

Gut mycobiota alterations in patients with COVID-19 and H1N1 and associations with immune and gastrointestinal symptoms

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Abstract

The relationship between gut microbes and COVID-19 or H1N1 flu is not fully understood. Here, we compared gut mycobiota of 67 COVID-19 patients, 35 H1N1 patients and 48 healthy controls (HCs) using internal transcribed spacer (ITS) 3-ITS4 sequencing. Fungal richness decreased in COVID-19 and H1N1 patients compared to HCs, but fungal diversity decreased in only H1N1 patients. Fungal mycobiota dysbiosis in both COVID-19 and H1N1 patients was mainly characterized by depletions of fungi such as *Aspergillus*, *Penicillium*, but several fungi, such as *Candida parapsilosis*, and *Malassezia yamatoensis*, were enriched in H1N1 patients. The altered fungal taxa were strongly associated with clinical features such as the incidence of diarrhoea, albumin. Gut mycobiota between COVID-19 patients with mild and severity symptoms are not different, as well as between COVID-19 patients in and out hospital. Therefore, gut mycobiota dysbiosis occur in covid-19 or H1N1 patients and do not improve until discharge.

Introduction

Although the infectivity and mortality of SARS-CoV-2 are much higher than those of influenza viruses and their treatment methods are quite different,¹⁻³ patients with coronavirus disease 2019 (COVID-19) caused by SARS-CoV-2 and influenza viruses such as influenza A (H1N1) have many very similar symptoms, such as fever, cough, vomiting, and diarrhoea.⁴⁻⁶ This will pose greater challenges to the prevention and treatment of COVID-19 when flu season arrives.

The normal gut mycobiota plays important roles in host immune homeostasis, metabolism, and infection prevention.⁷⁻⁹ In contrast, gut fungal dysbiosis and fungal infections are related to a large number of diseases, such as inflammatory bowel disease, colorectal cancer, and asthma.¹⁰⁻¹³ In particular, the development of some infectious diseases is also closely associated with gut fungi, such as the potential link between the enrichment of enteric fungi and hepatitis B virus infection.^{14,15} Furthermore, some fungi, such as *Candida albicans*, were able to decrease host susceptibility to colitis and H1N1 virus.¹⁶

The rate of fungal co-infection in COVID-19 patients was significantly higher in severe cases than in mild cases;¹⁷ this was also observed in H1N1 patients. Moreover, fungal co-infection strongly affected the prognosis of H1N1 patients.¹⁸⁻²⁰ H1N1 virus infection causes an impaired host immune response against fungi, which in turn leads to fungal proliferation.²¹ However, it is still unclear whether alterations in the gut mycobiota occur and whether they are associated with health and diseases in patients with COVID-19 and H1N1. This work compared clinical characteristics, gastrointestinal symptoms, inflammation, and the structure and predicted functions of the gut mycobiota among COVID-19 patients, H1N1 patients and healthy individuals. Furthermore, the associations and potential mechanisms that drove these observations were explored.

Results

Baseline characteristics

Nearly 54% of COVID-19 patients and 68% of H1N1 patients had severe disease (Table 1). The most common underlying diseases were hypertension, diabetes mellitus and liver diseases, while the most common symptoms were fever, cough and diarrhoea in both COVID-19 and H1N1 patients. All patients with H1N1 or COVID-19 were treated with antiviral drugs; some were treated with glucocorticoids, but no patients received antifungal drugs, probiotics or prebiotics. No patients died, but the median hospital stay of COVID-19 patients was approximately 16 days, which was much higher than the median hospital stay of 7 days of H1N1 patients.

Table 1 Baseline characteristics and clinical course

	COVID-19 (n=67)	H1N1 (n=35)	HCs (n=48)
Median (interquartile range) age (years)	52 (42, 60)	53 (43, 62)	59 (40, 78)
Males	43 (46.27%)	21 (60.00%)	25 (52.08%)
Subtype			
Mild	31 (46.27%)	14(40.00%)	—
Severe	36 (53.73%)	24(68.57%)	—
Underlying diseases			
Hypertension	16 (23.88%)	3(8.57%)	—
Diabetes mellitus	10 (14.92%)	2 (5.71%)	—
Heart disease	4 (5.97%)	0	—
Liver diseases	2 (2.99%)	3(8.57%)	—
Symptoms			
Fever	61 (91.04%)	31(91.43%)	—
Cough	54 (80.59%)	33(95.71%)	—
Diarrhoea	7(10.44%)	4(11.43%)	—
Vomiting	5(7.46%)	5(14.28%)	—
Abdominal pain	2(3.08%)	2(3.08%)	—
Stool with SARS-CoV-2	23 (34.32%)	—	—
Medication use			
Antivirals	67 (100%)	35 (100%)	—
Glucocorticoids	49 (73.13%)	10 (28.57%)	—
Antibiotics	—	—	—
Antifungals	—	—	—
Pro/prebiotics	—	—	—
Outcomes			
Mortality	0	0	—
Median (interquartile range) of hospital days	16 (13, 22)	7 (6, 9)	—

Inflammation in H1N1 and COVID-19 patients

The absolute lymphocyte count and neutrophil count in peripheral blood were not significantly different between COVID-19 patients and H1N1 patients, but the lymphocyte count was lower and the neutrophil count was higher in both patient groups than in HCs (Fig. 1A). C-reactive protein (CRP), an index of

bacterial infection, was significantly higher in COVID-19 patients and H1N1 patients than in HCs, while procalcitonin, an index of bacterial and fungal infection, was higher in H1N1 patients than in COVID-19 patients and HCs (Fig. 1D). Additionally, compared with HCs, both H1N1 patients and COVID-19 patients had significant increases in IL-2, IL-6, and IL-10, suggesting that patients were in an inflammatory state (Fig. 1C). IL-4 and TNF- α were higher in COVID-19 patients than H1N1 patients and HCs, suggesting COVID-19 patients experienced more inflammation. Compared with HCs, albumin decreased and ALT and GGT increased in both H1N1 patients and COVID-19 patients, suggesting of the patients experienced liver injury. Interestingly, AST increased in only H1N1 patients, and was even higher than that in COVID-19 patients, suggesting that more serious liver injury might occur in H1N1 patients. ALP decreased in only COVID-19 patients compared to HCs, suggesting that kidney injury and anaemia may occur in these patients (Fig. 1B). Compared with HCs, the absolute erythrocyte count in peripheral blood decreased in both COVID-19 patients and H1N1 patients, while haemoglobin decreased in only the COVID-19 group, suggesting that H1N1, and especially COVID-19, may cause erythrocyte damage (Fig. 1A). In addition, compared with HCs, cholesterol and uric acid decreased in COVID-19 patients and H1N1 patients, while platelets decreased in only H1N1 patients. Furthermore, platelets and cholesterol were lower in H1N1 patients than in COVID-19 patients (Fig. 1A and 1D).

Fungal dysbiosis in H1N1 and COVID-19 patients

In fungal ITS sequencing, 1,587 OTUs were identified, including 557 OTUs unique to HCs, 232 OTUs unique to H1N1 patients, and 285 OTUs unique to COVID-19 patients (Fig. 2A). The OTU richness, as reflected by the Chao1 index, was not significantly different between COVID-19 and H1N1 patients, but that of each patient group was lower than that of HCs (Fig. 2B). The OTU diversity, as measured by the Shannon index, was similar between COVID-19 patients and HCs and was higher in both groups than in the H1N1 patient group (Fig. 2C). In the principal coordinate analysis (PCoA) plots, HCs, COVID-19 patients and H1N1 patients were separately clustered (Fig. 2D), indicating that their compositions were significantly different; similar results were observed in non-metric multi-dimensional scaling (NMDS) plots (Fig. 2E). These conclusions were also confirmed by permutational multivariate analysis of variance ($P = 0.001$).

Compared with HCs, all significant alterations of the gut mycobiota in COVID-19 patients were depletions of certain fungal taxa, such as members of Ascomycota and Basidiomycota, which was observed in both the discovery and validation cohorts. In the phylum Ascomycota, most of the depleted taxa belonged to *Aspergillaceae*, such as *Penicillium citrinum*, *Penicillium polonicum*, and *Aspergillus*, together with its five species (Fig. 2B-2E). Furthermore, *Candida parapsilosis*, *Talaromyces wortmannii*, and two unclassified species that separately belonged to Didymellaceae or Onygenales were also depleted in COVID-19 patients. In the phylum Basidiomycota, five species, which included *Malassezia yamatoensis*, *Rhodotorula mucilaginosa*, *Moesziomyces aphidis*, *Trechispora sp.* and *Wallemia sebi*, were significantly depleted (Fig. 2C to 2E). Similar results were observed in the phylum Mucoromycota and the species *Mucor racemosus* (Fig. 2A and 2E).

Compared with HCs, H1N1 patients were mainly characterized by the enrichment of the phylum Ascomycota and the depletion of an unclassified fungus, which was observed in both the discovery and validation cohorts (Fig. 2A). In the phylum Ascomycota, *Cladosporium*, *Candida glabrata*, *Fusarium proliferatum*, and two species separately belonging to Helotiales and Sordariales were enriched; meanwhile, fungi such as *Aspergillus*, *Penicillium*, *Aspergillus niger*, and *Penicillium polonicum* were depleted in H1N1 patients. In the phylum Basidiomycota, an unclassified species of *Exidiaceae* was enriched, while *Trechispora*, *Rhodotorula mucilaginosa*, *Moesziomyces aphidis*, and *Wallemia sebi* were depleted (Fig. 2B-2E). Interestingly, the phylum Chlorophyta (belonging to the kingdom Plantae) and its species *Trebouxia decolorans* (Fig. 2A, 2C) as well as an unclassified species belonging to the kingdom Chromista were enriched in the gut mycobiota of H1N1 patients (Fig. 2D).

Compared with H1N1 patients, the alterations in the gut mycobiota of COVID-19 patients was mainly characterized by the depletion of the Ascomycota taxa, as observed in both the discovery and validation cohorts (Fig. 2A). The phylum Ascomycota, as well as its member *Candida glabrata*, and five unclassified species separately belonging to Helotiales, Pleosporales, Sordariales, *Microscypha* or *Emericellopsis* were depleted, while *Candida parapsilosis* was enriched in COVID-19 patients (Fig. 2C-2E). In the phylum Basidiomycota, *Cystobasidium* was enriched, while an unclassified species of *Exidiaceae* was depleted. Moreover, *Trebouxia decolorans* and an unclassified species belonging to the kingdom Chromista were also depleted in COVID-19 patients compared to H1N1 patients (Fig. 2C-2E).

The p value of the ANOSIM analysis comparing the gut mycobiota of mild and severe COVID-19 patients ($p = 0.69$), as well as comparing the first sample and a sample after a treatment period in COVID-19 patients ($p = 0.93$), were higher than 0.05. This indicates that the composition of the gut mycobiota was similar in these groups and was not significantly influenced by COVID-19 severity or treatment.

Ability of the gut mycobiota to distinguish HCs, H1N1 patients and COVID-19 patients

In ROC curve analysis, when the area under the ROC curve (AUC) of both the discovery and validation cohort higher than 0.7 (which is indicative of a discriminatory effect) was taken as a threshold, no signal fungal taxa could reliably distinguish COVID-19 patients from HCs or H1N1 patients. *Trebouxia decolorans* distinguished H1N1 patients from HCs or COVID-19 patients, but no signal fungus was able to accurately distinguish COVID-19 patients from H1N1 patients. Remarkably, only *Penicillium polonicum* could distinguish HCs from both H1N1 patients and COVID-19 patients, suggesting that this species is a potential health marker of the gut mycobiota.

Excellent discriminatory ability of the gut mycobiota can be acquired using combined ROC curves. A combination of *Aspergillus proliferans*, *Aspergillus rugulosus*, *Candida parapsilosis*, *Moesziomyces aphidis*, *Mucor racemosus*, *Penicillium steckii*, *Talaromyces sp.*, and two unclassified species separately belonging to Onygenales or an unclassified phylum of Fungus could distinguish COVID-19 patients from

HCs, with an AUC of 0.974 and 0.967 in the discovery and validation cohorts, respectively (Fig. 4A). Furthermore, a combination of *Aspergillus penicillioides*, *Moesziomyces aphidis*, *Penicillium polonicum*, *Wallemia sebi*, and three unclassified species separately belonging to Ascomycota, Helotiales or an unclassified phylum of Fungus could distinguish H1N1 patients from HCs, with an AUC of 0.986 and 0.974 in the discovery and validation cohorts, respectively (Fig. 4B). Moreover, a combination of *Aspergillus penicillioides*, *Candida glabrata*, *Candida parapsilosis*, *Mucor racemosus*, *Penicillium sp.*, *Trebouxia decolorans* and four unclassified species separately belonging to Sordariales, Helotiales, Ascomycota or an unclassified phylum of Fungus could distinguish COVID-19 patients from H1N1 patients, with an AUC of 0.986 and 0.974 in the discovery and validation cohorts, respectively (Fig. 4C).

Functional dysbiosis in the gut mycobiota of H1N1 and COVID-19 patients

In total, 905 functional genes were predicted based on the KEGG database. Compared with HCs, 295 functional genes, such as that encoding allantoate deiminase, were depleted, while 117 genes, such as that encoding saccharolysin, were enriched in the gut mycobiota of H1N1 patients (Fig. 5A). Compared with HCs, 260 functional genes, such as that encoding nardilysin, were enriched and 56 genes, such as that encoding pectin lyase, were enriched in COVID-19 patients (Fig. 5B). Compared with H1N1 patients, 262 functional genes, such as that encoding choline kinase, were depleted while 69 genes, such as that encoding L-lactate dehydrogenase, were enriched in COVID-19 patients (Fig. 5C).

Using a metabolic pathway database (MetaCyc), we identified 75 fungal pathways. Compared with HCs, 7 pathways, such as stearate biosynthesis III, were enriched and 47 pathways, such as fatty acid beta-oxidation, were depleted in the gut mycobiota of COVID-19 patients (Fig. 5D). Compared with HCs, 4 pathways were enriched and 34 pathways were depleted in H1N1 patients. Compared with COVID-19 patients, 5 pathways were enriched and 26 pathways were depleted in H1N1 patients. Pathways such as chitin degradation to ethanol were depleted, while pathways such as L-methionine biosynthesis III were enriched, in H1N1 patients compared to both HC and COVID-19 patients. In addition, pathways such as pyrimidine deoxyribonucleotides de novo biosynthesis I were depleted, while pathways such as 4-amino-2-methyl-5-phosphomethylpyrimidine biosynthesis were enriched, in only H1N1 patients compared to HCs (Fig. 5E and 5F). In contrast, pathways such as the pentose phosphate pathway were enriched, while those such as palmitate biosynthesis I were depleted, only in H1N1 patients compared to COVID-19 patients.

Links between the gut mycobiota and symptoms of H1N1 or COVID-19

To explore the links between the gut mycobiota with COVID-19 or H1N1, we conducted Spearman's rank correlation analysis (Fig. 6). The incidence of diarrhoea, albumin, the peripheral blood absolute

lymphocyte count, erythrocytes, haemoglobin and uric acid were positively correlated with *Aspergillus*, *Penicillium* and its members, such as *Aspergillus proliferans* and *Penicillium polonicum*, indicating that these fungi are closely linked with intestinal homeostasis, immunity and metabolism. Albumin and the peripheral blood absolute lymphocyte count were also positively correlated with *Talaromyces wortmannii*, *Rhodotorula mucilaginosa* and *Wallemia sebi*, suggesting that these two factors are closely linked with the gut mycobiota. ALT, an indicator of liver disease, was negatively correlated with *Moesziomyces aphidis*, while AST was positively correlated with an unclassified species of Sordariales. CRP was negatively correlated with gut fungi such as *Penicillium polonicum*, *Rhodotorula mucilaginosa* and *Wallemia sebi* and positively correlated with *Chlorophyta*, *Emericellopsis sp.* and *Trebouxia decolorans*, indicating that intestinal fungi were closely linked with bacterial infection and inflammation. Interestingly, infection symptoms were positively correlated with only an unclassified species of Chromista that was also the only taxa negatively correlated with platelet counts. The inflammatory cytokines IL-2, IL-4 and TNF- α were positively correlated with only an unclassified species of Ascomycota. Cholesterol was positively correlated with fungi such as *Penicillium* and negatively correlated with fungi such as *Trebouxia decolorans*, indicating that intestinal fungi are closely linked with cholesterol metabolism.

Discussion

Gastrointestinal symptoms such as diarrhoea, vomiting, and abdominal pain were frequently observed in patients with COVID-19 or H1N1. The proportion of our COVID-19 patients with gastrointestinal symptoms was 20.89%, within range from 16.1% to 33.4% in studies from China and other countries, respectively.^{22,23} Meanwhile, gastrointestinal symptoms were observed in 31.43% of our H1N1 patients, which was similar to 30.9% for A(H1N1)pdm09 in a meta-analysis.²⁴ The faecal positivity rate of SARS-CoV-2 RNA in our samples was 34.32%, which ranged from 15.3% to 59%, as previously reported.²⁵ Although we did not measure the positivity rate of H1N1 viruses in faeces, a meta-analysis showed that it was 20.6%.^{22,23} Remarkably, we found that the faecal positivity rate of SARS-CoV-2 RNA was positively correlated with diarrhoea and disease severity.

Gut mycobiota homeostasis plays an important role in maintaining host immune and metabolic functions. In general, *Candida*, *Malassezia*, *Aspergillus*, *Epicoccum*, *Saccharomyces*, *Alternaria*, and *Cladosporium* are the most common gut fungi.²⁶ On the one hand, the long-term colonization of symbiotic fungi such as *Candida albicans* in the gut stimulates the proliferation of systemic fungal-specific Th17 cells and IL-17 feedback through the circulation of neutrophils in the blood, which can help the host fight infections by exogenous pathogens.²⁷ The destruction of lung immunity by antifungal agents such as fluconazole aggravated allergic airway disease in model mice when it reduced gut fungi.²⁸ These results are consistent with our findings showing that most depleted gut fungi are positively correlated with a reduction in blood lymphocytes. On the other hand, symbiotic gut fungi can protect local and systemic immunity by providing complementary microbial stimulation in place of stimulation by bacteria. After the eradication of symbiotic bacteria, the oral administration of *Candida albicans* or *Saccharomyces cerevisiae* can significantly protect mucosal tissue from damage, calibrate the

responsiveness of circulating immune cells, and decrease host susceptibility to colitis and H1N1 virus.¹⁶ Therefore, the depletion of commensal gut fungi in COVID-19 patients or H1N1 patients may lead to the loss of their beneficial functions.

Our results show that some opportunistic pathogenic fungi were enriched in COVID-19 patients, and some of these fungi were particularly enriched in H1N1 patients compared to HCs. For example, *Candida glabrata* is not only the second most common cause of invasive candidiasis of skin, mucosa and even viscera but also accounts for up to 30% of *Candida* bloodstream infections.²⁹ *Candida parapsilosis* is the second or third most frequently isolated *Candida* species from patients with invasive candidiasis.²⁷ *Cladosporium* species were reported to cause various superficial and invasive fungal infections, such as brain abscess and keratitis.³⁰ *Fusarium* species, including *Fusarium proliferatum*, are causative agents of human respiratory disorders, superficial infections and urinary tract infection. Furthermore, extracts of *F. proliferatum* induced a strong release of IL-8 in human lung epithelial cells (BEAS-2B).³¹ Undoubtedly, most of these fungi also live in the gut of some HCs. However, in the case of systemic inflammation, the gut barrier may be damaged, and the enrichment of these fungi increases the risk of infection.

In addition to the lung, the receptors of SARS-CoV-2 and H1N1 are also abundant in the gut, suggesting that viral infection and alterations of mycobiota may be linked in the gut. Angiotensin I-converting enzyme 2 (ACE2), the receptor of both SARS-CoV and SARS-CoV-2, is a key regulator of the renin-angiotensin system (RAS), which is involved in acute lung failure, cardiovascular function and SARS infections.²² Moreover, ACE2 can regulate intestinal amino acid homeostasis, the expression of antimicrobial peptides, and the ecology of gut microbes. The depletion of ACE2 in mice results in highly increased susceptibility to intestinal inflammation induced by epithelial damage. In reverse, the transplantation of the microbiota of *Ace2* mutant mice to germ-free wild-type mice can transmit the increased propensity to develop severe colitis.³² Importantly, SARS-CoV-2 RNA was detected in gastric, duodenal, and rectal epithelia by intracellular staining, demonstrating that SARS-CoV-2 infects these epithelial cells.³³ Similarly, it was reported gastrointestinal epithelial cells were susceptible to H1N1 viruses using sialic acid (SA)- α 2,6-galactose (Gal)-terminated saccharides as the receptor and became apoptotic after infection.³⁴ This observation indicates that gastrointestinal symptoms and alterations in gut mycobiota during SARS-CoV-2 or H1N1 infections may form a pathogenic feedback loop, causing the disease to worsen.

The gut-lung axis plays a key role in linking alterations of the gut mycobiota and lung infection in SARS-CoV-2 and H1N1. The gut-lung axis mainly refers to the cross-talk between the gut microbiota and the lungs.³⁵ On the one hand, components and products of the gut microbiota, such as lipopolysaccharides and short-chain fatty acids, which can be transported via the circulatory system, are important means of communication between the gut microbiota and the lungs. Furthermore, immune cells such as intestinal group 2 innate lymphoid cells, cytokines and even hormones can migrate from the gut to the respiratory system via the circulation.³⁶ Therefore, the depletion of gut fungi with important functions and the enrichment of opportunistic pathogenic fungi may contribute to the development of pneumonia.

Moreover, since more than 60% of human immunity is derived from the gut, the relationship between blood cytokine storms, gut inflammation, and alterations in gut mycobiota in infections such as COVID-19 deserves more attention. On the other hand, the lung can also affect intestinal health and the homeostasis of gut mycobiota. The gut-lung axis resembles a loop that can be stimulated from both sites. There is evidence that some pulmonary infections can directly affect gut immunity. For example, the infection of mice with H1N1/PR/8/34 increased the number of lung-derived CC-chemokine receptor 9-positive CD4⁺ T cells that preferentially migrated to the gastrointestinal tract under the guidance of gut-derived C-C motif ligand 25. This resulted in the outgrowth of *E. coli*, the induction of aberrant Th17 responses, and intestinal damage. Therefore, in addition to direct viral infection in the gastrointestinal tract, pneumonia during SARS-CoV-2 or H1N1 infections may be one of the most important causes of gastrointestinal symptoms and alterations in the gut mycobiota.

The gut mycobiota may be involved in the damage of other organs in addition to the lung during SARS-CoV-2 or H1N1 infections. For example, increased and altered components and products of gut microbes enter the liver through the portal vein, thus causing or aggravating liver damage, especially when the gut barrier is damaged. Furthermore, alterations in the gut mycobiota may impair the enterohepatic circulation of bile acids and damage the liver. Our results showed that the levels of AST, ALT and GGT were significantly increased while albumin was decreased in patients with COVID-19 and/or patients with H1N1, which was reflective of liver injury. Regardless of the causal relationship between liver injury and alterations in the gut mycobiota, their coexistence may form a devastating feedback loop. It was reported that the severity of overall illness and the level of care were associated with AST levels at the time of presentation in a COVID-19 cohort from California, USA.³⁷ In addition, gut microbes are closely related to the health and diseases of organs such as the heart and brain. How alterations in the mycobiota in patients with COVID-19 and H1N1 affects future health deserves further study.

In conclusion, compared with HCs, some fungi with important functions were depleted and several opportunistic pathogenic fungi were enriched in patients with COVID-19 or H1N1, although the alterations differed in these two groups of patients. These altered gut fungi were strongly associated with clinical characteristics such as diarrhoea and the levels of lymphocytes and albumin. Alterations in the gut mycobiota may act as an important indicator of gut homeostasis, which is of great significance for evaluating health. Alterations in the gut mycobiota may have causal relationships with the infection of gut by viruses as well with as gastrointestinal or systemic inflammation due to viral pneumonia. Alterations in the gut mycobiota may not only aggravate pneumonia but may also affect the health of other organs, such as the liver. This study provides new perspectives on the pathogenesis, diagnosis, prevention and treatment of pneumonia after infection with SARS-CoV-2.

Methods

Diagnosis

SARS-CoV-2 or H1N1 infections were diagnosed by reverse-transcriptase polymerase chain-reaction assay using respiratory tract samples. Severe patients were classified according to the Chinese COVID-19 prevention and control guidelines (7th edition)³⁸ or the WHO surveillance case definitions for severe acute respiratory infection.³⁹

Participants and sample collation

This study was approved by the ethics committee of the First Affiliated Hospital, Zhejiang University (IIT2020-136 and 2018-447). All participants provided written informed consent. Sixty-seven COVID-19 patients, 35 H1N1 patients, and 48 healthy controls (HCs) were recruited. Their clinical characteristics were collected. Samples were collected and stored at -80 °C until use.

Analysis of haematological variables and liver, kidney and heart function

Haematological variables, such as lymphocytes, neutrophils, and erythrocytes were tested using a Sysmex XN-2000 (Sysmex, Japan). Serum IL-2, IL-4, IL-6, IL-10 and TNF- α were determined by enzyme-linked immunosorbent assays. Liver function, such as albumin (ALB), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and glutamyl transpeptidase (GGT), were assessed by standard methods using a 7600-210 analyser (Hitachi, Tokyo, Japan), as were kidney and heart function.

Therapeutic regimen

The therapeutic plan for patients was based on the Chinese COVID-19 prevention and control program (7th edition). The anti-viral treatment included oral lopinavir/ritonavir (200 mg/500 mg, bid) and oral Arbidol (200 mg, tid). For some patients, glucocorticoids such as methylprednisolone (40 mg, prn) were used.

Faecal microbial DNA extraction, amplification, sequencing and analysis

Faecal samples were kept at 56 °C for 30 minutes to inactivate viruses. Microbial DNA was extracted using a PowerSoil Pro Kit (Qiagen, California, USA). The partial internal transcribed spacer (ITS) of fungal ribosomal DNA was amplified using ITS3F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4R (5'-GCTGCGTTCTTCATCGATGC-3'). Prepared DNA libraries were sequenced using an Illumina MiSeq platform (San Diego, CA, USA). The taxonomy of each operational taxonomic units (OTUs) representative sequence was identified using the UNITE database.

Study design for mycobiota comparisons

To compare fungal taxa and analyse receiver operating characteristic (ROC) curves, participants were divided into age- and sex-matched discovery cohorts (COVID-19, n = 34 versus HC, n = 23; H1N1, n = 20 versus HC, n = 30; COVID-19, n = 34 versus H1N1, n = 20) and validation cohorts (COVID-19, n = 33 versus HC, n = 23; H1N1, n = 12 versus HC, n = 16; COVID-19, n = 33 versus H1N1, n = 12). In addition, fungal taxa were compared between 36 severe COVID-19 patients and 31 mild COVID-19 patients and between 67 COVID-19 patients and 37 patients who received further treated for nearly one week.

Functional predictions of the gut mycobiota

Functional genes and pathways were predicted using PICRUSt2 based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and the MetaCyc database.

Statistics

If clinical characteristics; haematological variables; liver, kidney and heart function; the Chao1 index; Shannon index; and functional genes and pathway were normally distributed based on the Kolmogorov–Smirnov test, differences between cohorts were compared using one-way ANOVA followed by the Student–Newman–Keuls method. For parameters that were not normally distributed, the Mann-Whitney U test was used. Analysis of similarities (ANOSIM) was performed to determine whether intergroup differences were higher than the intragroup differences. The Wilcoxon rank-sum test was used to evaluate differences in the relative abundance of fungal taxa between groups, and only taxa that were significantly different in both the discovery and validation cohorts are discussed. A two-sided $P < 0.05$ was considered significant.

Declarations

Acknowledgements

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Author contributions

Prof. L. Li and L. Lv designed the study. SG, Yanfei C, RL and CH collected the samples. HJ, HZ and JX performed the molecular biology analysis. L. Lv, RY and BZ analyzed the data and draft the manuscript. LH, Yunbo C, LT, GS contributed to data analysis and modify the manuscript.

Competing interests

The authors declare no conflict of interest.

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Figures

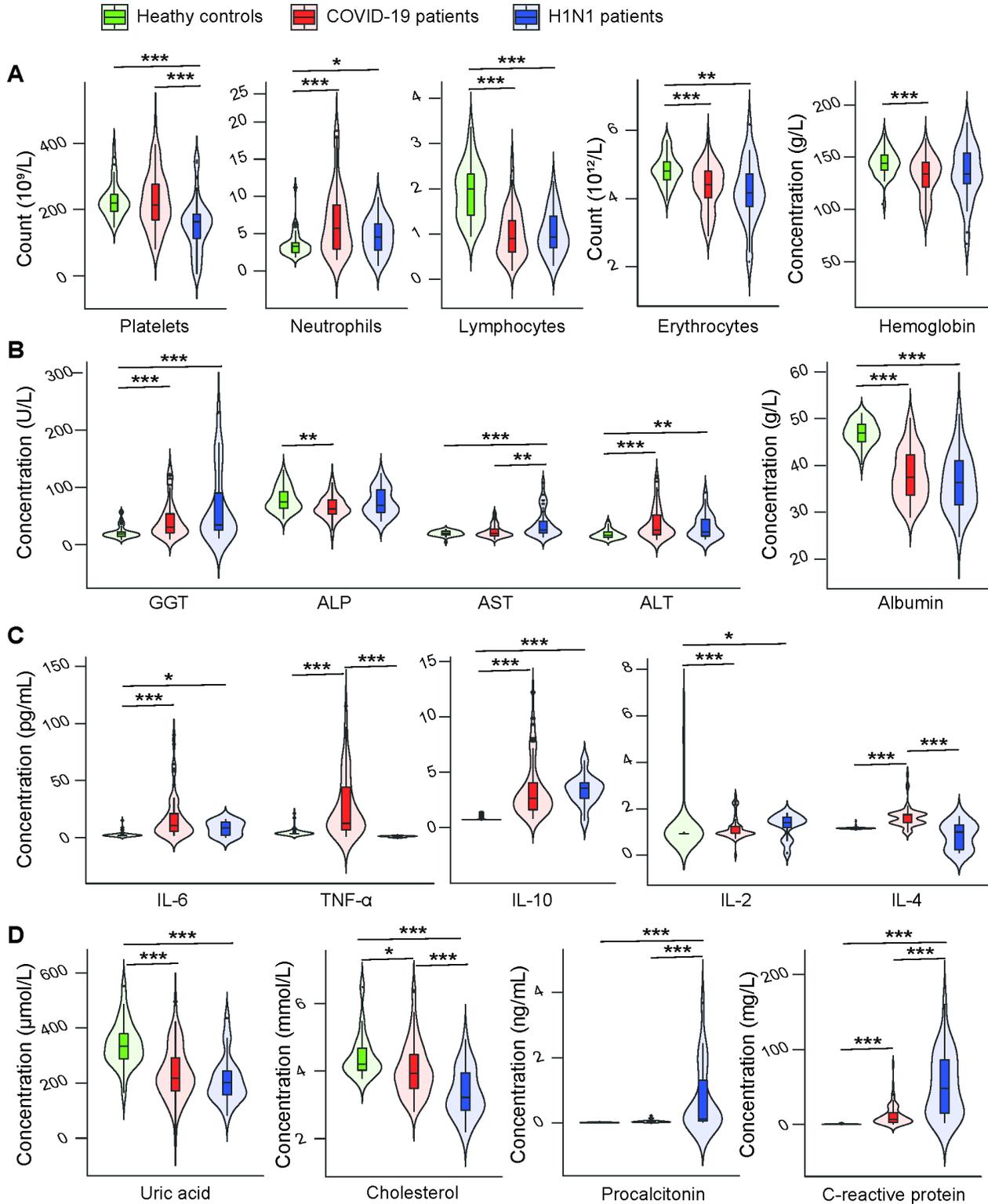


Figure 1

Alterations in inflammatory, hepatic, metabolic and infectious biomarkers in the blood of patients with COVID-19 or H1N1. Significantly altered (A) blood haemocytes, haemoglobin, (B) liver function indicators, (C) inflammatory cytokines, and (D) metabolic and infectious biomarkers in the blood of patients with COVID-19 or H1N1 compared with HC. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

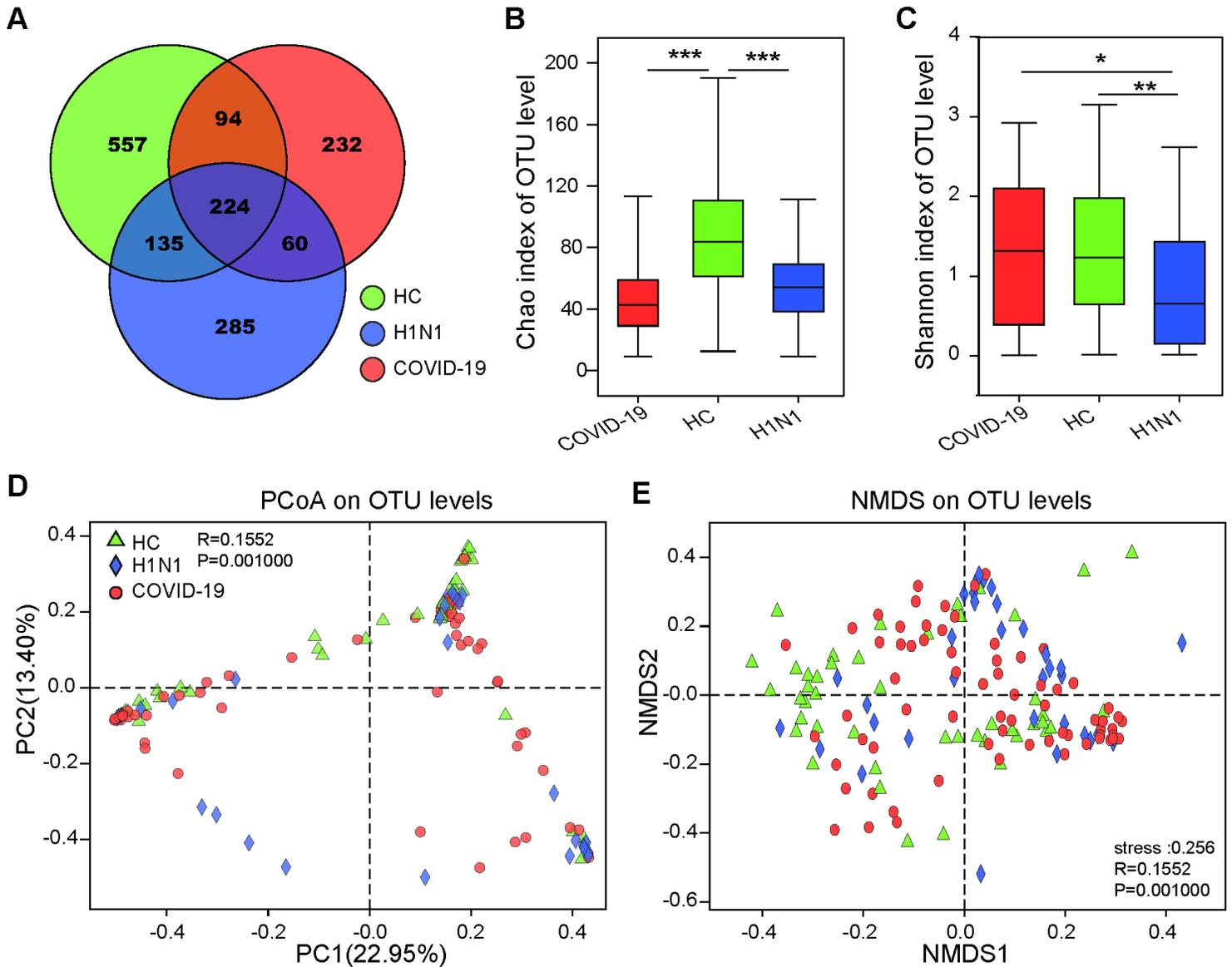


Figure 2

Alterations in the gut fungal diversity in patients with COVID-19 or H1N1. (A) Venn diagram, (B) Chao 1 index plot, (C) Shannon index plot, (D) PCoA plot, and (E) NMDS plot based on the gut fungal OTU of patients with COVID-19, patients with H1N1 and HCs. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

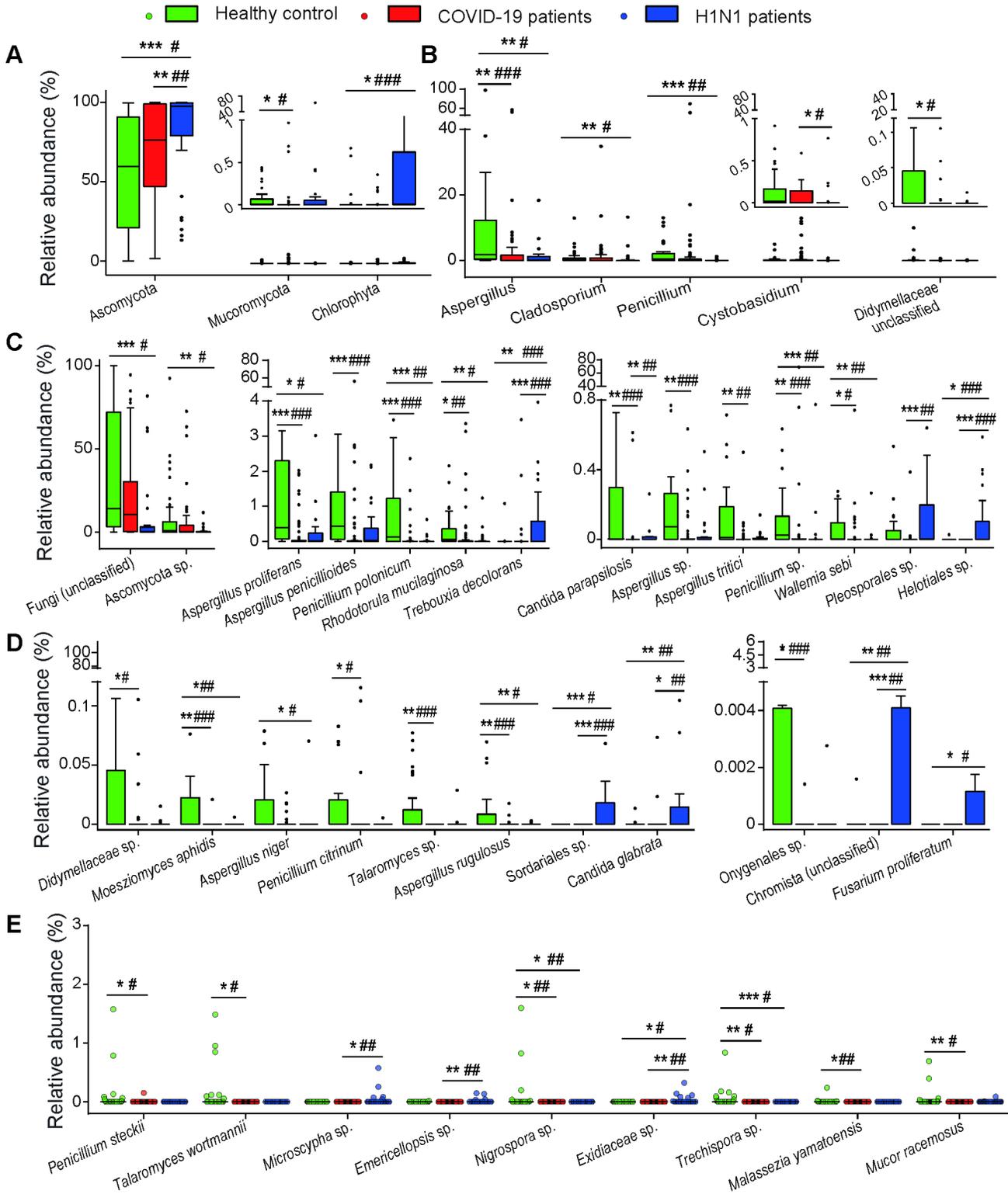


Figure 3

Alterations in gut fungal taxa in patients with COVID-19 or H1N1. (A) Phyla, (B) genera, and (C-E) species that were differently distributed between at least two groups (patients with COVID-19, patients with H1N1 and HCs). The figure was generated by combining data from the discovery cohort and the validation cohort. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ in the discovery cohort; #, $P < 0.05$; ##, $P < 0.01$; and ###, $P < 0.001$ in the validation cohort.

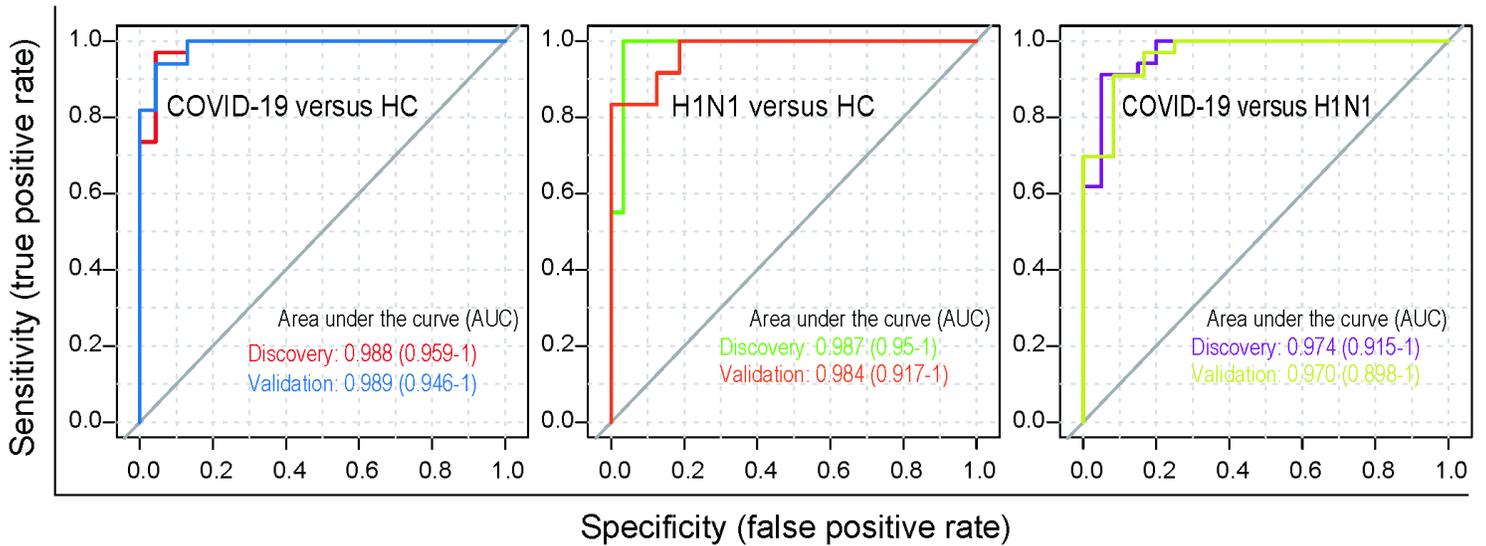


Figure 4

ROC curves of certain combinations of faecal fungal genera and species. ROC curves for classifiers based on (A) 9 faecal fungal genera and species differentiating patients with COVID-19 and HCs, (B) 7 faecal fungal genera and species differentiating patients with H1N1 and HCs, and (C) 10 faecal fungal genera and species differentiating patients with COVID-19 and patients with H1N1 in the discovery and validation cohorts.

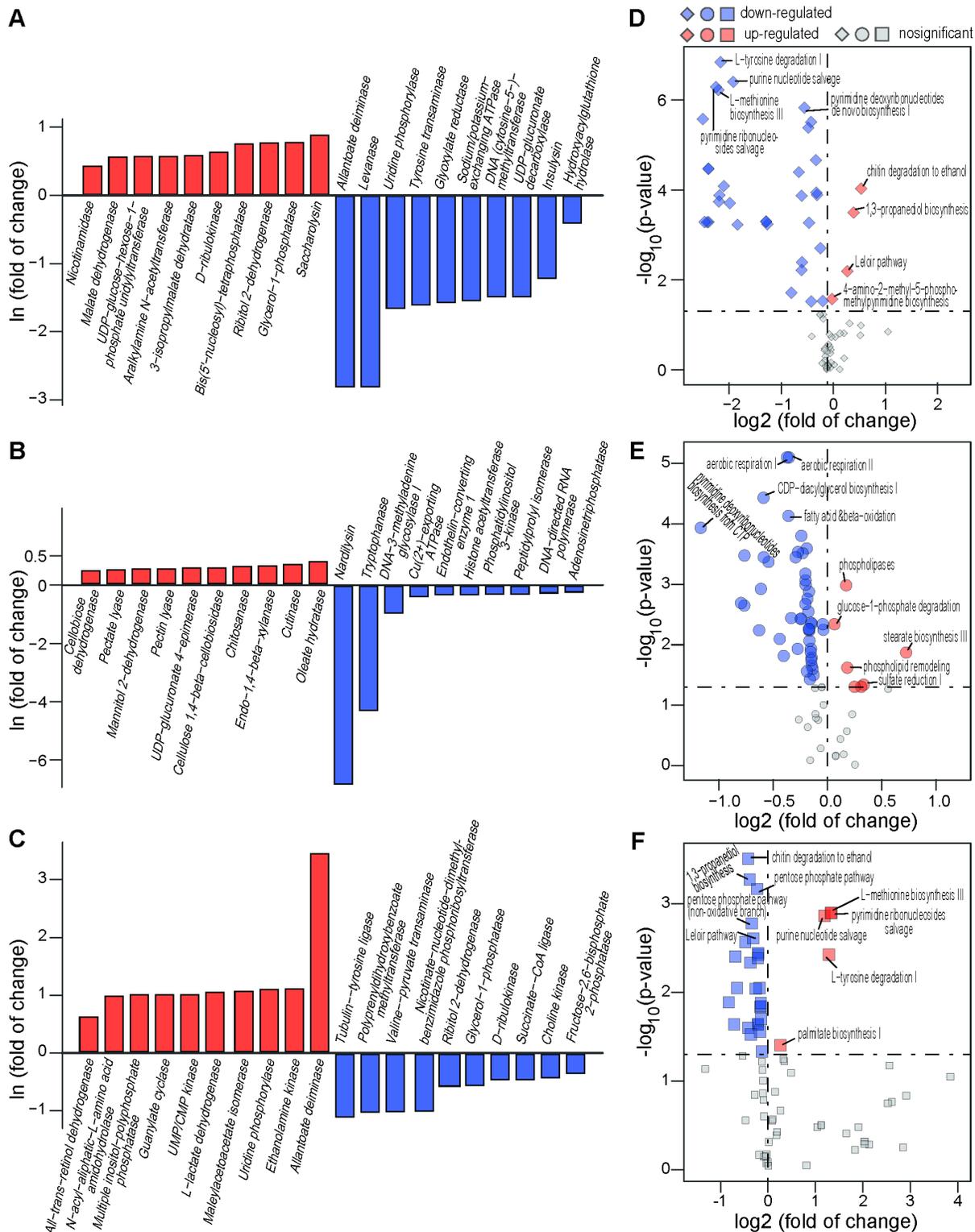


Figure 5

Altered functions in the gut mycobiota of patients with COVID-19 or H1N1. Alterations in functional proteins of the gut mycobiota predicted based on the KEGG database (A) in COVID-19 patients versus HCs, (B) in H1N1 patients versus HCs, and (C) in COVID-19 patients versus H1N1 patients. Only the top 10 most significantly enriched or depleted proteins are shown. Alterations in metabolic pathways of the gut mycobiota predicted based on the MetaCyc database (D) in COVID-19 patients versus HCs, (E) in H1N1

patients versus HCs, and (F) in COVID-19 patients versus H1N1 patients. Only the top 5 most significantly enriched or depleted pathways are listed.

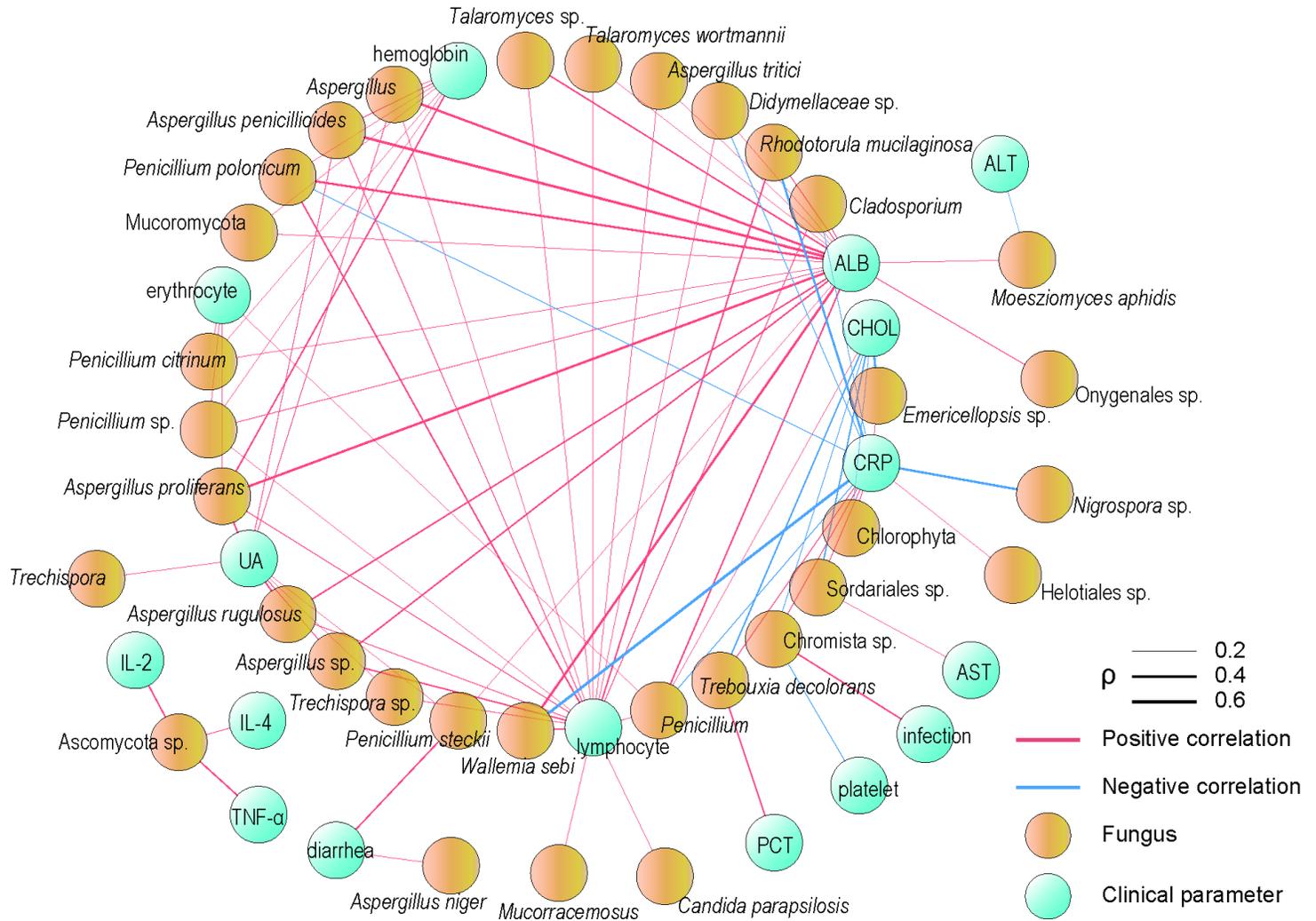


Figure 6

Altered fungal taxa are strongly linked with inflammatory, liver, infectious and metabolic biomarkers. Only correlations of fungal taxa with symptoms or biomarkers, but not correlations among different fungal taxa and among different biomarkers, are shown.