runny nose, shortness of breath), neuropsychiatric (headache, dizziness, loss of taste, loss of smell, anxiety, difficulty in concentration, difficulty in sleeping, sadness, poor memory, blurred vision), gastrointestinal (nausea, diarrhoea, abdominal pain, epigastric pain), dermatological (hair loss), musculoskeletal (joint pain, muscle pain) and fatigue. We tested associations of single bacteria taxa with different categories of symptoms using multivariate association with linear model (MaAsLin2). Different symptomatology was associated with different gut microbiome patterns (figure 5B, online supplemental table 14). A total of 81 bacterial species were associated with different categories of PACS and many of the bacteria species were associated with more than two categories of persistent symptoms (figure 5C, online supplemental table 15). At 6 months, gut microbiome composition in patients with persistent respiratory symptoms was positively correlated with a number of opportunistic pathogens including *Streptococcus anginosus, Streptococcus vestibularis, Streptococcus gordonii* and *Clostridium disporicum*, whereas abundance of nosocomial pathogens linked to opportunistic infections including *Clostridium innocuum* and *Actinomyces naeslundii*, were correlated with neuropsychiatric symptoms and fatigue. Butyrate-producing species such as *Roseburia inulinivorans* and *Faecalibacterium prausnitzii* were significantly depleted in patients who had persistent hair loss at 6 months (n=23), compared with non-COVID-19 controls (p<0.05, online supplemental table 16). Furthermore, relative abundance of multiple bacterial species known to be beneficial to

host immunity including *Bifidobacterium pseudocatenulatum, F. prausnitzii, R. inulinivorans* and *Roseburia hominis* showed the largest inverse correlations with PACS at 6 months (figure 5C, online supplemental tables 15 and 16.

The 6 min walk test is frequently used to determine functional capacity in patients.[4] Previous work has shown that post-convalescence patients with lower microbiota richness had impaired lung function,[6] but the cause of reduced 6 min walk distance after COVID-19 remains unknown. Of the 68 patients with COVID-19 who had provided stool samples at 6 month follow-up, 52 of them had a 6 min walk test assessment at 6 months. The median walking distance at 6 min in patients with PACS was significantly lower than those without PACS (mean 382 m vs 464 m, p<0.001, online supplemental figure 7A). We observed significant inverse associations of walking distance with pathogenic bacteria species (for example, *Clostridium innocuum, Clostridium bolteae*) which might confer pathogenicity or were associated with disease risk in different populations[17 18] (p<0.05, online supplemental figure 7B). The walking distance was positively correlated with several short-chain fatty acids and butyrate producers of the gut microbiome including *Bifidobacterium pseudocatenulatum,*[19] *Roseburia inulinivorans*[20] and *Bacteroides ovatus,*[21] implying beneficial symbiosis between human and gut–lung–microbiome axis after clearing the virus.

Gut microbiota composition at admission predicts PACS

We determined whether gut microbiome profile at admission affects development of PACS. We observed two significantly distinct bacteria clusters in patients with and without PACS by their admission samples (figure 6A, p<0.05). Compared with patients with PACS, baseline gut bacteria composition of those without PACS was enriched in 19 bacteria species and characterised by the genera including *Bifidobacterium, Blautia* and *Bacteroides* (figure 6B,C, p<0.05, linear discriminant analysis effect size >2, online supplemental table 17). At admission, Shannon diversity and richness of gut bacteria in patients who developed PACS were significantly lower than in non-COVID-19 controls (figure 4C). A total of 13 bacteria species including *Blautia wexlerae* and *Bifidobacterium longum* at admission negatively
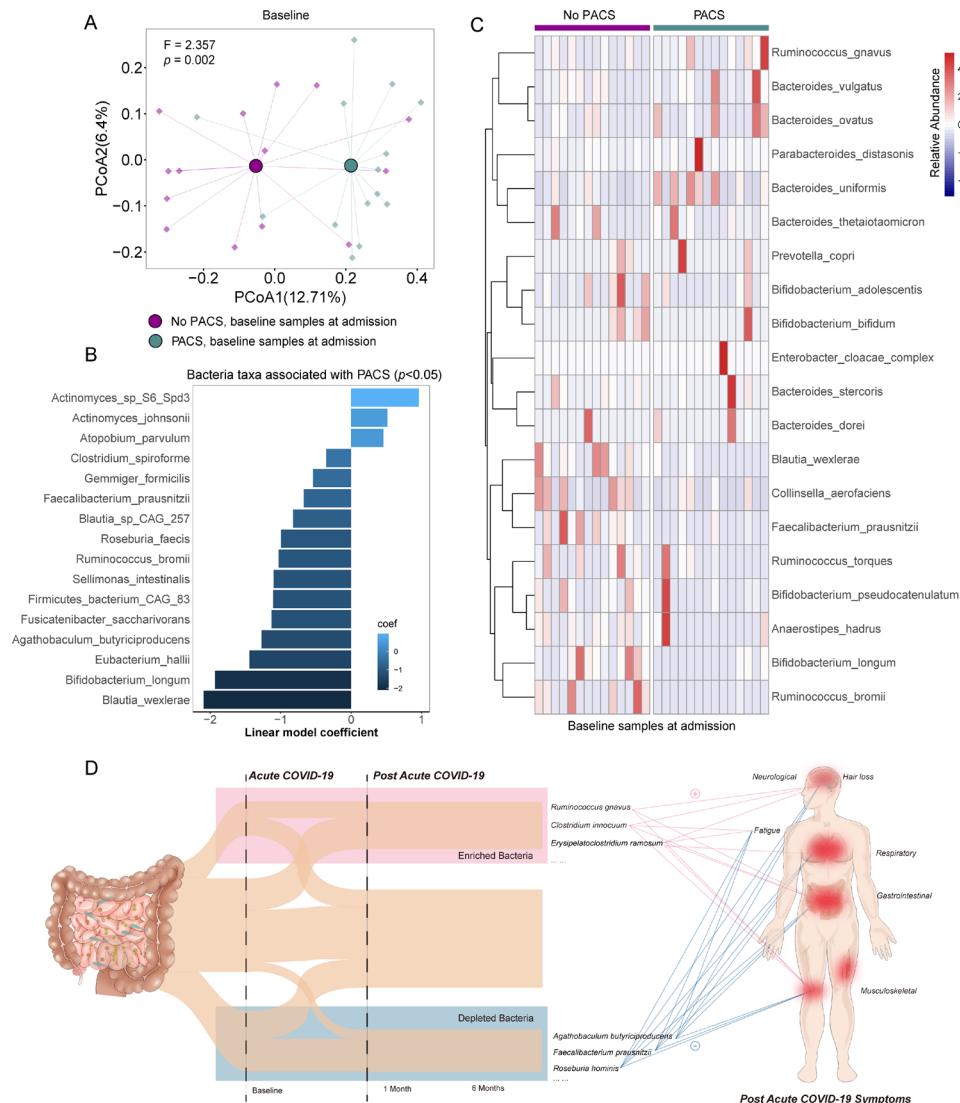
Figure 6 Gut microbiota composition at admission of patients with COVID-19 who had or had not any persistent symptoms at 6 months. (A) Principal coordinates analysis (PCoA) of gut microbiota composition of patients with COVID-19 who had or had not any persistent symptoms at month 6 after clearing SARS-CoV-2. (B) Gut microbiota composition of first stool samples after confirmed positive reverse transcriptase-quantitative polymerase chain reaction for SARS-CoV-2 during hospitalisation. (C) Associations between persistent symptoms in recovered patients with COVID-19 and baseline microbial features as determined by multivariate association with linear model (p<0.05). (D) Schematic summary of associations between gut microbiome and development of post-acute COVID-19 syndrome.

correlated with PACS at 6 months, indicating the putative protective role of these species in the recovery from SARS-CoV-2 infection (figure 6B,C), whereas *Actinomyces sp S6 Spd3*, *Actinomyces johnsonii* and *Atopobium parvulum* were positively correlated with PACS. We found overlap of bacteria species such as *R. gnavus, C. innocuum, Erysipelatous ramosum*, which remained altered from baseline to follow-up and exhibited association with several PACS symptoms, further implying the link between altered gut microbiome and the recovery process in patients with COVID-19 (figure 6D). These findings altogether suggest that an individual's gut microbiome configuration at admission may affect the subject's susceptibility to long-term complications of COVID-19.

DISCUSSION

To the best of our knowledge, this is the first study to demonstrate persistent gut dysbiosis at 6 months after recovery from COVID-19 and the link between altered gut microbiota and

common lingering symptoms. Our study is novel in assessing persistent symptoms after acute COVID-19 and their association with altered gut microbiota in patients with different COVID-19 severities (including mild disease). A strength is the prospective assessment of gut microbiota using shotgun metagenomic sequencing from admission to 6 months and complete clinical data collection. Few COVID-19 related deaths occurred in Hong Kong and subjects were recruited from three regional hospitals that manage most patients with COVID-19 in Hong Kong, hence there is unlikely to be selection bias in the cohort. The small sample size is a limitation of this study and our findings should be confirmed in larger cohorts across different populations.

Specific gut microbiome profiles were associated with the presence of PACS, suggesting that the human gut microbiome may play an important role in development of PACS. The potential role of gut microorganisms in acute lung injury, via gut–lung translocation of bacteria,[22] and regulation of immunology and inflammation,[23] suggests the possibility of microbiome-based

profile in risk stratification for PACS. The abundance of multiple commensal bacteria beneficial to host immunity, including *C. aerofaciens*, *F. prausnitzii*, *E rectale* and *B. obeum,* showed significant inverse correlations with development of PACS. *F. prausnitzii* depleted in patients with PACS is known to have immunomodulatory properties and can contribute to host defence, including downregulating inflammatory responses by inhibiting the NF-kB pathway,[24] interfering with interleukin-8 synthesis and suppressing interleukin-8 secretion,[25] whereas *Blautia obeum* from the genus *Blautia* can exert an anti-inflammatory effect.[26 27] Different Firmicutes bacteria have diverse roles in upregulating or downregulating ACE2 expression in the murine gut[28] and *F. prausnitzii*-derived molecule correlated with the capacity to induce interleukin-10, an anti-inflammatory cytokine.[29] Furthermore, the presence of a series of butyrate-producing bacteria strongly correlated with the 6 min walk distance test, implying the potential beneficial roles of these microorganisms in reducing systemic complications after the acute infection. As the respiratory tract is the main target organ for COVID-19, our finding of an association between certain streptococcus bacteria in the gut and persistent pulmonary symptoms, including breathlessness, is not surprising.

Our data are in line with previous studies that reported perturbations of gut microbiome in chronic diseases, ranging from gastrointestinal, inflammatory and metabolic diseases to neurological and respiratory illnesses.[30] Hair loss has been reported in approximately 20% of COVID-19 survivors. The composition of the faecal microbiota in patients who developed hair loss following acute COVID-19 had a reduction in butyrate-producing species such as *R. inulinivorans* and *F. prausnitzii*. Butyrate has been previously shown to produce beneficial adaptations in brain plasticity and function,[31] and butyrate-producing microorganisms may protect the host from many negative effects of stress, including hair loss and anxiety-like behaviours.[32 33] Our findings of bacteria taxa and their association with specific post-acute symptoms suggest that different microbial patterns may contribute to development of different PACS symptoms. Thus the microbiome could potentially serve as a proxy for prediction of development of specific post-acute COVID-19 symptoms.

In this study, we identified a strong negative correlation between abundance of multiple beneficial bacterial species and development of PACS at 6 months. Loss of several symbionts, including the genera *Bifidobacteria*, *Roseburia* and *Faecalibacteria* known to have immunomodulatory functions, were especially associated with persistent symptoms among recovered patients with COVID-19. The last two bacteria are important several short-chain fatty acid producers and major players in maintenance of immune homeostasis. These fatty acids have been shown to alter chemotaxis and phagocytosis, induce reactive oxygen species, change cell proliferation and function, and have antimicrobial and anti-inflammatory effects. Although the observed association is encouraging, the power of this study might be limited by unrecognised factors that are unrelated to COVID-19 but may affect the bacterial microbiome, including host milieu, diet, lifestyles, medication (though all subjects were not taking any antibiotics in the follow-up period, and there were no differences in their comorbidities) and possible bias of patients' subjective replies to the questionnaire. As the patients did not have repeated viral measurement after discharge, we were unable to determine the duration of viral activity in those with and without PACS. Further studies are required to understand whether and how microbiota modulation could reduce the burden of PACS. Studies that incorporate dietary, serological and immunological data to further elucidate mechanisms

of persistent symptoms after acute COVID-19 infection will be needed.

In summary, altered gut microbiome composition is strongly associated with persistent symptoms in patients with COVID-19 up to 6 months after clearance of SARS-CoV-2 virus. Considering the millions of people infected during the ongoing pandemic, our findings are a strong impetus for consideration of microbiota modulation to facilitate timely recovery and reduce the burden of post-acute COVID-19 syndrome.

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