The faecal metabolome in COVID-19 patients is altered and associated with clinical features and gut microbes

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HIGHLIGHTS

- Faeces of COVID-19 patients were enriched with important nutrients and harmful metabolites.
- COVID-19-altered faecal metabolites were correlated with clinical features, serum metabolites and gut microbes.
- Some faecal metabolites (e.g., sucrose) were important in predicting COVID-19 infection and its severity.
- Most COVID-19-altered faecal metabolites did not reverted to normal levels upon patient discharge.

GRAPHICAL ABSTRACT
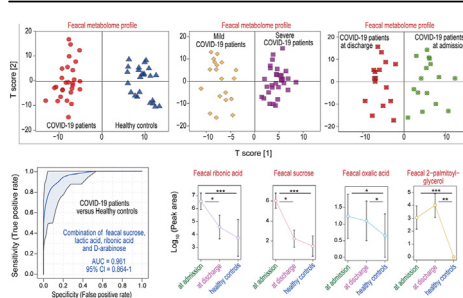
ABSTRACT

Although SARS-CoV-2 can invade the intestine, though its effect on digestion and absorption is not fully understood. In the present study, 56 COVID-19 patients and 47 age- and sex-matched healthy subjects were divided into a discovery cohort and a validation cohort. Blood, faeces and clinical information were collected from the patients in the hospital and at discharge. The faecal metabolome was analysed using gas chromatography-mass spectrometry, and Spearman's correlation analyses of clinical features, the serum metabolome, and the faecal micro- and mycobiota were conducted. The results showed that, the faeces of COVID-19 patients were enriched with important nutrients that should be metabolized or absorbed, such as sucrose and 2-palmitoyl-glycerol; diet-related components that cannot be synthesized by humans, such as 1,5-anhydroglucitol and D-pinitol; and harmful metabolites, such as oxalate, were also detected. In contrast, purine metabolites such as deoxyinosine and hypoxanthine, low-water-soluble long-chain fatty alcohols/acids such as behenic acid, compounds rarely occurring in nature such as D-allose and D-arabinose, and microbe-related compounds such as 2,4-di-tert-butylphenol were depleted in the faeces of COVID-19 patients. Moreover, these metabolites significantly correlated with altered serum metabolites such as oxalate and gut microbesincluding *Ruminococcaceae*, *Actinomyces*, *Sphingomonas* and *Aspergillus*. Although levels of several faecal metabolites, such as sucrose, 1,5-anhydroglucitol

and D-pinitol, of discharged patients were not different from those of healthy controls (HCs), those of oxalate and 2-palmitoyl-glycerol did differ. Therefore, alterations in the faecal metabolome of COVID-19 patients may reflect malnutrition and intestinal inflammation and warrant greater attention. The results of present study provide new insights into the pathogenesis and treatment of COVID-19.

1. Introduction

SARS-CoV-2, which causes COVID-19, has infected tens of millions of people worldwide. In addition to the respiratory system, SARS-CoV-2 can invade the gastrointestinal tract, which has a central role in nutrition absorption, toxin excretion, and immunity in humans [1,2]. As reported in different studies, 16−34% of COVID-19 patients experience gastrointestinal symptoms such as diarrhoea, abdominal pain, vomiting and anorexia [3]. However, the associated underlying mechanism remains incompletely understood.

Metabolites of food and other exogenous substances in the gastrointestinal tract not only provide energy and substrates but also play important roles in the regulation of the immune, nervous, and endocrine systems [4,5]. Indeed, gut metabolites have been recognized as key bridges of the gut-liver, gut-lung and gut-brain axes [6−8]. Metabolites that are not absorbed as well as toxins produced via metabolism are typically excreted through faeces. Therefore, alterations in the faecal metabolome reflect gastrointestinal metabolism and absorption, which is important for the exploration of pathogenesis, screening of diagnostic markers, early warning and prognosis of diseases, and treatment planning [9].

Metabolites in the gastrointestinal tract are the products of both the host and microbes such as bacteria and fungi [10]. Most of the absorbed metabolites, including nutrients and toxins, are typically first transported and processed by the liver and then transported to other organs, thereby impacting health and disease [6]. In our previous study, we showed that the serum metabolome and the gut microbiota and mycobiota of COVID-19 patients are significantly altered compared to those of healthy controls (HCs) [11,12]. In the present work, we explored alterations in the faecal metabolome and their related pathways and elucidated their relationship with gut microbes and clinical features of COVID-19 patients.

2. Methods

2.1. Diagnosis

This study was approved by the ethics committee of the First Affiliated Hospital, College of Medicine, Zhejiang University (IIT2020-136). COVID-19 patients were diagnosed by detecting SARS-CoV-2 RNA in respiratory specimens via quantitative RT-PCR. According to the diagnosis and treatment plan of COVID-19 (seventh edition) [13], adult patients with COVID-19 who met one of the following criteria were diagnosed as having severe disease: 1) shortness of breath, with a respiratory rate more than 30 times per min; 2) oxygen saturation less than 93% in the resting state; and 3) a ratio of arterial partial pressure of oxygen (PaO_2) to the fraction of inspired oxygen (FiO_2) of less than 300 mmHg. The discharge criteria were as follows: body temperature returned to normal for at least 3 days; respiratory symptoms improved significantly; acute exudative lesions improved significantly; and SARS-CoV-2 RNA in respiratory and faecal samples tested negative twice (sampling interval of more than 24 h).

2.2. Participants and sample collection

All participants signed formal informed consent. This study recruited 56 COVID-19 patients in our hospital from January 23 to February 18, 2020, as well as 47 age- and sex-matched healthy individuals. All participants' diets were required to be low-calorie, low-fat, low-sugar, no alcohol, etc., and inpatient diets were provided by the hospital during the study period. Respiratory (nasopharyngeal swabs, sputum, or endotracheal aspirates), blood and faecal samples of patients with COVID-19 were collected from admission to discharge. Blood and faecal samples were collected from the healthy individuals. Except for those used for immediate clinical testing, all samples were stored at −80 °C until use.

2.3. Clinical testing

Blood parameters, such as lymphocyte, neutrophil, and erythrocyte counts, were determined using a Sysmex XN-2000 haematology analyser (Sysmex, Tokyo, Japan). Liver, renal and cardiac function indicators such as alanine aminotransferase (ALT), aspartate transaminase (AST), creatinine, uric acid and lactate dehydrogenase were assayed using a Hitachi 7600-210 automatic analyser (Hitachi, Tokyo, Japan). Indexes of infection, C-reactive protein (CRP) and procalcitonin were detected using a Roche Cobas8000 system (Roche, Basel, Switzerland). In addition, cytokines, including IL-2, IL-4, IL-6, IL-10, and TNF-α, were measured via flow cytometry using Cytometric Bead Array (CBA) technology (BD Biosciences, San Jose, USA) [14].

2.4. Assay of the faecal metabolome

Faecal samples (50 mg) were added to 500 μL of methanol prechilled at 4 °C and 100 mg of ceramic beads (1 mm in diameter). The mixture was homogenized three times at 5000 rpm for 30 s with a 3-s interruption between each round using a Precellys Evolution (Bertin Technologies, USA). After centrifugation at 18,000 g for 15 min, the supernatant was filtered through a 0.22-μm filter membrane. Then, 20 μL of heptadecanoic acid (1 mg/mL) was added to 200 μL of the supernatant as an internal standard, mixed well, and dried with nitrogen at room temperature, after which 50 μL of methoxamine/pyridine solution (15 mg/mL) was added. The mixture was incubated at 37 °C for 24 h to inhibit the cyclization of reducing sugars and the decarboxylation of α-keto acid. Subsequently, 50 μL of N,O-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA + TMCS, Sigma-Aldrich, MO, USA) was added, and the new mixture was incubated at 70 °C for 2 h for trimethylsilylation. The prepared samples were assayed with an Agilent 7890A-5975C GC-MS system (Agilent, CA, USA) using an Agilent J&W Hp-5 MS column. All samples were analysed individually, but a quality control (QC) sample made by mixing and blending equal volumes (10 μL) of each faecal sample was used to estimate a mean profile representing all the analytes encountered during analysis. The general chromatographic conditions were as follows: 1 μL sample was injected in splitless mode at 250 °C; high purity helium was used as a carrier gas with constant

flow rate of 1 mL/min; the column temperature was ramped to 200 °C at 10 °C/min with an initial 2-min pause at 70 °C before being increased at 4 °C/min to 300 °C, with a final hold at 300 °C for 2 min. The general MS parameters were as follows: the temperatures of the transfer line and the electron impact (EI) ion source were set to 290 and 230 °C, respectively; the EI energy was 70 eV; and chromatograms were acquired in full scan mode (range 50−600 m/z) after a 5 min 25 s solvent delay.

2.5. Identification of metabolites and related pathways

The raw GC-MS data were processed using the Agilent software Qualitative Analysis (version B.07.00). Metabolites were identified on the basis of the National Institute of Standards and Technology (NIST) 17 mass spectral library, with matching scores higher than 80 used as a threshold. The matching result was then created and each metabolite was listed with its name, chemical formula, retention time, score and peak area. Then, an additional filtering step was manually performed to remove unidentified and low-confidence compounds and derivatization reagent from the raw results. Data were normalized with the internal standard prior to multivariate analysis and then analysed using Umetrics SIMCA version 14.1. Metabolite set enrichment and metabolic pathway analyses were conducted using the online platform MetaboAnalyst 4.0 (https://www.metaboanalyst.ca) based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) *Homo sapiens* (human) pathway library.

2.6. Data sources for microbiota and mycobiota

Sequencing data for the bacterial microbiota (accession number PRJNA636824) and fungal mycobiota (accession number PRJNA637034) were downloaded from the GenBank Sequence Read Archive repository.

2.7. Statistics

The Mann-Whitney U test was used to compare any two data sets that were not normally distributed based on the Kolmogorov-Smirnov test; otherwise, one-way ANOVA followed by the Student-Newman-Keuls method was used. Spearman's rank correlation test was applied to analyse correlations. Differential metabolites were selected according to variable importance in the projection (VIP) values obtained from the orthogonal partial least squares discriminant analysis (OPLS-DA) model. For all analyses, probabilities were two-tailed, and a two-tailed p value of <0.05 was considered significant, unless otherwise indicated.

3. Results

3.1. Clinical features

As shown in Table 1, 16.07% of COVID-19 patients enrolled in the present study experienced one or more gastrointestinal symptoms. Compared to the HCs, the levels of blood leucocytes, neutrophils, liver function indicators (e.g., alanine transaminase), infection indicators (e.g., C-reactive protein), and inflammation-related cytokines (e.g., IL-4) were significantly increased in patients, whereas the levels of lymphocytes, erythrocytes, haemoglobin and albumin were decreased. The average hospital stay of patients was 17.84 days, and no deaths occurred.

3.2. Dysbiosis of the faecal metabolome in COVID-19 patients

Two hundred five metabolites were identified by GC-MS in

faecal samples, among which 31 were detected only in the HCs and 3 only in the COVID-19 patients, the remaining 171 were observed in both groups. The COVID-19 patients and HCs were divided into an age- and sex-matched discovery cohort (COVID-19, n = 28; HCs, n = 24) and a validation cohort (COVID-19, n = 28; HCs, n = 23). In an OPLS-DA plot, the samples from COVID-19 patients were clearly separated from those from HCs in both the discovery and validation cohorts, indicating that the faecal metabolome profile between COVID-19 patients and HCs are different (Fig. 1A). Additionally, the VIP values of nine metabolites, namely, sucrose, ribonic acid, 2-palmitoyl-glycerol, 2,4-di-tert-butylphenol, arachidic acid, behenic acid, pseudouridine, 7H-purine and D-allose were higher than 1.5, indicating their important contributions in distinguishing the metabolome profile of COVID-19 patients from that of HCs in the OPLS-DA model.

Next, we compared the level of each faecal metabolite between the COVID-19 patients and HCs. The metabolites that were significantly ($p < 0.05$) enriched in both the discovery and validation cohorts or extremely significant ($p < 0.001$) in only one are shown in Fig. 1, while the remaining metabolites showing significant enrichment ($0.001 < p < 0.05$) in only one cohort are depicted in supplementary Figure S1. Nine metabolites were significantly enriched in COVID-19 patients (Fig. 1B). Among these metabolites, the mean levels of D-pinitol, oxalic acid, 2-palmitoyl-glycerol, sucrose and 1,5-anhydroglucitol increased by 130-, 112-, 56-, 55- and 38-fold, respectively, from the HCs to COVID-19 patients, while the remaining four metabolites, ribonic acid, pantothenic acid, lactic acid and malic acid, increased less than tenfold. In addition, 20 metabolites were depleted in the faeces of the COVID-19 patients (Fig. 1C and D). Importantly, the mean levels of 2,4-di-tert-butylphenol, deoxyinosine, 7H-purine and behenic acid decreased by nearly 90%. The remaining 16 metabolites depleted in the COVID-19 patients included three monosaccharides (D-allose, D-arabinose, and D-glucose), three nucleotides (hypoxanthine, inosine and pseudouridine), four amino acids (2-aminoheptanedioic acid, aminomalonic acid, L-tryptophan and L-tyrosine), and three alcohols (1-hexadecanol, 1-pentadecanol and beta-sitosterol) as well as deoxycholic acid, arachidic acid and orotic acid.

3.3. Differences in the faecal metabolomes between patients with mild and severe COVID-19

Subsequently, we compared the faecal metabolomes of patients with mild and severe COVID-19. As shown in the OPLS-DA plot, the sample dots for the mild COVID-19 patients were clearly separated from those of the patients with severe COVID-19, indicating that the faecal metabolome profiles of these patients differed (Fig. 2A). Among the observed metabolites, urea had the highest VIP value (3.1), suggesting that it can contribute the most to distinguishing between mild and severe COVID-19 in the OPLS-DA model. Furthermore, another 21 metabolites, including deoxycholic acid, pantothenic acid, monomethyl succinate, D-cellobiose and 1-pentadecanol had important contributions (VIP > 1.5) (Fig. 2B). Compared to mild COVID-19, cyclohexanecarboxylic acid, lactic acid and urea were enriched in the samples from patients with severe disease, whereas 1-pentadecanol, D-cellobiose, deoxycholic acid, monomethyl succinate and propanoic acid were depleted in those with severe COVID-19 (Fig. 2C).

3.4. Predictive ability of faecal metabolites for COVID-19

The ability of faecal metabolites to predict COVID-19 was evaluated based on receiver operating characteristic curve (ROC) analysis. The area under the curves (AUCs) of sucrose, lactic acid, ribonic acid and 2-palmitoyl-glycerol were between 0.7 and 0.85 in

Table 1
Comparison of clinical features between COVID-19 patients and healthy controls.

	COVID-19 (n = 56)	Healthy controls (n = 47)	p Value
Median (interquartile range) age (years)	54.00(47.25, 62.00)	39.00(53.00, 60.00)	1.77E-01
Males	32	25	6.89E-01
Subtype			
Mild	26	0	−
Severe	30	0	−
Underlying diseases			
Any	31	0	−
Hypertension	18	0	−
Diabetes mellitus	7	0	
Gastrointestinal symptoms			
Any	9	0	−
Vomiting	4	0	−
Abdominal pain	1	0	−
Diarrhoea	6	0	−
Leukocyte (10^9/L)	6.85(4.75, 10.23)	4.68(5.65, 7.05)	4.95E-02
Neutrophil (10^9/L)	5.50(3.03, 9.38)	2.40(3.25, 3.93)	1.86E-04
Lymphocyte (10^9/L)	0.85(0.50, 1.28)	1.42(1.99, 2.31)	6.66E-12
Erythrocyte (10^{12}/L)	4.30(3.90, 4.70)	4.54(4.83, 5.15)	1.29E-07
Haemoglobin (g/L)	130.00(113.25, 142.75)	136.50(145.00, 153.25)	2.00E-06
Albumin (g/L)	37.65(33.48, 41.28)	45.10(47.00, 49.40)	1.06E-19
Alanine transaminase (U/L)	26.00(6.25, 57.00)	12.00(16.00, 22.50)	3.42E-05
γ-Glutamyl transpeptidase (U/L)	29.50(20.00, 56.50)	14.00(18.00, 23.00)	6.84E-06
Alkaline phosphatase (U/L)	64.00(53.00, 78.00)	62.00(74.00, 92.00)	6.66E-03
Uric acid (μmol/L)	205.00(160.00, 288.00)	287.50(344.00, 402.00)	1.42E-08
C-reactive protein (mg/L)	6.36(2.12, 13.67)	0.15(0.37, 0.90)	5.82E-07
Procalcitonin (ng/mL)	0.04(0.02, 0.06)	0.01(0.01, 0.02)	5.88E-09
IL-2 (pg/mL)	0.95(0.88, 1.36)	0.93(0.93, 0.93)	9.98E-03
IL-4 (pg/mL)	1.40(1.40, 1.77)	1.16(1.16, 1.16)	2.68E-11
IL-6 (pg/mL)	9.49(4.00, 20.45)	2.01(2.01, 2.01)	4.79E-08
IL-10 (pg/mL)	2.63(1.70, 4.06)	0.73(0.73, 0.75)	7.24E-17
TNF-α (pg/mL)	14.30(6.71, 45.12)	3.56(3.56, 3.77)	3.77E-14
Outcomes			
Mortality	0	0	−
Median (interquartile range) in hospital stay (days)	17.00(14.00, 22.00)	−	−

both the discovery and validation cohorts, suggesting the good predictive abilities of these four metabolites (Fig. 3A). Subsequently, a composite prediction model was constructed using machine learning and logistic regression. The AUC of the combination of sucrose, lactic acid, ribonic acid and D-arabinose was 0.961 in the discovery cohort and 0.949 in the validation cohort (Fig. 3B), indicating excellent predictive ability. Although testing methods for SARS-CoV-2, such as molecular and antibody testing, may be more convenient, faecal metabolic testing may much better reflect the intestinal health status of COVID-19 patients.

3.5. Altered gut metabolic pathways in COVID-19 patients

According to pathway mapping using the KEGG database, COVID-19-altered faecal metabolites were significantly located in 17 pathways in both the discovery and validation cohorts. These pathways included biosynthetic pathways, such as for phenylalanine, tyrosine and tryptophan biosynthesis, aminoacyl-tRNA biosynthesis and glucosinolate biosynthesis, and metabolic pathways, such as for starch and sucrose metabolism and ABC transporters (Fig. 4). When only the human library of the KEGG database was selected, the pathways of glucosinolate biosynthesis, indole alkaloid biosynthesis, meiosis-yeast and the phosphotransferase system became insignificant, suggesting that the alteration of these pathways in COVID-19 patients may be primarily caused by gut microbes. Furthermore, using the HMDB database, we also notes that several COVID-19-altered metabolites were enriched significantly in the gluconeogenesis pathway, which was not observed during KEGG mapping, in both the discovery and validation cohorts. Regarding important metabolites to predict the COVID-19 features, sucrose was located in the pathways of galactose

metabolism, starch and sucrose metabolism, metabolic pathways, ABC transporters and the phosphotransferase system, while lactic acid was located in only the gluconeogenesis pathway. However, no significant pathways which related to ribonic acid, 2-palmitoyl-glycerol or D-arabinose were observed.

### 3.6. Network between altered metabolites and bacteria or fungi in faeces

Some correlations of altered faecal metabolites with bacteria or fungi in the COVID-19 patients suggest potential causal relationships between them. On the one hand, some COVID-19-enriched/depleted metabolites and bacteria or fungi were positively correlated. For example, D-pinitol and sucrose correlated with at least one among *Actinomyces*, *Sphingomonas*, *Rothia*, and *Streptococcus parasanguinis*; 1-hexadecanol, 1-pentadecanol and deoxycholic acid correlated with at least one Clostridiales member, such as *Ruminococcaceae*, *Eubacterium hallii* group, Family XIII AD3011 group, *Anaerostipes*, *Fusicatenibacter*, *Roseburia*, *Faecalibacterium* and *Ruminococcus* sp. 5139BFAA; D-arabinose correlated with *Aspergillus*; aminomalonic acid and L-tryptophan correlated with *Aspergillus rugulosus*; inosine correlated with *Aspergillus tritici* and *Penicillium* sp.; and beta-sitosterol correlated with *Penicillium citrinum* (Fig. 5A and B). In particular, 2,4-di-tert-butylphenol highly and positively correlated with an unclassified species of *Didymellaceae* (r = 0.73, p = 3.47E-07) (Fig. 5B). On the other hand, some COVID-19-depleted/enriched metabolites and bacteria or fungi were negatively correlated. For example, 1-hexadecanol, 1-pentadecanol and deoxycholic acid correlated with at least one among *Actinomyces*, *Sphingomonas*, *Rothia*, *Actinomyces odontolyticus* and *S. parasanguinis*; 1,5-anhydroglucitol correlated with
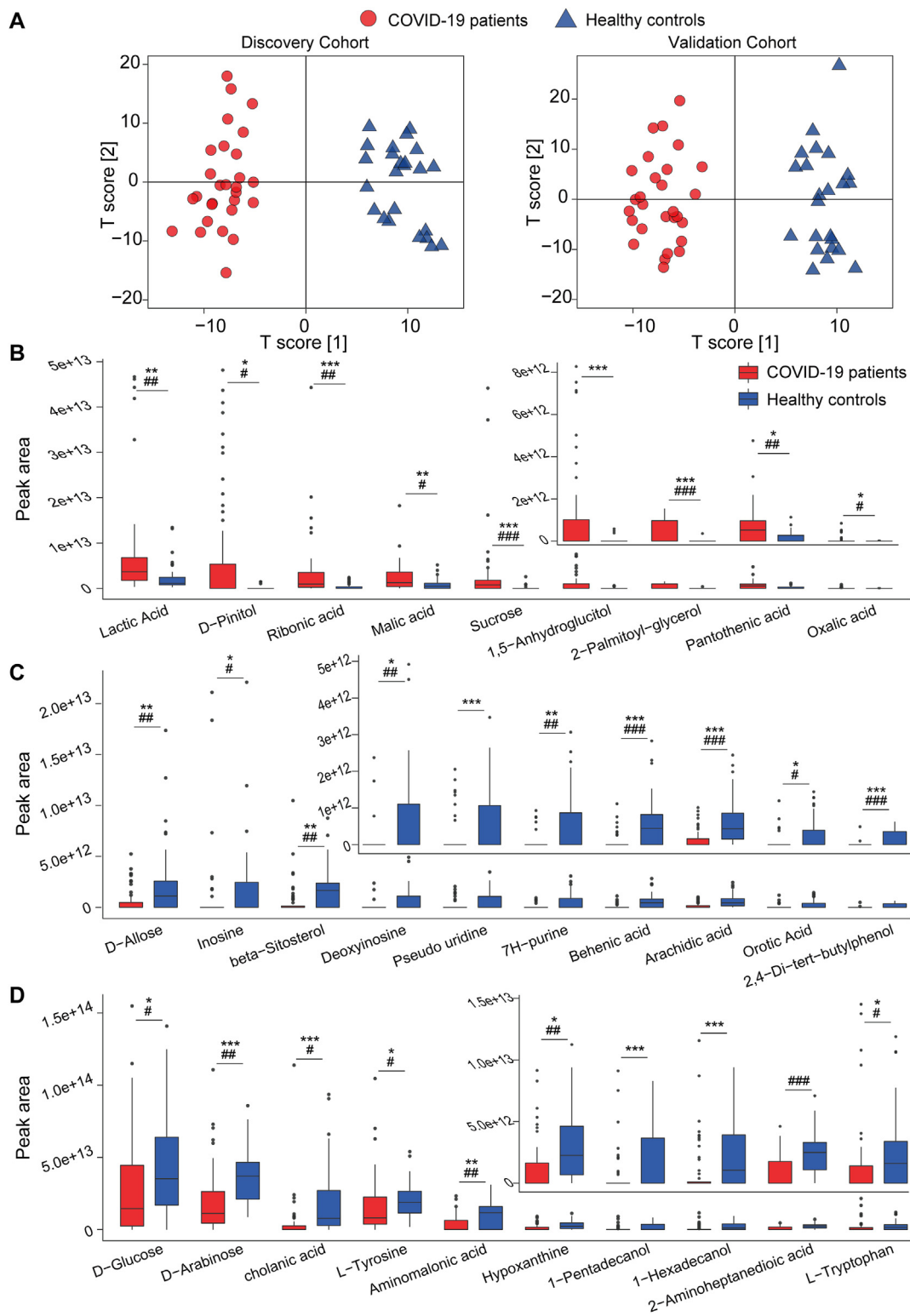
Fig. 1. The faecal metabolome of COVID-19 patients is distinct from that of HCs in the discovery and validation cohorts. (A) The OPLS-DA score plot shows that the metabolome profiles of the COVID-19 patients were clearly separated from those of the HCs in both the discovery and validation cohorts. (B) Nine metabolites were significantly enriched in the faeces of COVID-19 patients. (C, D) Twenty metabolites were significantly depleted in the faeces of COVID-19 patients. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ in the discovery cohort; #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ in the validation cohort).

Aspergillus and *Aspergillus penicillioide*; and 2-palmitoyl-glycerol correlated with *Aspergillus* sp.; and oxalic acid correlated with *Aspergillus* (Fig. 5A and B). Correlations of most of these metabolites

with bacteria but not fungi were observed in the HCs and were consistent with those detected in the COVID-19 patients (supplementary Figure S2A and S2B).
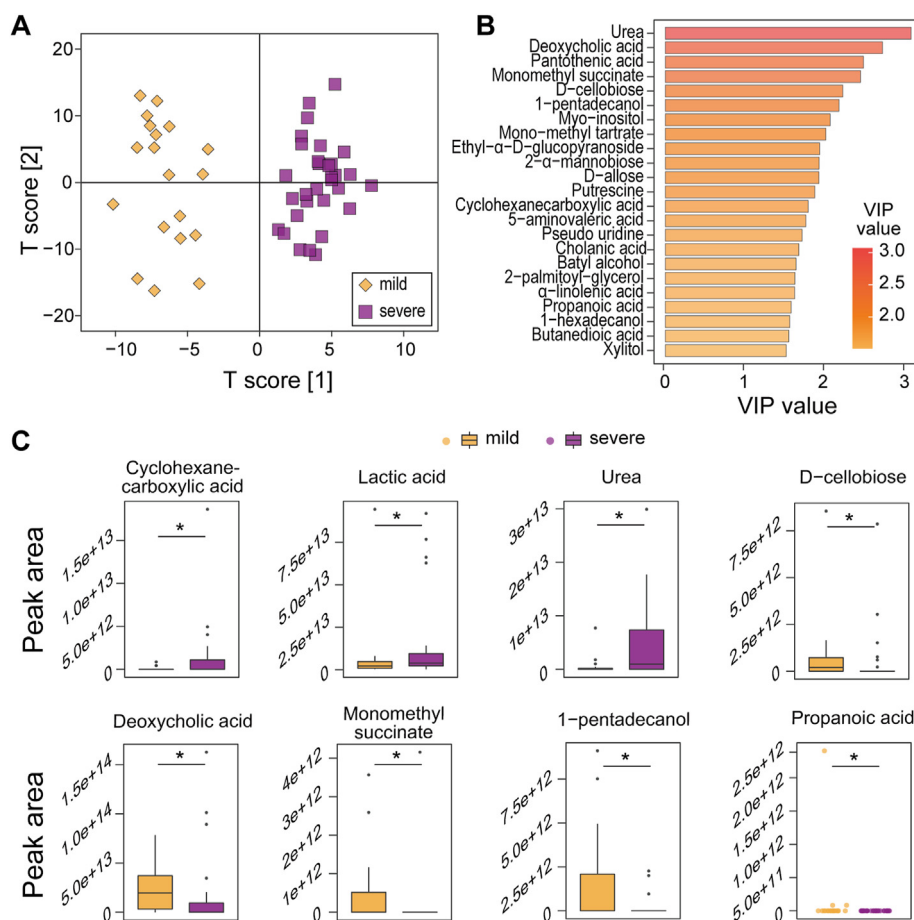
Fig. 2. The metabolome profiles of patients with mild and severe COVID-19 are clearly distinct. (A) Differences in faecal metabolome profiles between patients with mild and severe COVID-19 at admission based on the OPLS-DA model. (B) The VIP values of 22 metabolites that greatly contributed to distinguishing mild and severe COVID-19. (C) Eight metabolites were differentially distributed in mild and severe COVID-19. (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

Some correlations of faecal metabolites with bacteria or fungi in the COVID-19 patients appear to disagree with their observed alterations. For instance, COVID-19-enriched 1,5-anhydroglucitol negatively correlated with COVID-19-enriched *Streptococcus*, and COVID-19-enriched D-pinitol positively correlated with COVID-19-depleted *Peptostreptococcaceae*, as well as pantothenic acid, 1,5-anhydroglucitol and sucrose were positively correlated with *Eubacterium hallii* group, *Faecalibacterium* and *Rothia*, respectively. Furthermore, COVID-19-enriched D-pinitol and D-glucose positively correlated with COVID-19-depleted *Penicillium steckii*, whiloe COVID-19-depleted D-arabinose negatively correlated with COVID-19-depleted *Aspergillus rugulosus* (Fig. 5A and B). These correlations were not observed in the HCs, suggesting that they may not contribute to the mutual alterations in faecal metabolites or microbes associated with COVID-19 (supplementary Figure S2A and S2B).

3.7. Associations of altered faecal metabolites with clinical features and serum metabolites

In the present study, serum procalcitonin positively correlated with faecal oxalic acid and 2-palmitoyl-glycerol but negatively correlated with faecal 1-pentadecanol, whereas negative correlations were observed between blood neutrophils and faecal hypoxanthine and between blood alkaline phosphatase and faecal pantothenic acid (Fig. 5C). Furthermore, some COVID-19-enriched faecal and serum metabolites were positively correlated, such as

faecal sucrose and ribonic acid with serum ʟ-serine. Moreover, COVID-19-depleted faecal metabolites negatively correlated with COVID-19-enriched serum metabolites, such as faecal 1-pentadecanol and behenic acid with serum ʟ-alanine and faecal D-arabinose with serum 2-hydroxybutyric acid and 3-hydroxybutyric acid (Fig. 5C). COVID-19-enriched faecal ribonic acid also negatively correlated with COVID-19-depleted serum 2-palmitoylglycerol. Importantly, none of these correlations were observed in the HCs (supplementary Figure S2C).

3.8. Some faecal metabolites recovered to normal levels after patient discharge

OPLS-DA analysis showed that the metabolome profile of patients at discharge was significantly different from that while in the hospital (Fig. 6A). With VIP values higher than 1.5 as a threshold, 20 metabolites, such as 1,5-anhydroglucitol, 2-hydroxyisocaproic acid and sucrose, contributed greatly to distinguishing the metabolome profiles between in-hospital and discharged patients (Fig. 6B). Furthermore, compared to patients in the hospital, the levels of 1,5-anhydroglucitol, ribonic acid and sucrose decreased after discharge and even became not significantly different from those of the HCs (Fig. 6C). Nonetheless, although the level of beta-sitosterol significantly increased, it was still significantly lower than that of the HCs. In contrast, D-pinitol and malic acid enrichment levels were not significantly altered after patient discharge, yet the difference from the levels observed in the HCs became insignificant. Overall, most
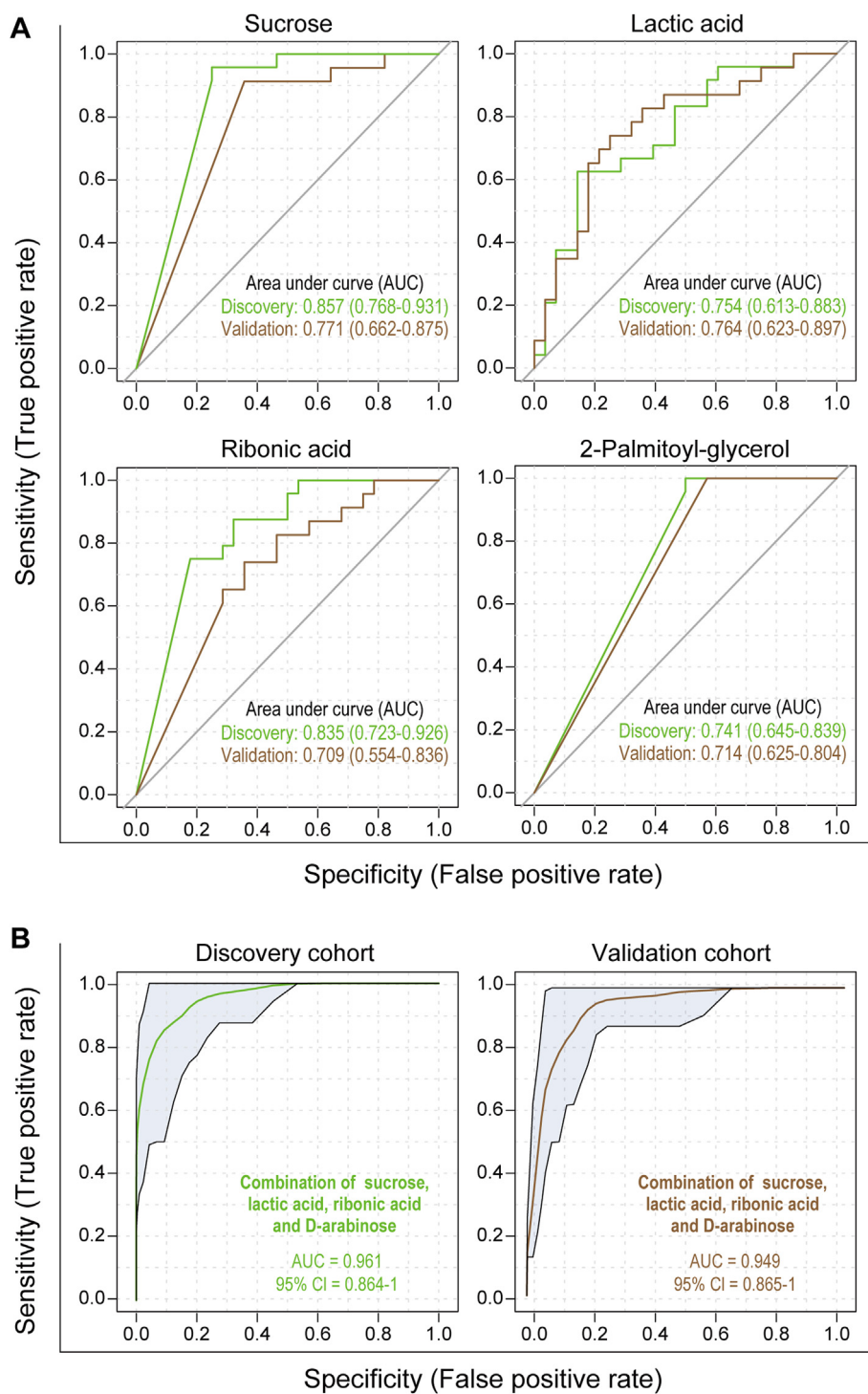
Fig. 3. Specific faecal metabolites are predictive of COVID-19. (A) ROC curves of sucrose, lactic acid, ribonic acid and 2-palmitoyl-glycerol for the prediction of COVID-19 in the discovery and validation cohorts. (B) ROC curve of the combination of sucrose, lactic acid, ribonic acid and D-arabinose for the prediction of COVID-19 in the discovery and validation cohorts.

metabolites exhibiting COVID-19-associated alterations, such as oxalic acid and 2-palmitoyl-glycerol, did not significantly recover after patient discharge (Fig. 6C).

4. Discussion

Gastrointestinal symptoms and active SARS-CoV-2 replication in the intestine are frequently observed in COVID-19 patients [15], but

their effects on gut metabolism remain unclear. In the present study, we observed that faecal metabolome profiles differed significantly not only between COVID-19 patients and HCs but also between patients with mild and severe disease. Several metabolites were observed to effectively predict COVID-19 patients from HCs. Furthermore, the COVID-19-altered metabolites were closely related to patients' clinical symptoms, gut microbes and serum metabolites. Importantly, when these COVID-19 patients were
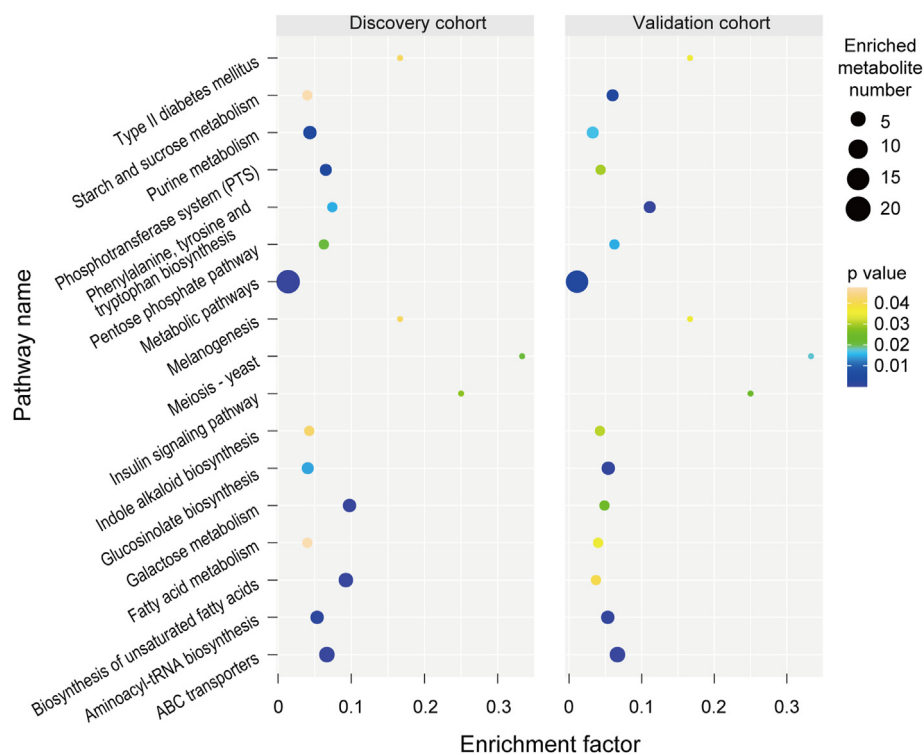
Fig. 4. Metabolic pathway analysis of COVID-19-altered faecal metabolites based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

discharged, only a small portion of the altered metabolites returned to normal levels. These results will aid in further understanding the mechanism by which SARS-CoV-2 affects health.

Our results showed that sucrose was enriched and glucose depleted in the faeces of COVID-19 patients. Sucrose is primarily metablized into fructose and glucose by sucrase-isomaltase in the small intestinal mucosa, and its enrichment in faeces suggests that sucrase-isomaltase activity may be impaired [16]. Coincidentally, intestinal symptoms caused by sucrase-isomaltase insufficiency typically manifest as osmotic-fermentative diarrhoea accompanied by vomiting, flatulence, and abdominal pain, which are also common symptoms of COVID-19 patients [2]. Among these symptoms, those such as flatulence are typically caused by the fermentation of unabsorbed carbohydrates in the intestine by bacteria. Correspondingly, we observed the level of sucrose to be significantly and positively correlated with the relative abundance of *Actinomyces* and *S. parasanguinis*, which were greatly enriched in the faeces of COVID-19 patients. In addition, the enrichment of sucrose will affect the absorption of other nutrients, thereby increasing the risk of malnutrition and disease deterioration [7]. Both intestinal SARS-CoV-2 infection and systemic inflammation may affect sucrase-isomaltase. Pro-inflammatory mediators [TNF-α (50 ng/mL), IL-1β (25 ng/mL) and MCP1 (50 ng/mL)] observed in patients with inflammatory bowel disease have been reported to induce a significant decrease in both the level and activity of sucrase-isomaltase in Caco2 cells [17]. Importantly, our results showed that the faecal sucrose levels of the COVID-19 patients at discharge were not significantly different from those of the HCs, further indicating that SARS-CoV-2 infection has an important effect on intestinal sucrose metabolism.

According to our results, oxalate was enriched in the faeces of COVID-19 patients. Faecal oxalate is primarily derived from plant foods or is produced and excreted into the gut via the catabolism of ascorbic acid and some amino acids [18]. Blood-associated oxalate

primarily originates from intestinal absorption and endogenous production, and it is not well metabolized in the human body. Indeed, approximately two-thirds of oxalate is excreted in urine and one-thirds in faeces [18]. In addition to faeces, we observed that oxalate was significantly enriched in the serum of COVID-19 patients and positively correlated with faecal oxalate, suggesting that COVID-19 patients have serious abnormalities in oxalate-related metabolism. However, as the intake of oxalate did not increase, assessing whether the level of oxalate in the urine of COVID-19 patients is altered is crucial for determining whether oxalate production and excretion are disrupted, which warrants further attention. Intestinal membrane-bound anion exchangers, belonging to the *SLC26* gene family, play important roles in transcellular oxalate absorption and secretion [19]. Interestingly, gastrointestinal disorders such as inflammatory bowel disease and/or surgery such as gastric bypass can lead to excessive oxalate absorption and secondary enteric hyperoxaluria, but compensatory oxalate secretion by the large intestine in chronic renal failure is triggered to increased faecal elimination of oxalate [20]. Moreover, alterations in the gut microbiota may affect oxalate metabolism, such as for the "specialist oxalotroph" *Oxalobacter formigenes* [21]. Although our results did not reveal a significant change in the relative abundance of this species, the effect of other significantly altered microorganisms on oxalate metabolism cannot be ruled out. Oxalate can bind to some minerals in the gut and prevent their absorption, and high oxalate levels may increase the risk of kidney stones and other health problems [19,20,22,23]. As our results showed that the elevation in the level of faecal oxalate was not significantly improved when the patients were discharged, the effect of oxalate on the development and recovery of COVID-19 needs further attention.

Our results showed that some metabolites mostly or completely derived from food, such as 1,5-anhydroglucose and D-pinitol, were enriched in the faeces of COVID-19 patients. D-Pinitol cannot be
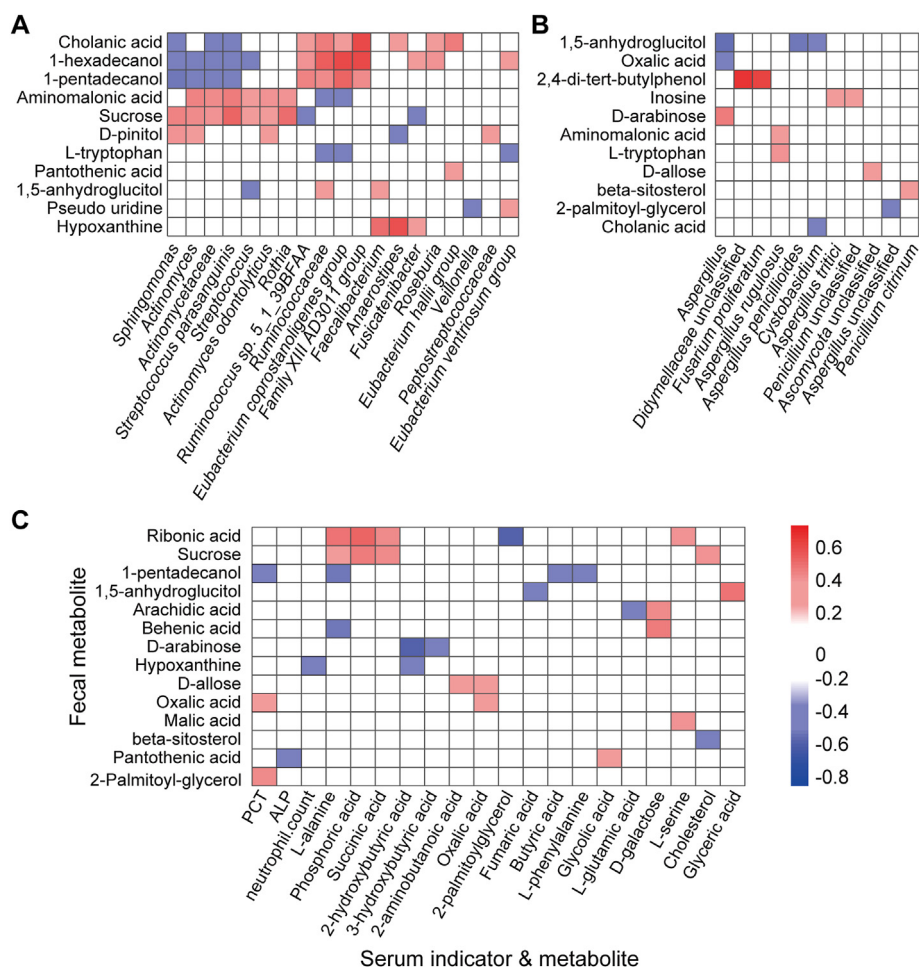
Fig. 5. Correlations of altered faecal metabolites with altered faecal microbes, haematological parameters, and serum metabolites in COVID-19 patients. (A) Correlations of faecal metabolites with faecal bacterial microbiota. (B) Correlations of faecal metabolites with faecal mycobiota. (C) Correlations of faecal metabolites with haematological parameters and serum metabolites.

synthesized by humans and is primarily derived from specific soybean and legume foods. Interestingly, D-pinitol has also been observed to exhibit anti-inflammatory and insulin-like effects [24]. 1,5-Anhydroglucitol is a glucose analogue that cannot be metabolized by humans, and nearly 99.9% of 1,5-anhydroglucitol is reabsorbed through glomerular filtration to maintain a stable serum level. Because serum 1,5-anhydroglucitol levels typically decrease when glucose levels increase, they can reflect the average blood glucose level in the past 1−2 weeks. On the one hand, because of the lack of additional intake of the above-mentioned compounds, their faecal enrichment may result (and thus reflect) from disruption of the intestinal absorption function in COVID-19 patients. Consistent with this idea, the serum 1,5-anhydroglucitol level decreased in COVID-19 patients, though the serum glucose level did not change. On the other hand, faecal enrichment of these metabolites may be related to alterations in the gut microbiota or mycobiota. The elimination of gut microbes has been reported to cause D-pinitol enrichment [25]. Similarly, the destruction of intestinal absorption and alterations in gut microbes may promote the presence of 2-palmitoyl-glycerol, a digestive product of dietary fat, which was observed to be enriched in faeces but depleted in serum. A similar situation may exist for pantothenic acid. Therefore, potential alteration of the intestinal absorption function and its impact on COVID-19 patients' health warrant further attention.

Our results showed that some metabolites that rarely occur in

nature, such as D-allose and D-arabinose, were depleted in the faeces of COVID-19 patients. D-Allose shows immunosuppressive and antiproliferation effects in animal experiments [26], whereas D-arabinose inhibits the growth of *Caenorhabditis elegans* cultured in monoxenic conditions [27]. Our results demonstrated strong positive correlations of D-arabinose with *Aspergillus* and of D-allose with an unclassified species of Ascomycota in the faeces of COVID-19 patients. Because D-allose and D-arabinose are rare sugars and both *Aspergillus* and the unclassified species of Ascomycota were depleted, their positive correlation indicates that gut microbes may contribute to D-allose and D-arabinose depletion. In addition, 2,4-di-tert-butylphenol, which has antibacterial, antifungal and anti-cancer activities and cannot be produced by humans [28], strongly and positively correlated with *Fusarium proliferatum* and an un-classified species of *Didymellaceae*, both of which were depleted in the faeces of COVID-19 patients, further indicating the important role of gut microbes in the depletion of metabolites rarely occurring in nature.

Our results showed that four purine metabolites, deoxyinosine, 7H-purine, hypoxanthine and inosine, were depleted in the faeces of COVID-19 patients. On the one hand, there is a substantial need for nucleotide genesis in the gut because intestinal epithelial cells have an extremely short lifetime and are almost completely renewed every 2−6 days [29]. Therefore, a reduction in faecal purine metabolites may result from increasing demands for nucleic
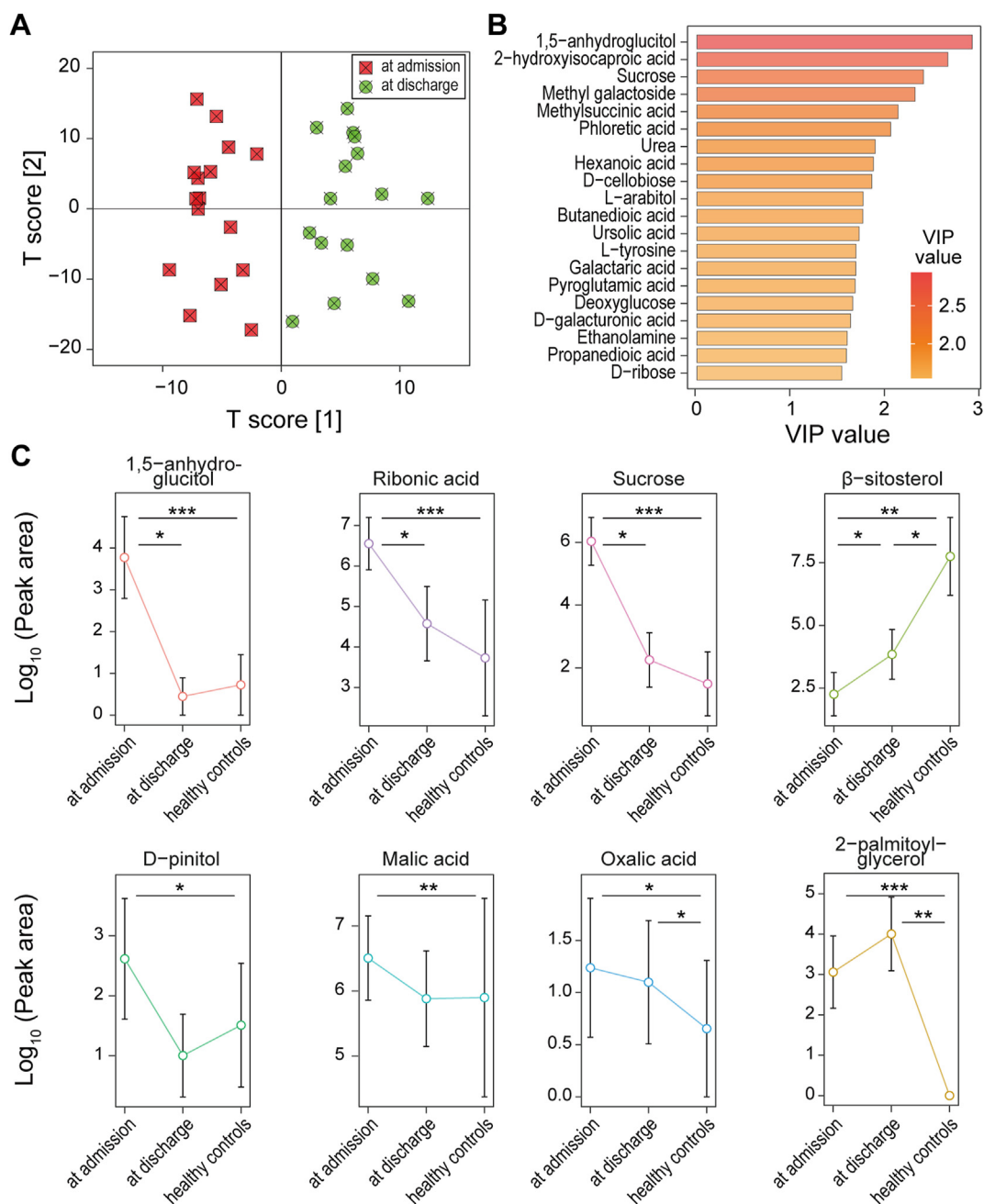
Fig. 6. Differences in metabolome profiles of COVID-19 patients in the hospital and at discharge. (A) The faecal metabolome profiles of patients in the hospital were clearly separated from those of patients at discharge in the OPLS-DA score plot. (B) The VIP values of twenty metabolites that importantly contributed to distinguishing patients in the hospital from patients at discharge. (C) Eight metabolites were differentially distributed between patients in the hospital and at discharge. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

acids to repair the intestinal epithelial damage caused by SARS-CoV-2 infection. On the other hand, as the gut microbiota can produce and release large quantities of purines [30], the reduction in faecal purine metabolites may also be related to alterations in the gut microbiota. Consistent with this phenomenon, our results revealed positive correlations between several COVID-19-depleted microbes and purine metabolites, such as between *Anaerostipes* and *Faecalibacterium* and hypoxanthine and between *Aspergillus tritici* and inosine.

Our results revealed the depletion of several low-water-soluble long-chain fatty acids or fatty alcohols, such as arachidic acid, behenic acid, and 1-hexadecanol, in the faeces of COVID-19 patients. Previous studies have demonstrated that long-chain fatty

acids such as arachidic acid are depleted in the faeces of patients with ulcerative colitis and Crohn's disease as well as in a mouse model of methionine-choline-deficient diet-induced nonalcoholic steatohepatitis [31,32]. In addition, decreases in arachidic and behenic acids were observed in the faeces of galacto-oligosaccharide-fed mice. Furthermore, our results showed that 1-hexadecanol and 1-pentadecanol positively correlated with the COVID-19-depleted faecal microbes such as *Ruminococcaceae*, *Eubacterium coprostanoligenes* and Family XIII AD3011. These results suggest that the depletion of these taxa may be related to gastrointestinal and liver inflammation as well as alterations in gut microbes.

Our results showed that ribonic and lactic acids were enriched

and important to predict the COVID-19 features, but were not significantly involved in altered pathways associated with COVID-19 infection. Ribonic acid can be generated from the dehydrogenation and dephosphorylation of ribose-5-phosphate, an intermediate of the pentose phosphate pathway [33]. Ribonic acid levels were previously reported be increased in the faeces of mice with faecal occult bleeding and defective gut epithelial cell proliferation induced by oral ampicillin and vancomycin asministration [34]. Therefore, the observed enrichment of ribonic acid in the faeces of COVID-19 patients may indicate the occurrence of intestinal injury. The other important metabolite enriched in COVID-19 patients, lactic acid, could be a chemical byproduct of cell anaerobic respiration or a product of bacterial metabolism in the gut [35]. Therefore, its enrichment in the faeces of COVID-19 patients may result from anoxia of intestinal cells or an increased abundance of lactic acid-producing bacteria, which needs to be further clarified in a future study.

Although we tried our best to control for diet and analyse the relationship between the faecal metabolome and microbes, there may still be confounding factors other than SASR-CoV-2 infection that affect faecal metabolism. In the future, multi-centre studies, especially those of large cohorts involving different countries and ethnic groups, can reduce the impact of unknown potential factors and more accurately elucidate the alterations in the faecal metabolome of COVID-19 patients due to SASR-CoV-2 infection.

5. Conclusion

In summary, the faeces of COVID-19 patients were enriched in important nutrients that should be digested or absorbed, such as sucrose and 2-palmitoyl-glycerol; with diet-related components that cannot be synthesized by humans, such as 1,5-anhydroglucitol and D-pinitol, and with harmful metabolites, such as oxalate. In contrast, purine metabolites, such as deoxyinosine and hypoxanthine; low-water-soluble long-chain fatty alcohols/acids, such as behenic acid; compounds rarely occurring in nature, such as D-allose and D-arabinose; and microbe-related compounds, such as 2,4-di-tert-butylphenol, were depleted in the faeces of COVID-19 patients. These metabolites significantly correlated with clinical features (e.g. blood neutrophils), altered serum metabolites (e.g., oxalate) and gut microbes (e.g., *Ruminococcaceae*, *Actinomyces*, *Sphingomonas* and *Aspergillus*) in COVID-19 patients. Moreover, the levels of several faecal metabolites, such as sucrose, 1,5-anhydroglucitol and D-pinitol, of discharged patients were not different from those of HCs, whereas oxalate and 2-palmitoyl-glycerol were. Therefore, alterations in the faecal metabolome of COVID-19 patients may reflect malnutrition and intestinal inflammation, which warrants greater attention. The results of the present study provide new insights into the pathogenesis and treatment of COVID-19.

Ethics statement

This study was approved by the ethics committee of the First Affiliated Hospital, College of Medicine, Zhejiang University (IIT2020-136).

CRediT authorship contribution statement

Longxian Lv: contributed equally to this work, conceived and designed the study. **Huiyong Jiang:** contributed equally to this work, performed the experiments and analysed the data. **Yanfei Chen:** contributed to sample collection and storage. **Silan Gu:** contributed equally to this work, performed the experiments and analysed the data. **Jiafeng Xia:** contributed to sample collection and storage. **Hua Zhang:** were responsible for clinical data collection. **Yingfeng Lu:** were responsible for clinical data collection. **Ren Yan:** conceived and designed the study, wrote the manuscript. **Lanjuan Li:** conceived and designed the study, wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2021.338267.

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