The immunomodulatory effects of Qushi Jianpi Hewei Decoction (QJHD) for patients with COVID-19 by metagenomics and transcriptomic sequencing

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ABSTRACT

Ethnopharmacological relevance: Several studies have confirmed that intestinal microflora dysbiosis correlates with the severity of COVID-19 patients. Clinical meta-analysis and our data show that the circulating miRNAs like miRNA-146 and the levels of serum cytokines in the peripheral blood are closely related to mild to moderate COVID-19 patients. Despite the widespread use of traditional herbal medicine for COVID-19 in China, the mechanisms remain largely uncovered.

Aim of the study: We conducted an observational case-control study to verify the efficacy and safety of traditional Chinese herbal medicine Qushi Jianpi Hewei Decoction (QJHD) for mild to moderate COVID-19 patients, and investigated the potential biomolecular mechanisms through metagenomics and transcriptomic sequencing methods.

Materials and methods: QJHD was given orally twice a day individually for 14 to 28 days. A total of 10 patients were enrolled in the study and given QJHD. We observed advantages in clinical cure time rate, and the relief of gastrointestinal symptoms as compared with reports in the literature. The metagenomics sequencing data of fecal microflora and transcriptomic sequencing data of blood cell in patients with SARS-Cov-2 infection patients were selected compared to the healthy control donors.

Results: No serious adverse events were reported. Meanwhile, the transcriptome analysis showed a decrease of the hsa-miR-21-5p expression in peripheral blood without QJHD. The species composition analysis showed an increase in the expression of *Faecalibacterium prausnitzii* in the intestinal tract; The interleukin-10 (IL-10) expression also in COVID-19 patient decreased in peripheral blood compared with healthy control donors. And we found an improvement in these parameters in patients taking QJHD.

Conclusions: Our findings show that QJHD could improve clinical outcomes of mild to moderate COVID-19 patients, probably through beneficial immunomodulatory effects by regulating *Faecalibacterium prausnitzii* in the intestinal tract and hsa-miR-21 and IL-10 expression in peripheral blood. (chictr.org.cn, ChiCTR2000030305)

Introduction

The novel coronavirus pneumonia has been diagnosed in more than 144 million cases worldwide, with a total death toll of 306 million according to the real-time statistics by Johns Hopkins University as of April 21, 2021. Traditional Chinese medicine, including acupuncture, has been widely used in the treatment of inflammatory diseases [1]. According to the research on virus infection and regulation of immune function, it is found that remdesivir may have therapeutic effect on pneumonia caused by SARS-Cov-2 infection [2]. Other experts found

that chloroquine, which regulates the immune function of the body, can inhibit the replication of SARS-Cov-2 [3]· However, the antivirus drugs have certain side effects, such as liver function damage, gastrointestinal reactions, which limits their clinical application. Intestinal microecology is the body's huge immune system, which plays an important role in regulating the body's immune function [4], suggesting that it should not be ignored in the prevention and treatment of COVID-19.

Traditional Chinese medicine has a variety of curative effects such as regulating intestinal micro-ecology, immune function, and antiinflammatory [5], with minor side effects, extensive safety range, and definite curative effect. According to traditional medicine, SARS-Cov-2 infection belongs to the category of "pestilence" or "Yidu-toxicity" [6]. It always hurts the lung and stomach, invades the upper and middle Jiao, traps the abdominal with dampness-evil, obstructs the stomach, and shows symptoms such as loss of appetite, fatigue, and diarrhea. In the traditional Chinese medicine treatment, our prescriptions such as Heat-clearing and Detoxifying, invigorating the intestinal immune organs and removing dampness-evil of gut, and harmonizing the liver and stomach are often used. A randomized controlled trial showed the use of Huoxiang Zhengqi dropping pills [7] and Lianhua Qingwen granules (LQG) combined with western medicine may have clinical advantages for COVID-19 patients in improving clinical symptoms, such as fever and coughing, reducing utilization rate of anti-infective drugs, and improving patient prognosis. Therefore, the LQG [8] and Qingfei Paidu decoction [9] already have been proved effective in treating the pandemic disease of COVID-19.

At present, more than half of the patients with SARS-Cov-2 infection are accompanied by digestive tract symptoms, especially severe patients [10]. Based on the traditional Chinese medicine prescriptions recommended by the national and Zhejiang Administration of traditional Chinese medicine, the prescription of Qushi Jianpi Hewei Decoction (QJHD) was made, and might affect the host immune system through reconciling conflicts of "Lung and Large intestine" and play a positive immunomodulatory role in treating of pneumonia. We thus speculate that QJHD may improve the antiviral ability of the body, but the mechanism of further specific efficacy is not clear, which is very worthy of in-depth study.

Previously multi-omics study has shown that angiotensin-converting enzyme 2 (ACE2) enriched in the intestinal tract more specifically, in the epithelium could mediate the entry of SARS-CoV-2 into cells to accumulate and cause cytotoxicity but does not respond to nonsteroidal anti-inflammatory drugs [11]. The peripheral blood mononuclear cells (PBMCs) changes of COVID-19 patients were found, that SARS-Cov-2 infection leads to systemic Mo/MΦ activation and then release of proinflammatory mediators [12,13]. Moreover, miR-146a-5p, miR-21-5p, miR-142-3p, and miR-15b-5p as potential contributors to the disease pathogenesis, which consistently the transcriptome profiles suggested hyper-activation of the immune response, loss of T-cell function, and immune dysregulation in severe patients [14]. Furthermore, a cohort study results data showed that serum concentration of miR-21, miR-155, miR-208a, and miR-499 were significantly increased in critically ill COVID-19 patients compared to healthy controls [15]. In addition, the host immune-response axis independently aligns with the major plasma composition changes, with clinical metrics of blood clotting, and with the sharp transition between mild and moderate COVID-19 [16]. Recently, the level of IL-10 expression in the serum of blood is considered to be useful prognostic biomarkers, to guide therapeutic strategies for COVID-19 [17,18].

The accomplished registered clinical trials on traditional Chinese medicine (TCM) for coronavirus disease 2019, such as Qingfei Paidu decoction, Huashi Baidu decoction, LQG and Xuebijing injection were to be tested for their therapeutic effects and symptoms relief [19]. However, the mechanism of Traditional Chinese medicine treatment on gastrointestinal tract symptoms of COVID-19 remains to be further elucidated.

Based on the clinical treatment and observation of SARS-CoV-2 infection in our hospital, this present study intends to investigate COVID-19 patients with QJHD combined with antiviral medication and the antiviral medication without QJHD in literature [20] at the same period and same location mainly by fecal metagenomics, whole peripheral blood transcriptomics, and enzyme-linked immunosorbent assay (ELISA) methods. A case-control study is adopted to observe the clinical characters of patients after treatment and analyze the correlation between these data and pulmonary symptoms. To verify the curative effect of invigorating the intestinal immune organs and removing dampness-evil of the gut, and forming a landscape of the immunomodulatory network for the patients with SARS-CoV-2 infection, it will have been great theoretical, practical, and scientific significance as promising therapeutic strategies and drug development to reveal the mechanism of the inflammatory reaction for COVID-19 patients.

Materials and Methods

Trial Design

The following Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) checklist and the Recommendations for Interventional Trials and 2013 statement for herbal interventions [21–23]. We rigorously followed the Consolidated Standards of Reporting Trials (CONSORT 2017) for Chinese herbal medicine recommendations [24]. The schematic diagram of study procedures as illustrated in Fig. 1.

Inclusion criteria: for patients with mild to moderate COVID-19 symptoms (inpatients) 1. Confirmed infection using PCR method. 2.Diagnosed mild to moderate pneumonia using CT imaging, requiring hospitalization. 3.Hospitalized ≤72 hours. 4. Patients with gastrointestinal symptoms.

Exclusion criteria: 1. Severe and critical pneumonia due to COVID-19. 2. Underlying diseases, including AIDS, asthma, carcinoma, neuropsychiatric disorders and severe liver and kidney disease. 3. Use of anticoagulants and ACE inhibitors (e.g., captopril). 4. History of drug allergy to QJHD. 5. Pregnancy or breastfeeding.

Diagnostic Criteria and different subtypes of COVID-19

Reverse transcription-polymerase chain reaction (RT-PCR) is an extremely common clinical method for COVID-19. The detection of the RNA from the novel coronavirus SARS-CoV-2 is the gold standard for establishing a COVID-19 diagnosis [25]. Patients with COVID-19 were divided into four subtypes according to the degree of disease severity, based on the diagnosis and treatment scheme for SARS-CoV-2 of Chinese (fifth edition). The mild type is defined as having slight clinical symptoms without pneumonia on radiography. The common type is defined as presenting with fever, respiratory tract, and other symptoms, pneumonia on radiography. The severe type is diagnosed according to dyspnoea (respiratory rate (RR) ≥30 times/min), resting finger oxygen saturation ≤93%, artery PaO2/FiO2≤300mm Hg (1 mm Hg=0.133kPa). The critical type is defined as respiratory failure with shock and multiorgan failure requiring mechanical ventilation and intensive care unit (ICU) admission [26].

Treatments

We used the basic prescriptions Qushi Jianpi Hewei Decoction as follows: *Pinellia* Tuberifera Tenore (Ban Xia, *Rhizome Pinelliae*) were collected from the province of Guizhou, China; Officinal Magnolia Bark (Hou Po, *Magnoliae Officinalis Cortex*) from the province of Sichuan, China; Indian Buead (Fu Ling, *Poria*) from the province of Anhui, China; Largehead Atractylodes Rh (Bai Zhu, *Atractylodis Macrocephalae Rhizoma*) from the province of Zhejiang, China; Ma - yuen Jobstears Seed (Yi Yi Ren, *Coicis Semen*) from the province of Guizhou, China; Cablin Potchouli Herb (Guang Huo Xiang, *Pogostemonis Herba*) from the province of Guangdong, China; Fortune eupatorium herb (Pei Lan, *Herba Eupatorii*) from the province of Jiangsu, China; Dan-Shen Root (Dan
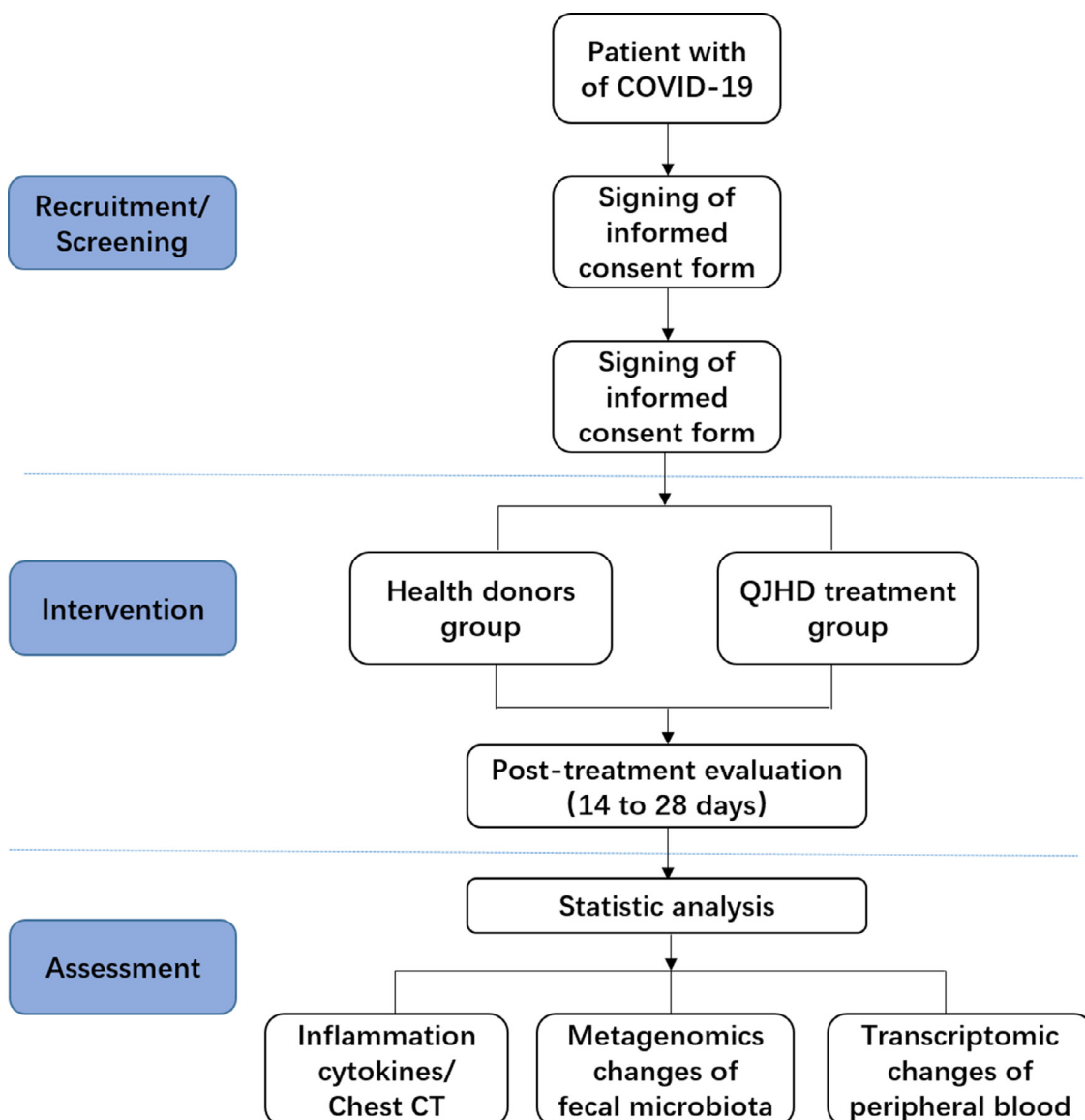
Fig. 1. Flow chart of the study design.

Shen, *Salviae Miltiorrhizae Radix et Rhizoma*) from the province of Gansu, China; Milkvetch Root (Huang Qi, *Astragali Radix*) from the province of Gansu, China; Baical Skullcap Root (Huang Qin, *Scutellariae Radix*) from the province of Neimenggu, China; Chinese Thorowax Root (Chai Hu, *Bupleuri Radix*) from the province of Shanxi, China; Gromwell Root (Zi Cao, *Arnebiae Radix*) from the province of Xinjiang, China; Coptis Root (Huang Lian, *Coptidis Rhizoma*) from the province of Sichuan, China; Chinese date (Da Zao, *Jujubae Fructus*) from the province of Xinjiang, China; Fresh Ginger (Sheng Jiang, *Rhizoma Zingiberis Recens*) from the province of Zhejiang, China. The quality of crude drugs was strictly performed according to Good Manufacturing Practice for Drugs to guarantee quality control (Chinese FDA). Furthermore, these species were authenticated by Prof. Yuyan Zhang (Zhejiang University of Chinese Medicine) before use. The Pharmacy Department provided all crude drugs of Qushi Jianpi Hewei Decoction (QJHD), Fourth Hospital Affiliated to Zhejiang University of Medical School (Yiwu, China). They were purchased from the Yiwu Sanxitang Pharmaceutical Co., Ltd. QJHD was administrated at the first 14 days with antiviral medication, as well as QJHD was used without antiviral medication in the last 14 days. The patients received antiviral treatment, including interferon-α2b sprays, arbidol hydrochloride two tablets three times daily and

lopinavir/ritonavir (400mg/100mg) twice daily [27]. The course of treatment was 14 to 28 days individually.

Preparation of QJHD extract

The dried herbs, including Ban Xia, Hou Po, Fu Ling, Bai Zhu, Yi Yi Ren, Guang Huo Xiang, Pei Lan, Dan Shen, Huang Qi, Huang Qin, Chai Hu, Zi Cao, Huang Lian, Da Zao and Sheng Jiang at the ratio of 3:4:5:10:10:4:3.33:3:3:3.33:3:5:2:5:3 (w/w/w/w/w/w/w/w/w/w/w/w/w/w/w), were firstly soaked in distilled water of 10-fold volumes of herbs (v/w) for 30 min and then extracted by decoction for 2 times. After filtration, the solution was evaporated with a final density of 0.05 g/mL, and stored at 4°C for further use.

Phytochemical characterization of QJHD extract by HPLC analysis

QJHD extract by ultrasonic was dissolved in water and/or ethanol and filtered by a 0.45 μm millipore filter for further use. HPLC analysis was implemented on Thermo Fisher U3000 (DAD/VWD) HPLC system equipped with a Welch Ultimate LP C18 column (4.6 mm × 250 mm,

5μm) at a wavelength of 294 nm, and the mobile phase was s gradient system consisted of water containing 0.1% (v/v) phosphoric acid (A) and methanol (B) with a flow rate of 1 mL/min: (30:70, v/v) for magnolol. The same HPLC system equipped with a Welch Ultimate LP C18 column (4.6 mm × 250 mm, 5μm) at a wavelength of 280 nm, the mobile phase was s gradient system consisted of water containing 0.1% (v/v) phosphoric acid (A) and methanol (B) with a flow rate of 1 mL/min: 0 min, A–B (60:40, v/v); 20min, A–B (35:65, v/v); 22-30 min, A–B (15:85, v/v); 31 min, A–B (60:40, v/v); 31–45 min, A–B (60:40, v/v) for baicalin.

Inflammatory Cytokines Expression in peripheral blood

Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-10 (IL-10) were detected by ELISA method; The blood cell count (WBC), lymphocyte count(LYM), and C-reactive protein (CRP), liver function, kidney function, electrolyte, and immunoglobulin were recorded and compared between the two different groups. Chest computed tomography imaging scan was performed for observation of pulmonary inflammation [28].

To compare the metagenomics changes of fecal microflora in patients with SARS-Cov-2 infection in different groups

Collection of samples

In this study, the faecal samples were obtained from 10 COVID-19 patients (named as F1, 2, 6, 8, 13, 15, 16, 17, 21,25 respectively) and 4 healthy donors (named as F26, 27, 29, 30 respectively) at the Fourth Affiliated Hospital, School of Medicine, Zhejiang University.

Extraction of metagenomics DNA from feacal samples

The DNA of faecal samples (0.25 gram) were extracted from all donors by using PowerSoil ® DNA Isolation Kit (Mo-Bio, cat. no. 12888) . The faecal was place in PowerOil Bead Tube and mix gently. After that, put 60mL dissolving solution C1 at 60 ˚C. Next, Vortex for 1 minute and store vertically at -20 ˚C. Subsequently, the quantity and quality of DNA were assessed using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Qubit was used for accurate quantification of DNA concentration that above 15ng per μL. The size of the inserted fragment was detected by capillary electrophoresis. After the size of the inserted fragment met the expectation, the quantitative PCR method was used to quantify the effective concentration of the library accurately (> 3nM), to ensure the quality of the library. The sequencing libraries were created with a TruSeq DNA Sample Prep Kit (Illumina, USA) according to the manufacturer's protocol. The libraries were sequenced on an Illumina HiSeq X Ten platform. The metagenomic DNA from faecal samples was collected and stored in a faecal collector containing a DNA stabilizer and a refrigerator at - 80 ˚C.

Species notes

To study the species composition and diversity information of samples, we used kraken2 (v2.0.7-beta) [29] to annotate and classify all valid sequences of all samples. Clean data was compared with the species sequences in the database to analyze the composition of microbial communities (bacteria, archaea, eukaryotes, and viruses). After the classification data were classified, the level of the species abundance information was reestimated by Bayes posterior distribution, and namely Kingdom, phylum, class, order, family, genus and species.

Species composition analysis

The community structure composition and a pie or histogram chart of species composition was drawn in Fig. 2.

Species diversity analysis

Shannon index, or Shannon entropy index or Shannon Wiener index, takes into account species abundance and evenness. Simpson index also considers species richness and evenness, but it is more affected by evenness than the Shannon index. The microbial species diversity was characterized by the Shannon index and Simpson method.

Anosim analysis

Anosim analysis, also known as similarity analysis, is a nonparametric test, which is mainly used to analyze the similarity between groups of high-dimensional data. It is often used in ecological data analysis to evaluate the overall similarity of two groups of experimental data and whether the similarity is significant. The similarity between samples is calculated. The commonly used methods are Bray Curtis, Euclidean. The similarity matrix is obtained and R-value is calculated. Then calculate the p-value by permutation test.

Linear discriminant analysis effect Size

Linear discriminant analysis effect size (LEfSe) is an analysis tool for discovering and interpreting biomarkers (taxon, pathway, gene, et al) of high latitude data. It can realize the comparison between two or more groups, and can carry out comparative analysis among subgroups within groups, to find species with the significant differences in abundance between two groups. Firstly, a nonparametric Ruskal-Wallis rank sum was used to detect the species with the significant differences in abundance among different groups. Then, the Wilcoxon rank-sum was used to test the different consistency of different species in different subgroups. Finally, linear regression analysis (LDA) was used to estimate the impact of species abundance on the different effect.

Target genes assembly, genes prediction, and gene set construction

Megahit (v1.1.2) is a de-novo second-generation sequencing assembly tool [30]. It has the characteristics of fast assembly speed, longer overlap group N50 and average overlap group length. Megahit software was used to assemble clean data of all samples after removing host genes (default assembly parameters of megahit) to obtain contings. Metagenemark (v.4.30) is a member of GeneMark family of gene prediction tools [31]. It is a dedicated ab initio prediction tool for macro genome based on the Hidden Markov model (HMM). There are many redundant genes in the predicted gene set of each sample. Through CD hit (v4.5.7) software [32], the redundant gene set is obtained by removing the redundancy of the gene set. The clean data of each sample is compared with the gene set after the redundancy is removed, and the gene abundance is calculated for the subsequent function annotation analysis.

Species functional note analysis

The functional note analysis of species was selected in this study. Furthermore, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed for these species.

To compare and analyze the differences of transcriptomics in peripheral blood of patients with SARS-Cov-2 infection in different groups

Peripheral blood samples were collected from ten COVID-19 patients (named as X1~X10, respectively) classified to E group and four health control donors (named as X11~X14, respectively) classified to A group, and RNA sequencing technology was used to detect the transcriptome data at the Fourth Affiliated Hospital, College of Medicine, Zhejiang University.

RNA extraction and quantification

The whole blood (2 mL) was extracted from all donors by using BD PAXgene blood RNA tubes (BD, cat. no. 762165). The blood was mixed up and down 8-10 times. Then PAXgene tubes were incubated at room
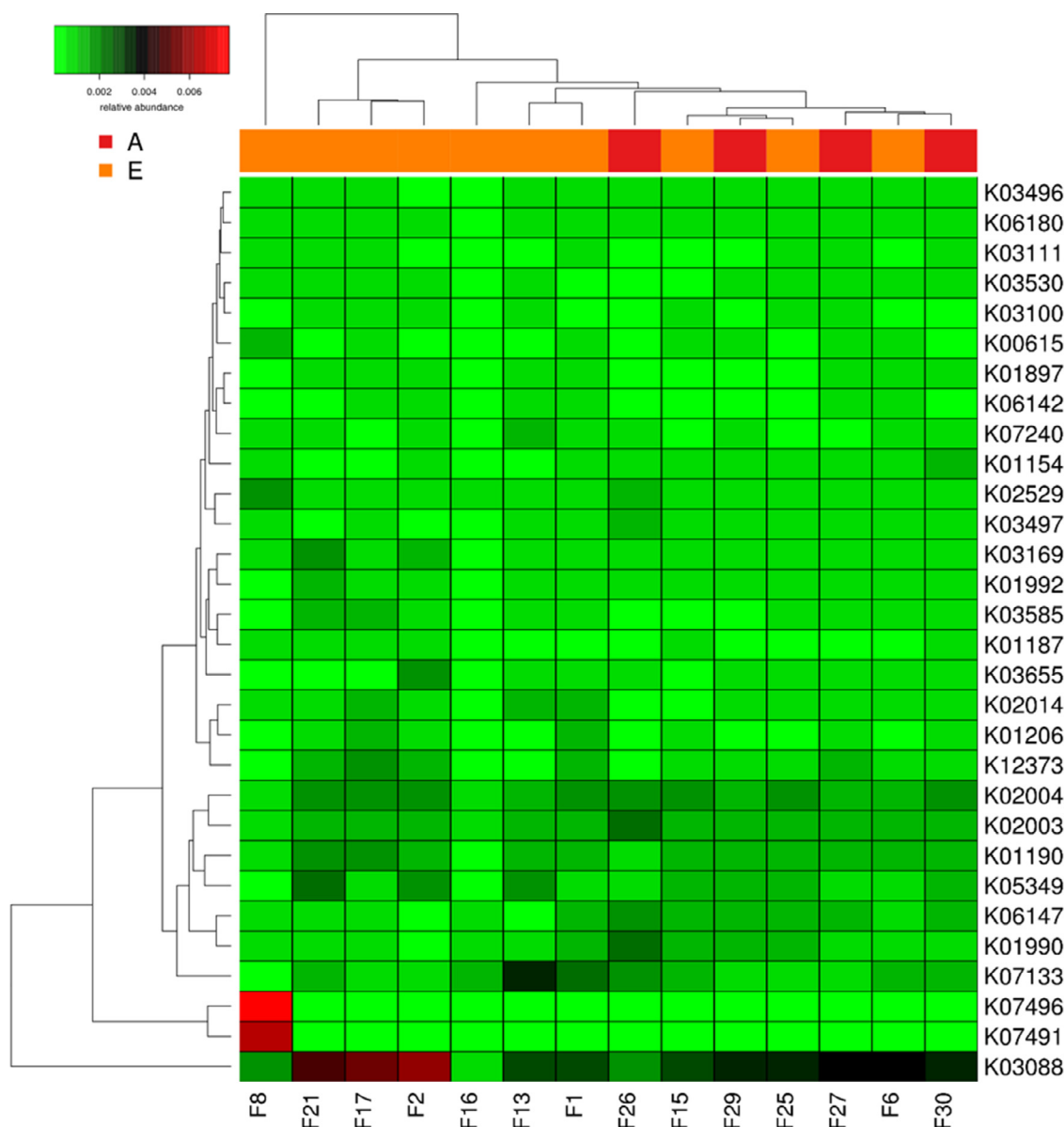
Fig. 2. The faecal microbiota function analysis Heatmap between Group A and Group E.

temperature for 2 hours at least. Next, the total RNA was extracted using the TRIzol reagent (Invitrogen, USA) following the manufacturer's protocol [28]. The purity of RNA was determined by the photometer. In addition, RNA integrity was evaluated by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA) and biochemical analyzer 2100 system (Agilent, CA, USA). Samples with RNA integrity score higher than 8 were used in this study.

Library preparation and sequencing

1 μ g qualified total RNA samples were ligated with 3 'adaptor; RT primers were added after ligation; 5′ adaptor was ligated after reaction; then the products which had been ligated on both sides were reverse transcribed; after reverse transcription, some products were taken for PCR amplification. After PCR, the product was detected by electrophoresis with 6% PAGE gel, and the target product was a single band of 140 BP. The qualified library was cut and recovered with 6% PAGE glue, and the recovered product was the final library. The sequencing platform was the Illumina HiSeq X Ten system and the sequencing mode was PE75.

Filtering of Raw data reads of miRNAs and mRNAs

The fastp software is used to filter raw data for quality control [33]. In order to ensure the accuracy of subsequent analysis, the miRNAs raw data needs to be filtered to remove the primers and adaptors, low-quality areas, and length less than 18 base pairs (bp) or more than 36 bp that affect the data quality and subsequent analysis. For mRNAs, the sequences with fragment length less than 35 bp and 12 bp from the front of R1 were removed.

Identification of miRNA and Screening of DE miRNAs and DE mRNAs

The expression level of each mRNA was calculated according to the fragments per kilobase of the transcript per million mapped read (FPKM) method. And the expression level of each miRNA was analyzed with the reads per million (RPM) method. The miRNAs were initially identified with miRdeep2 software (https://github.com/rajewsky-lab/mirdeep2) [34]. The expression level of each miRNA was normalized with the reads per million (RPM) method. Differential expression analysis of mRNAs and miRNAs was performed with the DESeq2 R package (version 3.12, http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html). Transcripts with a p value<0.05 and|log$_2$fold change(FC)|$\neq$0

Table 1
Epidemiologic and clinical characteristics of different treatments in patients with COVID-19.

	Health donors (n = 4)	Antiviral medication without QJHD (n = 62)	Antiviral medication with QJHD (n = 10)	Antiviral medication with LQG (n = 284)
Age (years, mean± SD)	34:75 ± 11:84	null	44:90 ± 19:94	
Male, no. (%)	1 (25)	35 (56)	4 (40)	
Female, no. (%)	3 (75)	27 (44)	6 (60)	
Signs and symptoms				
Fever		48 (77)	5 (50)	
Cough		50 (81)	6 (60)	
Myalgia or fatigue		32 (52)	4 (40)	
Sputum production		null	3 (30)	
Sore throat		null	1 (10)	
Stuffy nose		null	2 (10)	
Gastrointestinal (GI) symptom				
Abdominal pain		null	1 (10)	
Diarrhoea		3 (8)	null	
Bilateral involvement on chest radiography		52 (84)	3 (30)	
White blood cell count, (×10^9/L, mean ± SD)				
Leukocyte	7.40 ± 1.4	null	5.19 ± 1.7*	
Lymphocyte	37.35 ± 4.29	null	24.02 ± 8.58*	
C-reactive protein, (mg/L)	1.00 ± 0.75	null	12.61 ± 16.61	
Tumor necrosis factor α (TNF-α), (pg/mL)	10.35 ± 2.40	null	44.98 ± 47.67	
Interleukin-6, (pg/mL)	3.24 ± 2:82	null	15.09 ± 20.81	
Interleukin-10, (pg/mL)	5.13± 1.2	null	3.47± 0:89*	
Treatment				
Arbidol+interferon alpha inhalation		1 (2)	1(10)	
Arbidol+lopinavir/ritonavir		17 (28)	8(80)	
Arbidol+lopinavir/ritonavir+probiotics		null	4(40)	
Antibiotics		28 (45)	4 (40)	
Prognosis				
Clinical cure rate (%)			80	78.9
Time to recovery of fever			null	2
Time to recovery of coughing			7	3
Time to recovery of abdominal pain			3	null
Time to recovery of watery stools			10	null
Time to recovery of anorexia			5	null
Time to recovery of fatigue			6	7
Median time to symptom recovery after treatment (days)			6.5	10

were considered as DE miRNAs. The False Discovery Rate (FDR) level < 0.01 or 0.05 was taken as the default standard. And the hierarchical clustering analysis was drawn on the DE miRNAs by using the R package pheatmap.

Target Gene Prediction and Functional Enrichment analyses of DE miRNAs

To investigate the function of DE miRNAs, we predicted the target genes of all DE miRNAs. the miRanda (http://www.microrna.org/microrna/home.do) was used to predict the target genes of DE miRNAs [35]. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed for these DE miRNAs target genes and DE mRNAs by R package clusterprofiler (http://bioconductor.org/packages/release/bioc/html/clusterProfiler.html) [36].

Statistical Analysis

Statistical analysis will be performed using SPSS version 20.0 for Windows (Chicago, IL, USA). Professional statisticians who are independent of all the other processes of the study will perform the statistical analyses. Consistent with the CONSORT statement and intention to treat principle, the last observation carried forward method will be used for missing values. Inter-group comparisons will be analyzed by appropriate methods: independent t-tests (homogeneity of variance, normal distribution) for qualitative data. Results were expressed as the mean values±standard deviation. One-way ANOVA and Tukey's post hoc test was used to compare the counting data between groups *P*-value <0.05 will be considered statistically significant.

Results

Chemical characteristics of QJHD extract

High performance liquid chromatography diode-array detector (HPLC-DAD) was employed to isolate and collect QJHD extract, given the fact that flavone and saponin are the main potential bioactive components of QJHD. HPLC analysis was implemented in order to investigate the chemical characteristics of the obtained QJHD extract using 80% (v/v) ethanol as eluent. To acquire separation selectivity and high efficiency in HPLC analysis, the mobile phase composition (methanol), and its additives (0.1% phosphoric acid) were optimized. Specifically, the contents of magnolol and baicalin in QJHD extract were 3.81, and 39.44 mg/g, respectively.

Clinical characteristics of COVID-19 Patients

By the end of February 2020, 10 admitted hospital patients were identified as laboratory-confirmed SARS-CoV-2 infection in Yiwu city, in the middle of Zhejiang province, settled in the east of China in the current study. Fever (50%), cough (60%), and myalgia or fatigue (40%) were the most common symptoms. According to Chest computed tomography scan findings, 3 patients (30%) presenting with bilateral pulmonary infiltration. In blood cell counts, the numbers of leukocytes were significantly decreased in COVID-19 patients (P=0.041), compared to healthy control (CTR), while the lymphocytes were below the levels of healthy donors (P=0.013). There were no differences in the levels of CRP and TNF-α), and IL-6 between COVID-19 and CTR participants in this study. The level of IL-10 was decreased in COVID-19 patients compare to CTR (P=0.013) in Table 1.
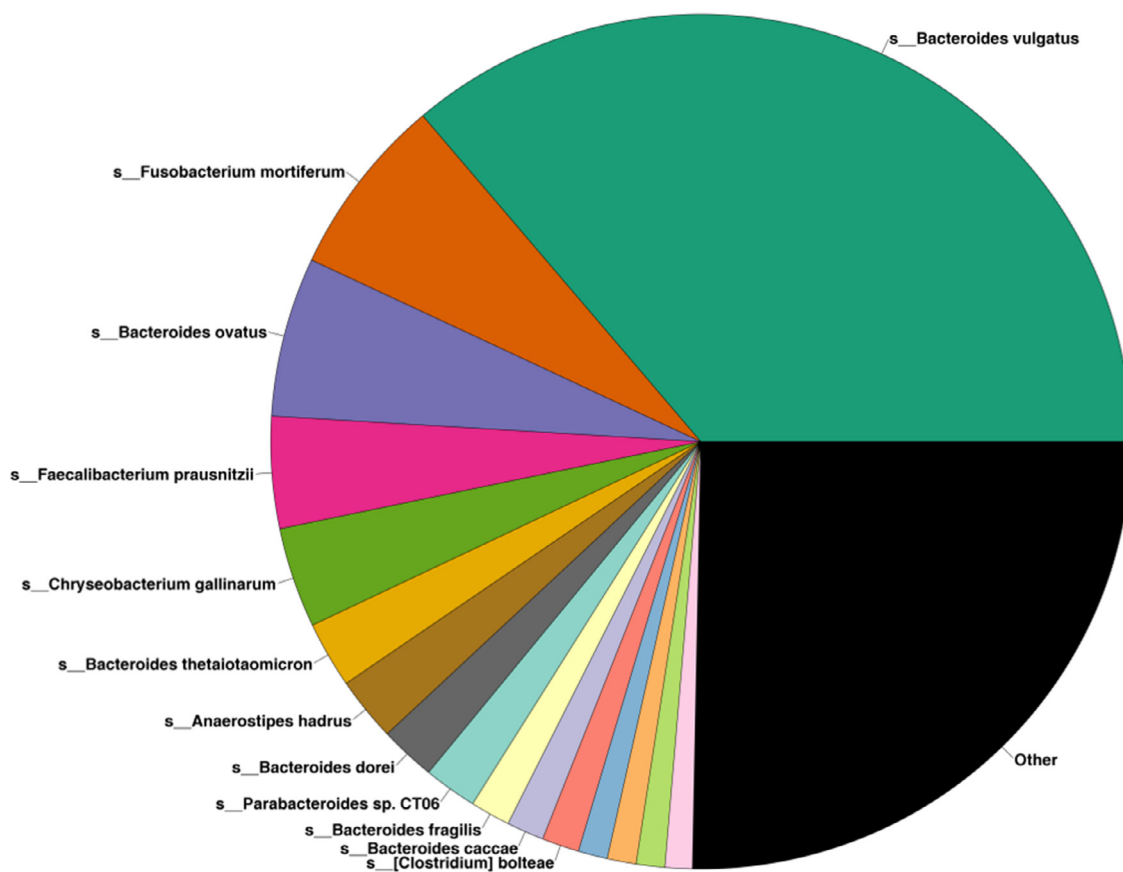
Fig. 3. Pie chart of species composition. The sector size indicates the proportion of the category.

The proportion of species notes

Species composition analysis showed that the order was *Bacteroides vulgatus*, then *Fusobacterium mortiferum, Bacteroides ovatus, Faecalibacterium prausnitzii, Chryseobacterium gallinarum, Bacteroides thetaiotaomicron, Anaerostipes hadrus, Bacteroides dorei, Parabacteroides sp.CT06, Bacteroides fragilis, Bacteroides caccae, Clostridium bolteae.* These data were shown in Fig. 2. The proportion of species composition from each different individual was also shown in Fig. 3. We deeply found that the *Fusobacteria* was largest one and the proportion was 38 percent, then *Actinobacteria* was 24 percent, *Verrucomicrobia* was 9 percent, *Euryarchaeota* was 7 percent, *Candidatus Korarchaeota* was 7 percent, *Firmicutes* was 5 percent, *Proteobacteria* was 5 percent, and the *Bacteroidetes* was 4 percent by Krona software method (https://github.com/marbl/Krona/releases/download/xl2.4/KronaExcelTemplate-2.4.zip). Multi-level pie chart of interactive exploration of hierarchical data also shown in **Supplementary Figure 1**.

Function analysis Wilcox Difference and LDA Effect Size analysis

The function analysis using Wilcox Difference was drawn in **Supplementary Figure 2** and linear regression analysis to estimate the impact of species abundance on the difference effect between healthy control donors and COVID-19, and was drawn in **Supplementary Figure 3**.

KEGG analysis of metagenomics data

In the KEGG pathway database, biological metabolic pathways were divided into six categories: cellular processes, environmental information processing, genetic information processing, human diseases, metabolism and organic system. The carbohydrate metabolism, amino acid metabolism, membrane transport, energy metabolism, and metabolism of cofactors and vitamins were the top KEGG classification. Each category was classified into two, three, and four layers. The second layer includes 57 seed pathways; the third layer was its metabolic pathway map; the fourth layer was the specific annotation information of each metabolic pathway map. According to the results of gene alignment to the KEGG database, the results of each class were counted in Fig. 4.

The DE miRNA expression profiling

A total of 73/390 DE miRNAs were identified between the COVID-19 group and the control group. A total of 35 DE miRNAs were significantly upregulated have a fold change ≥ of 1.5 and *P* value < 0.05, of which 9 were upregulated and 38 were downregulated (*P <0.05*). Importantly, whereas miR-627-5p was the most downregulated miRNA, with a 2.3-fold reduction compared to that of the control group. In addition, the expression of miR-183-5p, miR-627-5p, and miR-144-3p was reduced by more than 1.3-fold compared to that of the healthy control. The transcriptome analysis showed a decrease of the miR-21-5p expression in peripheral blood and the FDR=0.029 between group A and group C in **Supplementary Table 1**.

Gene ontology and KEGG enrichment analysis of DE miRNA target genes

After screening the differentially expressed genes between the COVID-19 patients and the healthy donors, the distribution of the GO of the differentially expressed genes was studied to predict their gene functions **in Supplementary Figure 4**. The GO classification analysis suggested that the blood vessel development, aromatic compound catabolic process, and positive regulation of secretion by cell were the most enriched biological processes with a significant difference between the
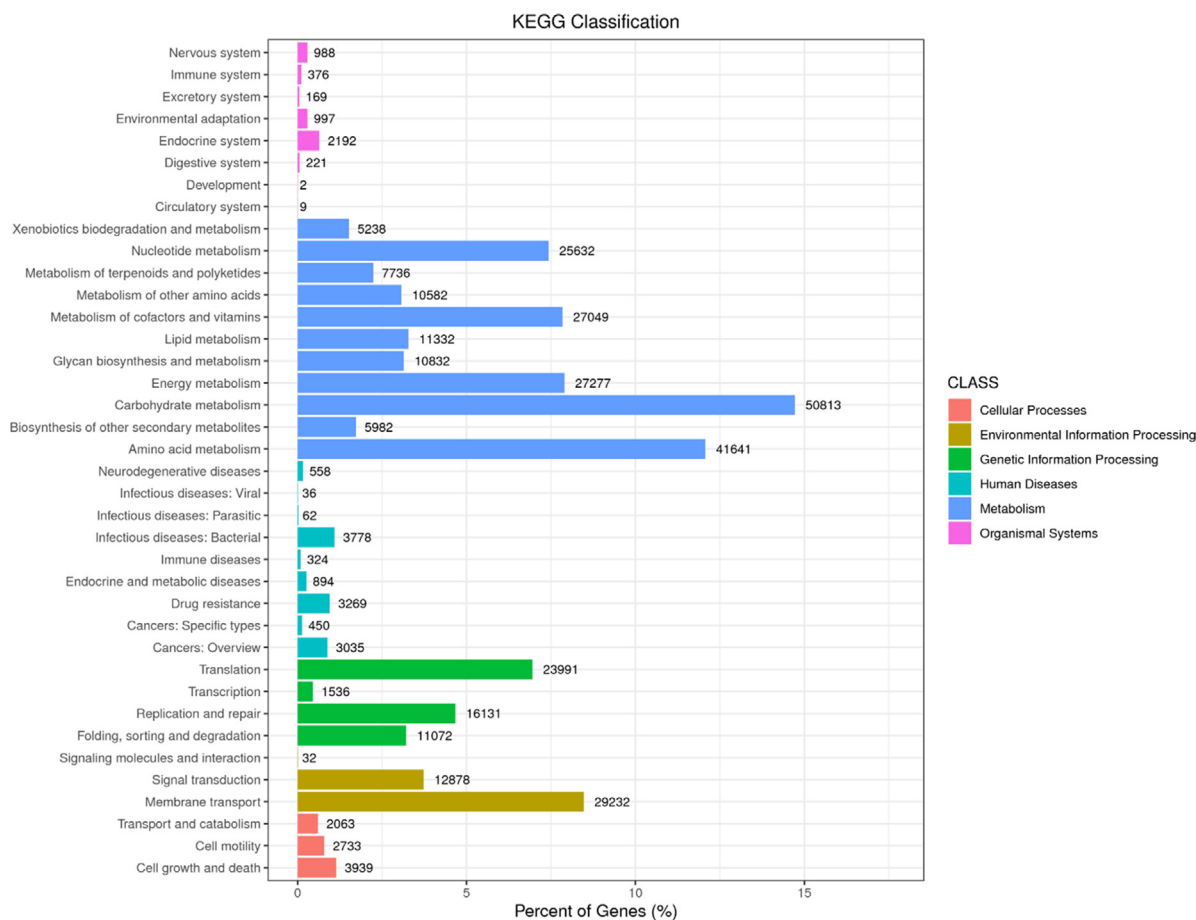
Fig. 4. KEGG classification mapping in health control donors and COVID-19 patient by metagenomics sequence from human feacal sample.

two groups. Subsequently, the nuclear chromosome part, organelle inner membrane, and nuclear envelope were cellular compartments with significant differences between the two groups. In addition, the molecular functions of Ras GTPase binding, purine Ribonucleoside triphosphate binding, and DNA−binding transcription activator activity, RNA polymerase II−specific were analyzed between the two groups **in Supplementary Figure 5**. KEGG enrichment analysis of Up/Downregulated DE miRNAs was shown in Fig. 5a and 5b respectively.

DE mRNA expression profiling

According to the results of differential gene detection, the pheatmap function in R software for hierarchical clustering analysis was used between the COVID-19 and healthy control donors were shown in Fig. 6.

KEGG enrichment analysis of Up/Downregulated DE mRNAs

We performed KEGG enrichment analysis on the up/downregulated DE mRNAs, respectively were shown in **Supplementary Figure 6**, and the FPKM-heatmap of DE mRNA between the COVID-19 patients and the healthy control donors was shown in **Supplementary Figure 7**.

Discussions

Traditional Chinese Herbal Medicine has been considered as a promising supplementary treatment in many severe diseases, like severe acute respiratory syndrome (SARS), that clinically proven to be effective [37]. The timely and appropriate measures for treating COVID-19 in China, which are inseparable from the contribution of TCM, have won much praise from the world [38,39]. Nevertheless, the use of herbal drugs to treat COVID-19 should be with caution [40].

In our present study, the mild to moderate COVID-19 had a normal level of IL-6, but they had a 1 to 2-fold increase of IL-10 with both leukopenia and lymphopenia of COVID. However, one study showed that mild patients had a normal level of IL-4 and IL-10 in peripheral blood, but they had a 1 to 2-fold increase of IL-6 [41].

The patients with COVID-19 with gastrointestinal (GI) symptoms showed its novel characteristics of increased family clustering and liver injury, severe/critical tendency, and a higher rate of body temperature above 38.5°C[26]. The presence of GI symptoms was associated with a high risk of ARDS, non-invasive mechanical ventilation, and tracheal intubation in patients with COVID-19 but not mortality [42]. The QJHD prescription has a better time to recovery of fatigue 6 days than LQG 7 days. Meanwhile, the QJHD might shorten time to recovery of abdominal pain, watery stools, and anorexia than LQG. It should be noted that the days of median time to symptom recovery after QJHD treatment of mild to moderate COVID-19 also shorten than LQG at the same period from February to March outside Wuhan, China. However, the time to recovery of coughing in QJHD treatment was longer than LQG.

According to the TCM illness classification, the COVID-19 was "an epidemic disease characterized by Dampness and Cold". Its etiology and pathogenesis manifested was the "Dampness and Cold invasion from oral and nasal and attack the Lung first" [43].

In the TCM Treatment Principle and Method Theory, our therapeutic regimen of COVID-19 "Qushi" might mean the pathogen dampness expelling. Moreover, available data suggest that TCM could be considered as an adjunctive therapeutic option in the management of all mild to critical type COVID-19 patients [44]. The severe and critical COVID-19 patients exhibit lymphopenia and high level of cytokines, especially impaired T cells, and increased IL-6 or IL-10, which are served as potential biomarkers for disease progression [45]. In the present
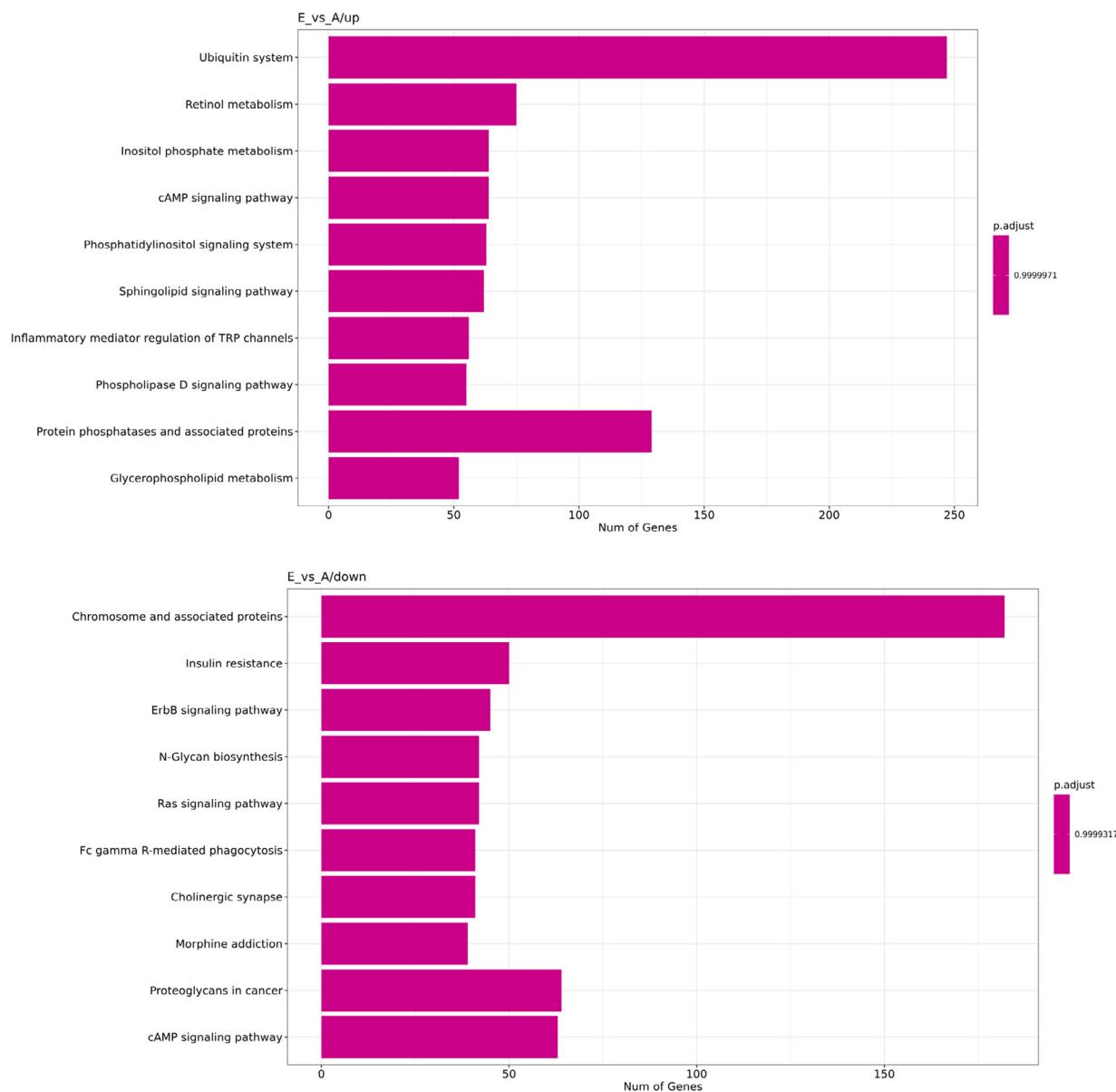
Fig. 5. a The barplot of KEGG enrichment analysis of up-regulated genes between COVID-19 and health control donors. Fig. 5b The barplot of KEGG enrichment analysis of down-regulated genes between COVID-19 and health control donors.

study, our research results demonstrated that lymphopenia and lower IL-10 level compare to the control group($P<0.05$), that partial inconsistent with a triad of interleukin-10, interferon-inducible protein-10 and interleukin-6, anticipate subsequent clinical progression [46]. In this study, our data showed that the leukocytes were significantly decreased in COVID-19 patients before treatment ($P<0.05$) in blood cell counts tests compare to healthy control (CTR), while the lymphocytes were below the levels of healthy donors ($P<0.05$). The related mechanism might be that, SARS-CoV-2 induced activation of apoptosis and associated with dysregulated P53 signaling pathway in lymphocytes may be the cause of patients' lymphopenia [13], that it is consistent with our results.

The precise contributions of elucidating changes of the microbiome are reliable biomarkers in the COVID-19 patient [47]. The gut microbiota's composition and function can be profoundly affected by COVID-19 disease [48]. The baseline abundance of *Coprobacillus, Clostridium ramosum,* and *Clostridium hathewayi* correlated with COVID-19 severity in 15 patients with COVID-19. Moreover, there was an inverse correlation between the abundance of an anti-inflammatory bacterium *Faecal-*

ibacterium prausnitzii and disease severity., *Bacteroides dorei, Bacteroides thetaiotaomicron, Bacteroides massiliensis, and Bacteroides ovatus*, which downregulate expression of ACE2 in the murine gut, correlated inversely with SARS-CoV-2 load in fecal samples from patients throughout hospitalization [49]. The dysfunction of ACE2 alters the composition of the gut microbiota [50]. The stimulatory mechanisms involve short-chain fatty acids (SCFAs) that are absorbed by and act on immune cells to reduce the inflammatory component of infection. SCFAs also enhance the effector activity of CD8+ T cells by stimulating cellular metabolism [51]. Therefore, the parallel bioinformatic predictions identified a *priori* potential B and T cell epitopes for SARS-CoV-2 infection in further bioinformatic-based diagnostic and prognostic estimations [52].

In this present study, we simultaneously detected bacteria in faecal from COVID-19 patients and healthy donors by using metagenomics sequencing. *Faecalibacterium prausnitzii* and other *Bacteroides* species might benefit to GI symptoms recovery of patients with COVID-19. In the TCM Treatment Principle and Method Theory, our therapeutic regimen of COVID-19 "Hewei" maybe mean to make gut harmonizing and cure diarrhea by enhancing gastrointestinal immune function.
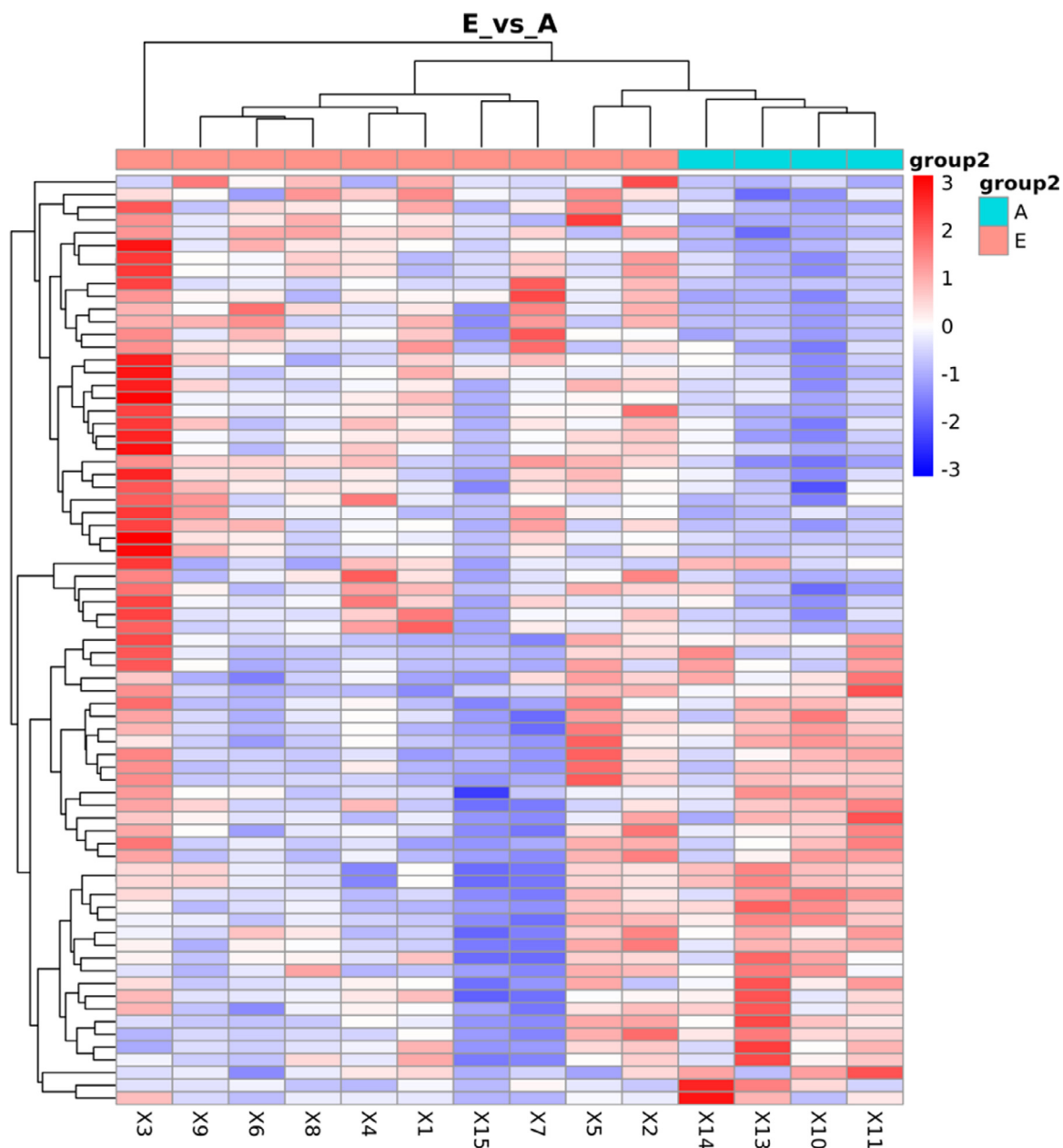
Fig. 6. The GO classification of differentially expressed miRNAs down-regulated genes between the COVID-19 patients and the health control donors.

A reported that induction effects of *Faecalibacterium prausnitzii* on toll-like receptor signaling pathway gene expression and cytokine level in human intestinal epithelial Caco-2 cells, with increase anti-inflammatory cytokines TNF-α, IL-4, IL-8, and IL-10 expression and significantly decreased inflammatory cytokines IL-1, IL-6, IL-17a, IFN-γ compared to the control group in tract [53].In our study, we found that the top five species after QJHD treatment are *Bacteroides vulgatus, Fusobacterium mortiferum, Bacteroides ovatus, Faecalibacterium prausnitzii, Chryseobacterium gallinarum*. Therefore, the QJHD prescription has potential efficacy against SARS-Cov-2 infection in the gut at 14 to 28 days by increasing the numbers of gastrointestinal symbiotic probiotics like *Faecalibacterium prausnitzii and Bacteroides*. However, another research data has shown that the faecal samples with a signature of high SARS-CoV-2 infectivity had higher abundances of bacterial species *Collinsella aerofaciens, Collinsella tanakaei, Streptococcus infantis, Morganella morganii*, and higher functional capacity for nucleotide de novo biosynthesis, amino acid biosynthesis, and glycolysis [54]. The results indicated that the proportion of symbiotic probiotics specie might be the change

in different geographic locations or gut environments. These probiotics may help decrease the inflammatory response of viral pathogenesis and respiratory symptoms by strengthening the host immune system, amelioration of gut microbiome, and improvement of gut barrier function [55]. The symbiotic probiotics indicated that less GI syndrome in our study. Interestingly, a high intake of vegetables is linked to lower IL-6 levels for white blood cells, and 20% of the effect is mediated by the genus *Collinsella* [56].

The 5 top miRNAs reduction in our previous study were listed below: hsa-miR-183-5p, hsa-miR-627-5p, hsa-miR-21-5p, hsa-miR-20a-5p, hsa-miR-146b-5p [57], in which hsa-miR-21-5p occupied four binding sites and was among the top miRNAs that targeted up-regulated differentially expressed genes DE mRNAs after QJHD treatment. In addition to miR-21, miR-16, let-7b, let-7e, and miR-146a were the top miRNAs targeting DE mRNAs. In our present study the transcriptome analysis showed a decrease of the hsa-miR-21-5p expression in peripheral blood after QJHD treatment. Moreover, miR-146a-5p as a blood-based biomarker can provide clues about the molecular link between inflammaging and

COVID-19 [58]. However, our results data results indicated that hsa-miR-146b-5p decreasing in QJHD treatment.

It is worth mentioning that the role of DEGs including STAT1, CCND1, CXCL-10, and MAPKAPK2 in SARS-CoV-2 should be investigated to identify the similarities and differences between SARS-CoV-2 and other respiratory viruses [59]. Another study revealed that distinct host inflammatory cytokine profiles to SARS-CoV-2 infection in patients, and highlight the association between COVID-19 pathogenesis and excessive cytokine releases such as CCL2/MCP-1, CXCL10/IP-10, CCL3/MIP-1A, and CCL4/MIP1B. As blot et al., report that CXCL10 could be one key mediator involved in the dysregulated immune response for critical type COVID-19 [60].

Taken together, the quality control data of QJHD extract was shown using HPLC-DAD analysis was shown in **Supplementary Figure 8.** These important biological processes and pathways of module targets played important roles in the inflammatory process of COVID-19, which strengthened our understanding of the underlying immunomodulatory mechanism of "GI symptoms" in COVID-19 and may provide a novel therapeutic regimen for COVID-19. The possible mechanisms of QJHD on treating COVID-19 patients were shown in **Supplementary Figure 9**.

Though our preliminary investigation showed the effectiveness of QJHD for patients with COVID-19, the limited sample size could not draw out reliable statistical power. Moreover, we lack the assessment of the long-term effects of QJHD prescriptions on primary outcome measures. The treatment period was only 14 days to 28 days' investigation, which is relatively short. Due to the limited time frame, the potential roles of prescriptions in reducing overall mortality and recrudescence events over the long term remain uncertain. Future RCTs should include a larger sample size and longer follow-up periods.

Conclusions

Our findings reveal a potential role of QJHD in COVID-19 treatment by regulation of improve clinical outcomes of mild or moderate COVID-19 patients, probably mainly through beneficial immunomodulatory effects by regulating *Faecalibacterium prausnitzii* in the intestinal tract, decreasing the level of miR-21-5p without QJHD treatment and decreasing the level of IL-10 expression in peripheral blood after QJHD. Thus, QJHD may be a promising therapeutic regime for protecting the intestine integrity of the immune barrier and treating SARS-Cov-2 infection with GI symptoms.

Statement of Contributions

LJ and YZ conceived and designed this study. LJ and YY wrote the manuscript with contributions from all authors. LJ, YY, LJ, BX and YZ refined the protocol. All authors contributed to the article and approved the submitted version.

Declaration of Competing Interest

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Also, the data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to.

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