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Short-term high-dose gavage of hydroxychloroquine changes gut microbiota but not the intestinal integrity and immunological responses in mice

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ABSTRACT

Aims: Hydroxychloroquine (HCQ), a widely used antimalarial drug, is proposed to treat coronavirus disease 2019 (COVID-19). However, no report is currently available regarding the direct effects of HCQ on gut microbiota, which is associated with the outcomes of elderly patients with COVID-19. Here, we first investigated the effects of HCQ on intestinal microecology in mice.

Main methods: Fifteen female C57BL/6J mice were randomly divided into two groups: HCQ group (n = 10) and control group (n = 5). Mice in the HCQ group were administered with HCQ at dose of 100 mg/kg by gavage daily for 14 days. The feces of mice were collected before and on the 7th and 14th days after HCQ challenge, and then analyzed by 16S rRNA amplicon sequencing. At the end of the experiment, the hematology, serum biochemistry and cytokines were determined, respectively. The mRNA expression of tight junction proteins in colonic tissues were also studied by RT-PCR.

Key findings: HCQ challenge had no effects on the counts of white blood cells, the levels of serum cytokines, and the gene expression of tight junction proteins in colon. HCQ also did not increase the content of serum D-lactate in mice. Notably, HCQ significantly decreased the diversity of gut microbiota, increased the relative abundance of phylum *Bacteroidetes* whereas decreased that of *Firmicutes*.

Significance: Short-term high dose HCQ challenge changes gut microbiota but not the intestinal integrity and immunological responses in mice. Special attention should be paid to the effects of HCQ on intestinal microecology in future clinical use.

1. Introduction

Hydroxychloroquine (HCQ), a wildly used antimalarial drug for more than 70 years, was also empirically prescribed to treat rheumatoid arthritis, systemic lupus erythematosus and other inflammatory diseases [1,2]. With the spread of novel coronavirus disease 2019 (COVID-19) outbreak, HCQ was one of the earliest proposed candidates which have therapeutic potential for the prevention and treatment of COVID-19 caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [3], mainly due to its potent antiviral activity against SARS-CoV-2 *in vitro* [4], although the clinical efficacy remains huge controversial [5]. Several large-scale clinical trials of prophylactic administration with HCQ to prevent COVID-19 were still undergoing [5].

Considering a large volume tissue distribution and a long half-life after oral administration [2], the medical safety of HCQ have quickly become a great concern worldwide. It is well documented that the cumulative toxicities after long-term use of HCQ include retinopathy, cardiomyopathy and rhythm disorders [6], whereas the most common side effects after short-term high-dose HCQ use are gastrointestinal (GI) side effects such as nausea, vomiting, diarrhea, and abdominal discomfort [7]. In fact, up to 20% of patients with COVID-19 had GI symptoms [8,9], and a part of whom with active SARS-CoV-2 GI infection were tightly associated with alterations of gut microbiota [10]. Recently, it has been proposed that gut microbiota dysbiosis is one of the underlying mechanisms responsible for the poor outcomes (higher disease severity and mortality rate) in elderly COVID-19 patients with

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pre-existing age-related diseases [11]. Apparently, identifying the impacts of HCQ, a potential drug for the treatment of several viral infectious diseases with GI side effects (including COVID-19), on intestinal microecology is urgently needed. Previously, it has been reported that patients after long-term treatment with doxycycline and HCQ displayed significant alterations of gut microbiota [12]. However, no reports are currently available regarding the direct effects of short-term high-dose HCQ application on intestinal microecology.

Therefore, the objective of this study was to investigate the effects of short-term high-dose HCQ administration on gut microbiota in mice, as well as the intestinal integrity and immunological responses.

2. Materials and methods

2.1. Chemical reagents

Hydroxychloroquine sulfate was purchased from Sigma-Aldrich (USA). TRIzol Reagent, TURBO DNA-free™ Kit, and SuperScript II Reverse Transcriptase were purchased from Thermo Fisher Scientific (USA). TB Green Premix EX Tap™ II (Tli RNaseH Plus) was purchased from TaKaRa (JAPAN). A Mouse Th1/Th2/Th9/Th17/Th22/Treg Cytokine Panel (17plex) was obtained from eBioscience (USA). All other chemicals and materials were obtained from local commercial sources.

2.2. Animals

Fifteen female C57BL/6J mice, specific-pathogen free grade, weighing 19 to 21 g, were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China), and were housed in groups of five mice per cage at the Experimental Animal Center, under a 12-h light/dark cycle at a temperature of 23 ± 2 °C and relative humidity of 40 ± 5%, with free access to water and food. All animal procedures were carried out in accordance with the Declaration of the National Institutes of Health Guide and Use of Laboratory Animals and approved by the animal care and use committee of Beijing Institute of Microbiology and Epidemiology (No. SCXK-2020-01070108).

After adaptive feeding for one week, the mice were randomly divided into two groups: HCQ group (n = 10) and control group (n = 5). Mice in the HCQ group were treated with HCQ by gavage daily for 14 days. Considering the commonly recommended intake of HCQ should not exceed 800 mg per day, the dosage of HCQ in the current study was therefore calculated to be 100 mg/kg, according to body surface area method. Mice in the control group were given at the same volume of sterilized saline. Body weights were monitored every day before gavage throughout the study.

2.3. Sample collection

Fresh stool samples of mice were harvested in a sterilized tube before gavage at the beginning of the experiment and at the days 7 and 14 after HCQ administration, and then immediately stored at −80 °C for microbiome analysis. At the end of the study, blood from each mouse was respectively collected. The EDTA-anticoagulated blood sample was quickly used for basic hematology determination. The other whole blood samples were centrifuged at 1000*g* for 15 min at 4 °C, and the isolated serum was stored at −80 °C for subsequent biochemical parameters and cytokines measurements. Colonic tissues were taken, rinsing well with saline, snap-frozen in liquid nitrogen, and then stored at −80 °C for molecular biology analysis.

2.4. Hematology and biochemical parameters determination

The contents of hemoglobin (HGB) and the counts of red blood cells (RBC), white blood cells (WBC), platelets (PLT), neutrophils and lymphocytes in EDTA-anticoagulated blood samples were determined by an IDEXX ProCyte Dx® Hematology Analyzer (IDEXX Laboratories, Inc. USA). The activities of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatine kinase (CK), and lactate dehydrogenase (LDH), as well as the concentrations of serum urea (UREA), creatinine (CREZ), cholesterol (CHO), triglyceride (TG), high density lipoprotein (HDL), and low density lipoprotein (LDL) were respectively measured by a Clinical Chemistry Analyzer (AU 480, Beckman Coulter, USA).

2.5. Cytokines assay

The levels of serum inflammatory cytokines were analyzed using ProcartaPlex™ Multiplex Immunoassays through Bio-Plex 200 system (Bio-Rad Laboratories, USA), according to the manufacturer's instructions. A Mouse Th1/Th2/Th9/Th17/Th22/Treg Cytokine Panel (17plex, eBioscience, USA) was used to detect cytokines. They are listed as follow: GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17A, IL-18, IL-22, IL-23, IL-27 and TNF-α.

2.6. Serum D-lactate measurement

Serum sample (30 μL) was added with 30 μL of propionic acid (as an internal standard) and 140 μL acetonitrile, vortexed and centrifuged at 13,200 rpm for 5 min at 4 °C to precipitate proteins, and then the supernatant was subjected to determine by a high-performance liquid chromatography system (Ultimate3000, Dionex, USA) coupled to a mass spectrometry system (API 3200 Q-TRAP, Applied Biosystems, USA) in multiple reaction monitoring (MRM) mode using negative electrospray ionization (ESI) at MSLab (Beijing, China).

2.7. Quantitative real-time PCR detection

Total RNA was extracted from colonic tissue using TRIzol reagent and then treated with RNase-free DNase I to remove DNA contaminants. Resulting RNA concentrations were tested using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA), and 2 μg of

Table 1
Primers used for Quantitative-PCR analysis.

No.	Target mRNA	Direction sequence (forward)	Direction sequence (reverse)	Accession number	Product size (bp)
1	Gapdh	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA	NM_008084.2	95
2	Claudin 1 (Cldn1)	ATGCCTTCAACTGTTCTGTATCTC	AATCCAGGTCTACCAATGTCAATG	NM_016674.4	194
3	Claudin 2 (Cldn2)	ATGTCCTCGCTGGCTTGTATTATCTCT	GCCATGAAGATTCCAAGCAACTG	NM_016675.4	159
4	Claudin 4 (Cldn4)	TCGTGGGTGCTCTGGGGATGCTTC	GCGGATGACGTTGTGAGCGGTC	NM_009903.2	170
5	Claudin 5 (Cldn5)	GCTCTCAGAGTCCGTTGACC	CTGCCCTTTCAGGTTAGCAG	NM_013805.4	235
6	Claudin 8 (Cldn8)	TCAGTATGTGTAGTTGTG	ATAATACCTCATTCTGCTAA	NM_018778.3	190
7	Claudin 14 (Cldn14)	CTGGGCTTCATCTCCTCATC	CATTCAGCCTGTACCCACTGT	NM_019500.4	214
8	Occludin (Ocln)	CTGCTGCTGATGAATATAATAG	CCTCTTGATGTGCGATAA	NM_008756.2	110
9	Tight junction protein 1 (Tjp1/ZO1)	CATAGTTCAACACAGCCTCCAG	CCATCCTCATCTTCATCTTCTTCC	NM_001163574.1	155
10	Cadherin 1 (Cdh1)	TTGTTCGGCTATGTGTCT	TGTGTACCTAAGAATCTGAGA	NM_009864.3	82
11	Mucin 2 (Muc2)	GATGGCACCTACCTCGTTGT	GTCCTGGCACTTGTTGGAAT	NM_023566.4	246

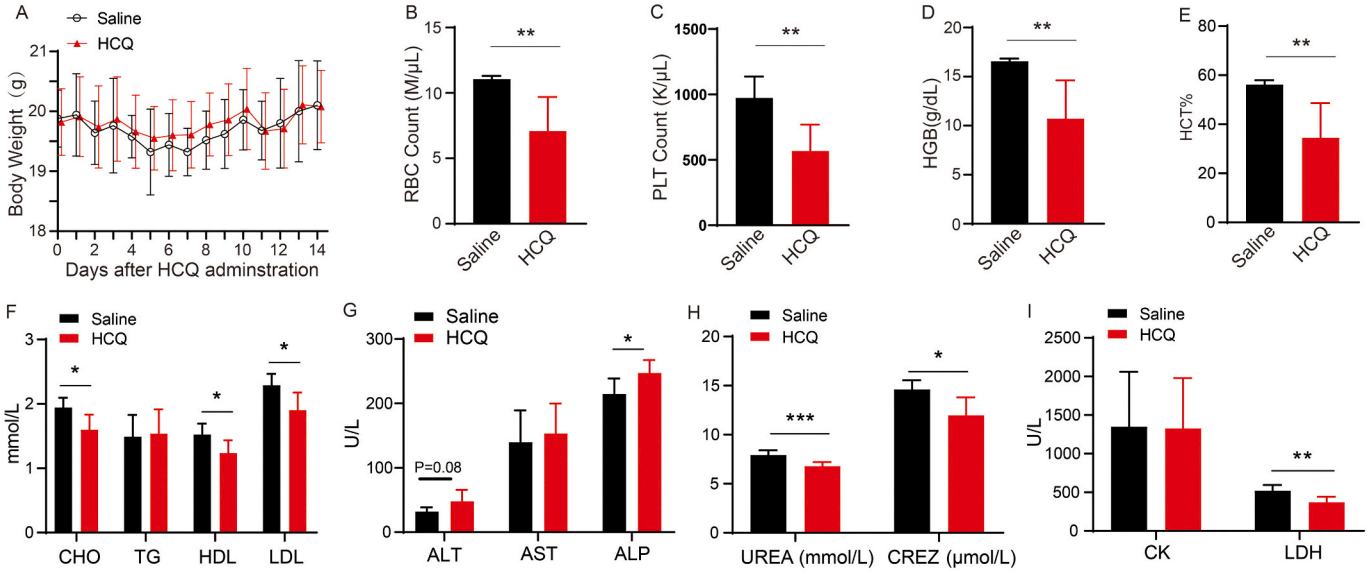

Fig. 1. Effects of HCQ on blood hematological and biochemical parameters in mice.
A: Effects of HCQ on body weight in mice throughout the study. Female C57BL/6J mice (n = 10) were gavage of HCQ at dose of 100 mg/kg for 14 days. Mice (n = 5) treated with saline were set as control. Body weight was monitored daily before gavage. B–E: Effects of HCQ on hematology in mice. At the end of the experiments, EDTA-anticoagulated blood from each mouse was respectively collected. The counts of red blood cells (RBC, B) and platelets (PLT, C), the content of hemoglobin (HGB, D), and hematocrit (HCT%, E) were detected by a hematology analyzer, respectively. F–I: Blood from each mouse was harvested and centrifuged at 1000*g* for 15 min at 4 °C. The isolated serum was used to determine biochemical parameters. The levels of lipoproteins (F), liver function (G), renal function (H), and markers of myocardial damage (I) were measured by a Clinical Chemistry Analyzer, respectively. Abbreviations: HCQ (hydroxychloroquine), EDTA (ethylenediamine tetraacetic acid), CHO (cholesterol), TG (triglyceride), HDL (high density lipoprotein), LDL (low density lipoprotein), ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALP (alkaline phosphatase), UREA (urea), CREZ (creatinine), CK (creatine kinase), LDH (lactate dehydrogenase). Data were expressed as mean ± S.D. Statistical significance (*$P < 0.05$, **$P < 0.01$; ***$P < 0.001$; one-way analysis of variance followed by Student's *t*-test) was performed by GraphPad Prism 8.0 software. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

each sample was reverse-transcribed to cDNA using SuperScript II reverse transcriptase. PCR amplifications were performed in a LightCycler 480 instrument (Roche, Switzerland) using the primers listed in Table 1. Relative mRNA expression levels of the tight junction-associated proteins containing claudins (1, 2, 4, 5, 8, and 14), occludin, cadherin 1, mucin 2, and zonula occluden-1 (ZO-1) were then respectively calculated and normalized to the expression of the glyceraldehyde-3-phosphate dehydrogenase (Gapdh) gene. The advanced relative quantification mode was employed to analyze RT-PCR data using proven algorithms (Software Version:LCS480 1.5.0.39.), according to the Instrument Operator's Manual. The PCR efficiency was defined as 2. This analysis compares two ratios: (1) the ratio of a target DNA sequence to a reference DNA sequence in an unknown sample, and (2) the ratio of the same two sequences in a standard sample called a "calibrator". The result of each sample is expressed as a normalized ratio, *i.e.*, ratio (1) divided by ratio (2). The formula is listed below.

$$\text{Normalized Ratio} = \left(\frac{\text{Conc. Target}}{\text{Conc. Reference}}\right)_{Sample} \div \left(\frac{\text{Conc. Target}}{\text{Conc. Reference}}\right)_{Calibrator}$$

$$= (2^{\text{Cp(Reference)}-\text{Cp(Target)}})_{Sample} \div (2^{\text{Cp(Reference)}-\text{Cp(Target)}})_{Calibrator}$$

(Conc. represents the DNA concentration for each sample by the end of the amplification, Cp represents the crossing point).

2.8. Fecal microbiome analysis

The mouse gut microbiome was analyzed by 16S rRNA gene amplicon sequencing. Briefly, total bacterial genomic DNA from fecal samples was extracted using hexadecyltrimethylammonium bromide (CTAB, Sigma-Aldrich, USA) method. The quality of the isolated DNA was checked by 1% agarose gel electrophoresis and the concentration was measured using a Nanodrop Spectrophotometer (ND-2000, Thermo Scientific, USA). The obtained DNA at the concentration of 1 ng/μL was used as template, and the V4 hypervariable regions of the bacterial 16S rRNA gene was amplified by specific primer (515F: GTGCCAGCMGCC GCGGTAA; 806R: GGACTACHVGGGTWT-CTAAT) with the barcode. The polymerase chain reaction (PCR) amplifications were conducted by Phusion® High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs, USA). After quality control by 2% agarose gel electrophoresis, PCR products were purified with QIAquick Gel Extraction Kit (Qiagen, Germany). The sequencing libraries were constructed using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) according to the manufacturer's instructions. After quality testing by Qubit, the library was run in an Illumina NovaSeq 6000 platform at Novogene Company (Tianjin, China), according to standard operating protocols.

2.9. Sequencing data processing

After removing the barcode and primer sequences, and assembling by FLASH software [13], the raw reads were filtering and chimera removal by QIIME software to generate the high-quality clean effective reads [14]. All effective reads were clustered into operational taxonomic units (OTUs) with 97% identity by Uparse software [15]. Representative sequence for each OTU was screened for further annotation using Mothur method and SSUrRNA database of SILVA to obtain taxonomic information [16,17]. Before bioinformatics analyses, sequence alignment with high accuracy and high throughput was performed by MUSCLE software [18]. Finally, the data of each sample were normalized for subsequent analysis. Taxonomic α-diversity such as the Observed species and Shannon indices were calculated with QIIME software. The bacterial community discrepancy (β-diversity) between groups was evaluated by principal coordinates analysis (PCoA) based on unweighted UniFrac distances using QIIME software.

Sequencing dataset was deposited to the NCBI Sequence Read Archive under BioProject accession number PRJNA650014.

2.10. Statistical analysis

Physiological data were expressed as mean ± SD. Figures were made by GraphPad Prism 8.0 software, comparisons between groups were also performed by GraphPad Prism 8.0 software using one-way analysis of variance (ANOVA) followed by Student's *t*-test. For sequencing data analysis, figures were made by R software, diversity comparisons among groups were carried out by one-way ANOVA followed by Tukey's HSD means comparisons. Metastats analysis was employed to evaluate significant differences in relative abundance of bacterial genus among groups by R software [19]. A P value below 0.05 ($P < 0.05$) was considered to be statistically significant.

3. Results

3.1. Effects of HCQ on blood hematological and biochemical parameters in mice

We first compared the dynamic changes of body weight in mice between groups, and no significant difference was observed throughout the study (Fig. 1A), indicated that HCQ challenge for 14 days did not affect the body weight of mice. At the end of the experiment, we detected the alterations of hematological indicators, and found that there was a marked decline in the counts of PLT and RBC, as well as the content of HGB, and hematocrit (HCT%) in HCQ group (Fig. 1B–E). Compared with the control group, the results also showed that HCQ challenge significantly decreased the levels of total cholesterol (CHO), high-density lipoprotein (HDL), and low-density lipoprotein (LDL), but not the level of total triglyceride (TG) (Fig. 1F). The effects of HCQ on RBC, PLT, CHO, and serum lipoproteins were in line with previous reports [20,21], suggesting a significant impact of HCQ on hematology and lipids metabolism.

For biochemical parameters, HCQ failed to influence the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), although there was a mild increase of the alkaline phosphatase (ALP) activity after HCQ challenge (Fig. 1G). HCQ also significantly reduced the concentrations of serum urea (UREA) and creatinine (CREZ) in mice (Fig. 1H). Moreover, since the myocardial damage is one of the most commonly reported side-effects after long-term administration of HCQ [22], we therefore detected the effects of HCQ on the activities of creatine kinase (CK) and lactate dehydrogenase (LDH), two indicators mostly related to myocardial injury. We showed that short-term HCQ intervention had no effects on the activity of CK, but decreased that of LDH (Fig. 1I).

According to the normal reference values of physiological parameters in mice, we noticed that the statistically significant changes of ALP, LDH, UREA, and CREZ were still within the range of physiological fluctuations, indicating that short-term high-dose HCQ administration had no pathological effects on liver and renal functions, and did not induce apparent myocardial injury.

3.2. Short-term high dose HCQ had no effects on immunological responses in mice

Through the hematology analysis, we found that there were no changes in the counts of WBC, neutrophils, lymphocytes, monocytes, and eosinophils between two groups (Fig. 2A). No statistically significant differences were also observed in all detected cytokines. Among them, the serum levels of GM-CSF, IL-18, IL-9 and IL-22 were almost under the limit of detection (data not shown), and the levels of the other 13 kinds of cytokines were showed in Fig. 2B. These results suggested that short-term challenge with HCQ at high dose did neither influence the number of immunocytes in circulation nor the secreted functions of immune cells including Th1, Th2, Th9, Th17, Th22, and Treg cells.

3.3. Short-term high dose HCQ did not affect the intestinal integrity in mice

It is well recognized that the increased serum level of D-lactate is a useful biomarker for intestinal hyperpermeability and gut barrier dysfunction [23]. We thus compared the serum contents of D-lactate between groups, and found that a decline of the D-lactate level was observed in mice after 14 days administration with HCQ (Fig. 3A). This result demonstrated that HCQ challenge did not increase the intestinal permeability. We also detected the effects of HCQ on gene expression of tight junction-associated proteins in colonic tissue, and there was no statistically significant change in the mRNA expression of claudins (1, 2, 4, 5, 8, and 14), occludin, cadherin 1, mucin 2, and zonula occludens-1 (ZO-1) after HCQ challenge for 14 days (Fig. 3B). Collectively, we speculate that short-term challenge with HCQ at high dose fails to affect the intestinal integrity in mice.

3.4. HCQ changed the gut bacterial community in mice

Although there was no significant change in intestinal permeability, we further investigated the effects of HCQ on gut microbiota in mice. The dynamic alterations of fecal microbial composition at the phyla level (Top 10) was summarized in Fig. 4A. The dominant phyla of these groups were *Bacteroiddetes* and *Firmicutes*. Compared with the bacterial phyla before challenge with HCQ (HCQ.Base), a significant increase of the relative abundance of *Bacteroiddetes* and *Actinobacteria* was observed, whereas the relative abundance of *Firmicutes* and *Tenericutes* were significantly decreased after HCQ challenge for 14 days (HCQ.D14) (Fig. 4B). Compared with HCQ.Base group, the α-diversity indices, Shannon and the observed species, were both significantly decreased in a time-dependent manner (Fig. 4C and D), suggesting that short-term challenge with HCQ could decrease the bacterial species richness and community diversity in stool of mice. β-Diversity was represented by principal co-ordinates analysis (PCoA) of the bacterial communities that derived from the weighted UniFrac distance matrix, and it showed that the distance between the HCQ.Base and HCQ. D14 groups was relatively large, which indicating a largely different of the bacterial community composition before and after HCQ challenge (Fig. 4E).

Further analysis of OTUs abundance revealed that bacteria from five genus (*Dialister*, *Negativicoccus*, *Ruminobacter*, *Atopostipes*, and *Sutterella*) sharply decreased and even disappeared after HCQ challenge (Fig. 5A–E), bacteria from three families (six genus: *Adlercreutzia*, *unidentified_Ruminococcaceae*, *Oscillospira*, *Fournierella*, *Harryflintia* and *Candidatus_Arthromitus*) decreased significantly in a time-dependent manner (Fig. 5F–K), whereas three genus *Bifidobacterium*, *Parabacteroides*, and *Acetitomaculum* increased in a time-dependent manner after HCQ challenge (Fig. 5L–N). Additionally, we found that the relative abundance of *Turicibacter* was firstly increased after HCQ challenge for 7 days and then recovered to normal level of pre-administration with HCQ (Fig. 5O). Taken together, our results indicated that short-term challenge with HCQ at high dose significantly altered the structure, richness, and the community diversity of the gut microbiota in mice.

4. Discussion

Recent studies have shown that gut microbiota is closely associated with human health [24]. The dynamic crosstalk between the host and its gut microbiota is important for maintaining whole-body homeostasis [25]. An altered gut microbiome (gut microbiota dysbiosis) has been identified to play critical roles in the development of several metabolic disorders in human [26]. Actually, the composition, function and metabolic activity of the gut microbiota were vulnerable to diet, lifestyles, environmental factors, physical stressors, and drug abuse [27]. In this field, it is well known that antibiotic drugs are no longer considered only beneficial, but also potentially harmful, owing to their undesirable

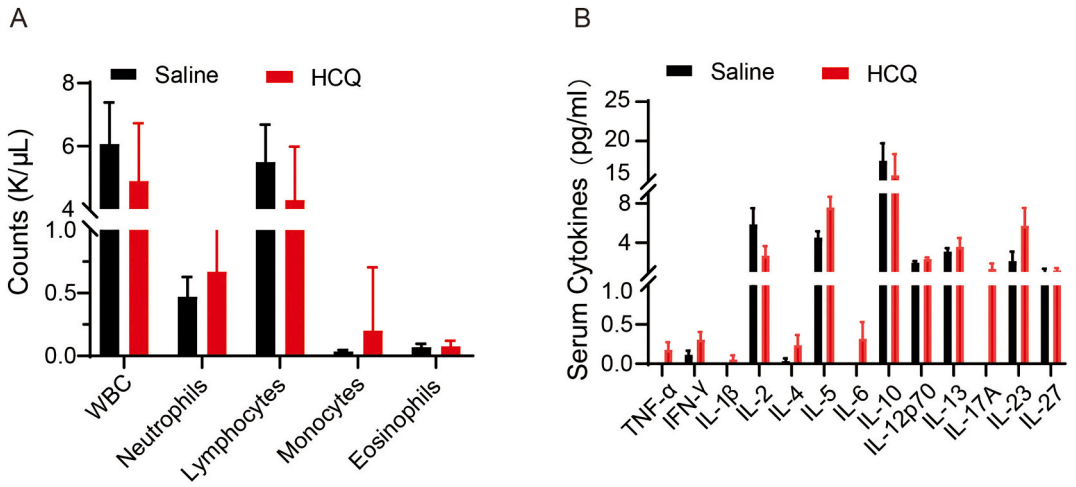

Fig. 2. HCQ had no effects on immunological responses in mice.
Female C57BL/6J mice (n = 10) were gavage of HCQ at dose of 100 mg/kg for 14 days. Mice (n = 5) treated with saline were set as control. A: The EDTA-anticoagulated blood from each mouse was used to detect the counts of white blood cells (WBC) containing neutrophils, lymphocytes, monocytes, and eosinophils, by a hematology analyzer. B: Blood from each mouse was harvested and centrifuged at 1000*g* for 15 min at 4 °C. The levels of serum cytokines were determined using multiplex immunoassays through Bio-Plex 200 system. Abbreviations: HCQ (hydroxychloroquine), EDTA (ethylenediamine tetraacetic acid), TNF-α (tumor necrosis factor-alpha), IFN-γ (interferon-gamma), IL (interleukin). Data were expressed as mean ± S.D. There was no statistically significant difference between both groups by GraphPad Prism 8.0 software (one-way analysis of variance followed by Student's *t*-test).

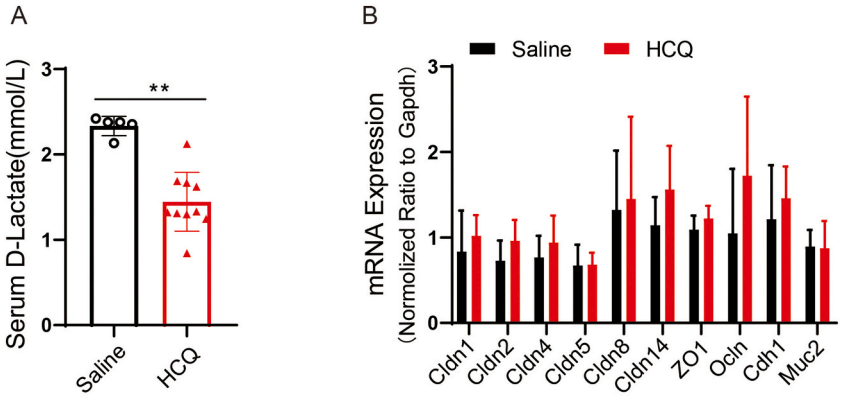

Fig. 3. HCQ did not affect the intestinal integrity in mice.
Female C57BL/6J mice (n = 10) were gavage of HCQ at dose of 100 mg/kg for 14 days. Mice (n = 5) treated with saline were set as control. A: Blood from each mouse was harvested and centrifuged at 1000*g* for 15 min at 4 °C. The serum level of D-lactate was determined by high-performance liquid chromatography combined with mass spectrometry system. B: Relative mRNA expression levels of the tight junction-associated proteins in colonic tissues of mice were measured by Quantitative real-time PCR method. The relative expression of target genes was calculated and normalized to that of Gapdh gene. Abbreviations: HCQ (hydroxychloroquine), PCR (polymerase chain reaction), Gapdh (glyceraldehyde-3-phosphate dehydrogenase), Cldn (claudin), ZO (zonula occludin), Ocln (occludin), Cdh (cadherin), Muc (mucin). Data were expressed as mean ± S.D. Statistical significance (*$P < 0.05$, **$P < 0.01$; one-way analysis of variance followed by Student's *t*-test) was performed by GraphPad Prism 8.0 software.

impairments on gut microbiota and intestinal microecology [28]. Nonetheless, studies focused on drug-induced changes of gut microbiota are still lacking. Since GI symptoms were the commonly observed side effects after administration with HCQ [7], we thought that if HCQ still used in COVID-19 patients with GI side effects, there is a possibility to accelerate the progression of disease. Unfortunately, no reports are currently available regarding the direct effects of HCQ on intestinal microecology. We thus hypothesized that there is a link between HCQ-induced GI symptoms and the alterations of intestinal microecology, especially for that of gut microbiota.

In present study, we found that treating with HCQ to simulate the maximum dose in clinical use for 14 days significantly decreased the counts of PLT, but not the liver and renal functions in mice, which was in line with previous report that HCQ given alone (400 mg per day) for 7 days resulted in a significant reduction in count of PLT in healthy human volunteers [20], without changing the activities of ALT and AST, and the concentrations of UREA and CREZ [20]. We also showed that HCQ decreased the counts of RBC, the level of HGB, and the contents of cholesterol and lipoproteins, which may be attributed to a higher dose and relatively longer administration time in this study, since a meta-analysis had shown that long-term treatment with HCQ significantly lowered the serum lipids in lupus erythematosus patients [21]. These hematological and biochemical results suggested that the mouse model used in current study could entirely mimic the clinical effectiveness after short-term high dose HCQ treatment in humans.

Several previous studies have suggested that HCQ could inhibit the productions of some pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α. However, most of which were based on *in vitro* studies, and the results are still controversial. In peripheral blood mononuclear cells, HCQ could inhibit phytohemagglutinin (PHA)-induced TNF-α and IFN-γ production, and lipopolysaccharide (LPS)-induced TNF-α and IL-6 production, but failed to affect PHA-induced IL-6 production [29]. Another study showed that HCQ inhibited production of IL-1α (monocytes) and IL-6 (T cells and monocytes), whereas failed to affect the productions of IL-2, IL-4, TNF-α and IFN-γ [30]. In cultured plasmacytoid dendritic cells (pDC) and natural killer (NK) cells from healthy donors, HCQ blocked TNF-α and IL-6 production in pDCs but not in NK cells after stimulation by RNA-containing immune complexes. Conversely, in cultured monocyte-depleted peripheral blood mononuclear cells from systemic lupus erythematosus (SLE) patients and healthy controls, HCQ both had no effects on TNF-α production [31]. Furthermore, the clinical efficacy of HCQ on cytokines observed in *in vivo* studies were also conflicting. In patients with SLE, two-month treatment with HCQ resulted in significant decrease in serum levels of

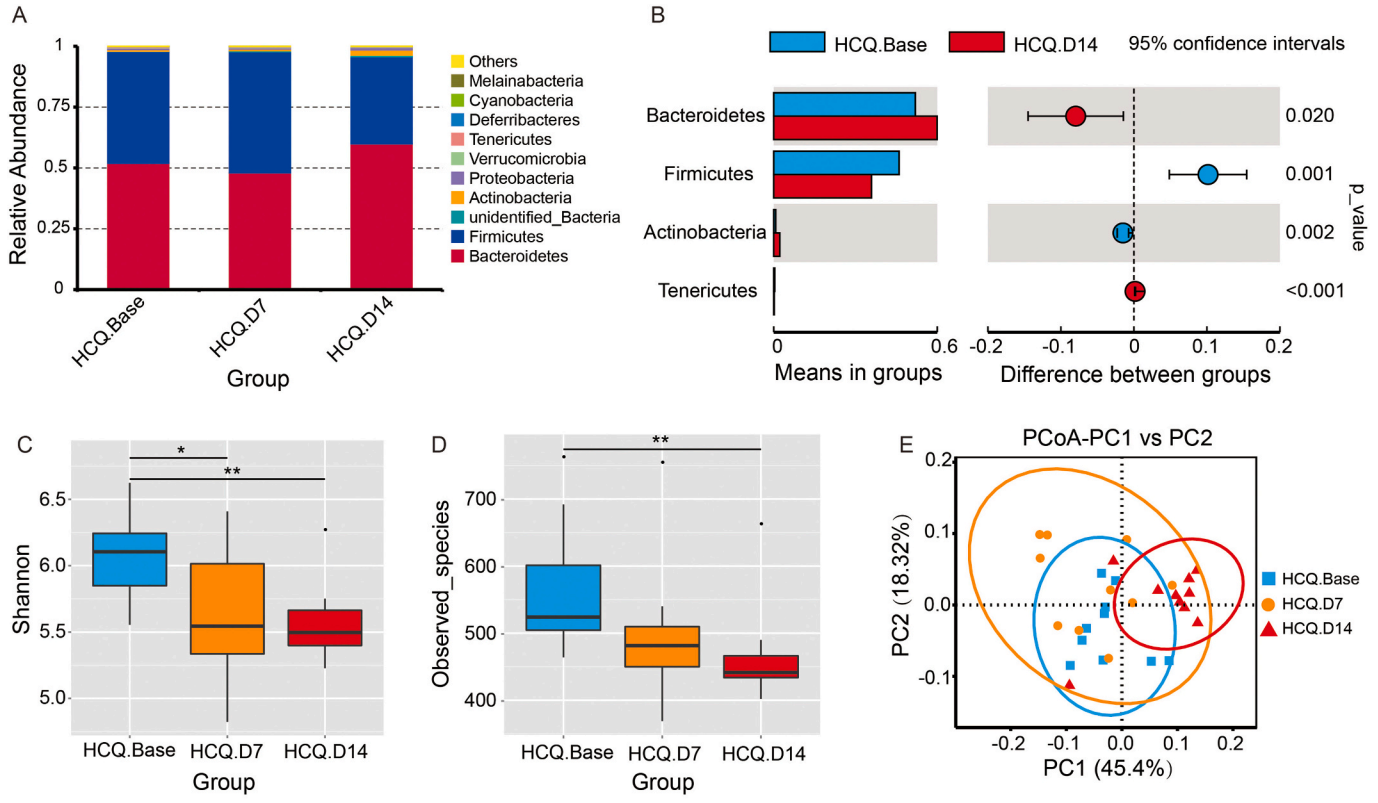

Fig. 4. HCQ changed the gut bacterial community in mice.
Female C57BL/6J mice (n = 10) were gavage of HCQ at dose of 100 mg/kg for 14 days. Fresh stool samples of mice were harvested at the beginning of the experiment (HCQ.Base), and at days 7 (HCQ.D7) and 14 (HCQ.D14) after HCQ challenge. Microbial community in stool of mice were analyzed by 16S rRNA gene amplicon sequencing. A: A summary of the relative abundance of gut microbial community at the phylum level (top 10). B: Comparisons of the dominant bacteria at phylum level between HCQ.Base and HCQ.D14 groups. C and D: The dynamic alterations of α-diversity indices (C: Shannon index, D: Observed_species). E: Differences of β-diversity among groups were represented by principal co-ordinates analysis (PCoA) of the bacterial communities that derived from the weighted UniFrac distance matrix. Figures were made by R software, statistical significance (*$P < 0.05$, **$P < 0.01$) was performed by one-way analysis of variance followed by Tukey's HSD means comparisons (among groups) or *t*-test (between groups).

IL-1β, IL-6, and TNF-α [32]. While another clinical investigation also regarding patients with SLE found that HCQ therapy just had a decreased trend on the serum levels of IL-6, IFN-α, IL-8 and TNF-α. Unfortunately, these changes were not statistically significant [33]. In fact, it has been reported that 3 months of monotherapy with chloroquine did not influence the mRNA skin expression of interleukin IL-1β, IL-6 and TNF-α in non-irradiated non-diseased skin of patients with SLE. However, the expression of all three cytokines mRNA levels in the irradiated sites were significantly higher than those in non-irradiated group, and chloroquine treatment significantly lowered the expression of IL-1β, IL-6 and TNF-α mRNAs in irradiated skin samples [34].

One reason accounting for this contradiction in that the inhibitive effect of HCQ on cytokines may be an indirect action, since these results were always observed after induction by exogenous or endogenous pro-inflammatory stimulators. No report is currently available about the HCQ on the cytokine productions in normal without induction. Thus, it has been suggested that the direct effect of antimalarial drugs on cytokine production requires further delineation [2]. Another possible reason is that significant inhibition of HCQ on cytokines may be reached for a relatively longer time through successive intracellular accumulation in *in vivo* studies. Because it has been demonstrated that very high concentrations (over 20 μmol/L) of chloroquine are required to achieve inhibition of cytokine induction in several *in vitro* studies [35].

Our results showed that HCQ failed to affect neither the productions of cytokines, nor the counts of WBC in normal mice. One possible reason for this is that challenge with HCQ for only two weeks is not long enough to induce immunological responses. Another potential reason is that it had been a remarkable pathologically increased production of pro-inflammatory cytokines before the establishment of an animal model with chronic debilitating inflammatory diseases, and HCQ might regulate them by several other pathways [2]. For example, HCQ is able to enter and accumulate in lysosomes, thereby increasing the pH value to prevent the activity of lysosomal enzymes, and finally reduces the productions of pro-inflammatory cytokines [2]. Thus, it is reasonable to infer that challenge with HCQ in a short period of time failed to change the immunological response in normal mice.

A growing body of evidence indicates that impaired gut barrier structure and function, characterized by reduced tight junction proteins density and intestinal hyperpermeability, may increase the passage of bacteria and/or microbiota-derived inflammatory components into the circulatory system, leading to endotoxemia and subsequent “cytokine storm”, and finally severely endanger life [36]. To our knowledge, elevated serum level of D-lactate, a carbohydrate fermentation metabolite almost produced by commensal bacteria in the colon, is a useful biomarker for dysbiosis and/or increased intestinal permeability [23]. Our results showed that HCQ could decrease, but not elevate the serum level of D-lactate in mice, and failed to affect the mRNA expression of several classical tight junction proteins in colonic tissue. These results suggested that no gut barrier structure and intestinal permeability were changed after HCQ short-term challenge.

The key finding of this investigation was that short-term challenge with HCQ at high dose significantly altered the gut microbiota. After taking HCQ for 7 days, the Shannon index showed that α-diversity was significantly decreased, whereas β-diversity was not changed according to the principal co-ordinates analysis. With the prolonged challenge of

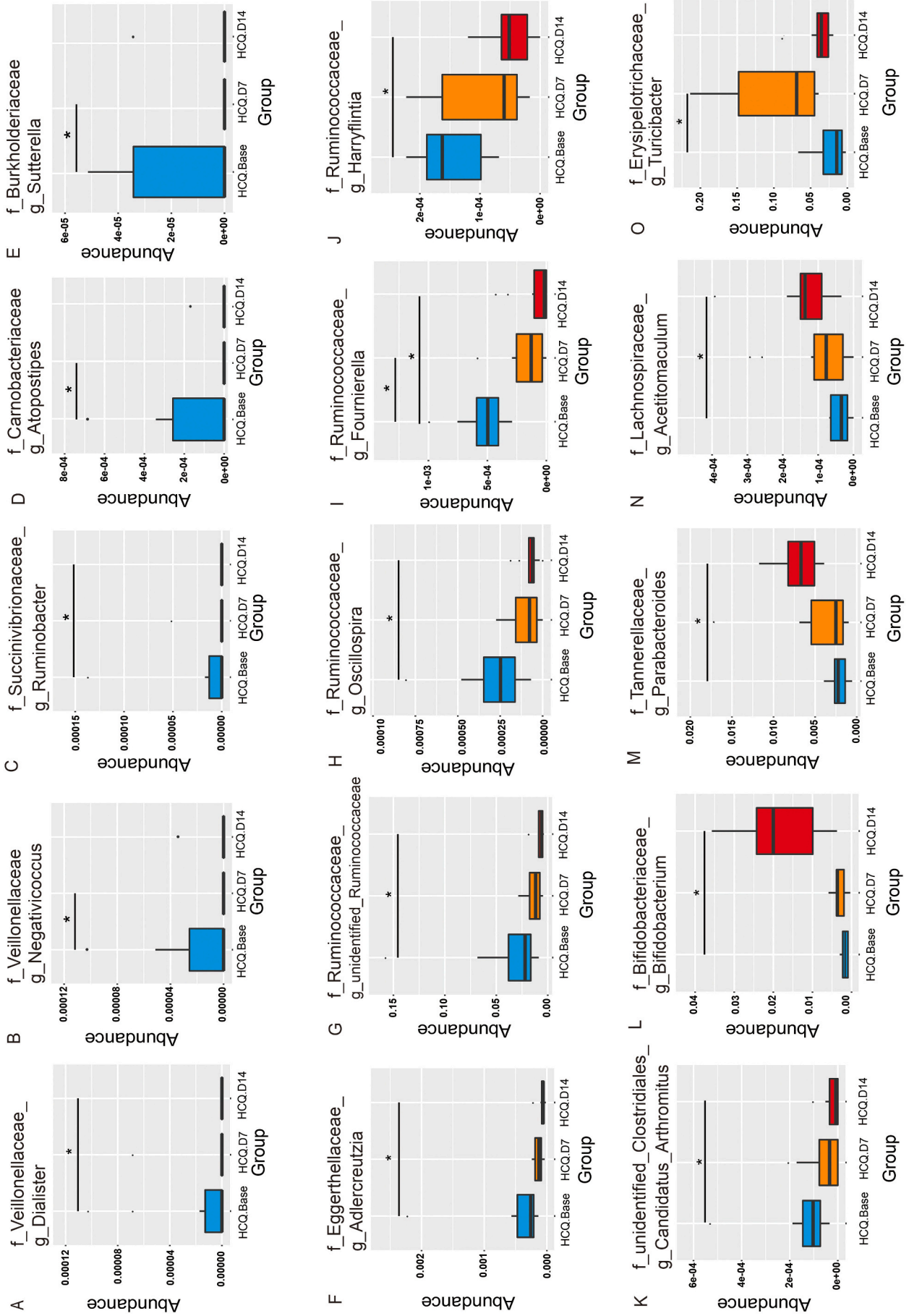

Fig. 5. HCQ-induced differentially abundant features in stool of mice.
Female C57BL/6J mice (n = 10) were gavage of HCQ at dose of 100 mg/kg for 14 days. Fresh stool samples of mice were harvested at the beginning of the experiment (HCQ.Base), and at days 7 (HCQ.D7) and 14 (HCQ.D14) after HCQ challenge. Microbial community in stool of mice were analyzed by 16S rRNA gene amplicon sequencing. Metastats analysis was employed to evaluate differences in relative abundance of bacterial genus among groups by R software. Statistical significance ($^*P < 0.05$) was performed by one-way analysis of variance followed by Tukey's HSD means comparisons.

HCQ, the α- and β-diversity were both reduced, accompanied by altered *Bacteroides* to *Firmicutes* ratio, indicating a significant alteration in gut microbial community.

Indeed, gut microbiota dysbiosis with a reduced diversity and an impaired prevalence of *Firmicutes* to *Bacteroidetes* ratio, was commonly observed in patients with obesity or type 2 diabetes mellitus [37]. Epidemiological investigation also showed that there was a higher disease severity and mortality rate in elderly COVID-19 patients with pre-existing age-related comorbidities containing obesity and diabetes [38]. In addition, several viral infectious diseases have been proved to have a reduced diversity of gut microbiome, which may increase susceptibility to secondary bacterial infections [39]. These findings suggested that a greater attention should be pain to the clinical application of HCQ to prevent and treat patients with COVID-19.

Further analysis regarding to the abundance altered species showed that four genus members in the family *Ruminococcaceae* were significantly decreased after HCQ challenge. One of them is *Oscillospira*, a genus that was associated with lower body mass index and leanness [40]. *Oscillospira* species appear to be a kind of potential probiotics because they are butyrate producers [41]. Among the abundance elevated members, the increased families of *Tannerellaceae* and *Lachnospiraceae* were found in toxigenic *Clostridioides difficile*-associated diarrhea [42]. The genus *Parabacteroides*, a member of the *Tannerellaceae* family, has been shown to be the most abundant in Crohn's disease [43]. Notably, we also found that the abundance of *Turicibacter*, a member of the *Erysipelotrichaceae* family, was elevated after HCQ challenge for 7 days. However, the *Erysipelotrichaceae* family has been implicated in several inflammation-related disorders of the GI tract [44], and was recently reported to augment SARS-CoV-2 infection in the host gut [11]. Altogether, these changes resulted from HCQ challenge seem to be harmful for healthy gut microbiota. In contrast, the relative abundance of *Bifidobacterium* genus, a well-known probiotic, was increased after HCQ challenge, indicating a comprehensive and complicated effects of HCQ on gut microbiota.

A latest meta-analysis on the clinical therapeutic role of HCQ in COVID-19 from the available literature has been reported [45]. Some of the studies demonstrated good clinical outcomes with HCQ alone or in combination with azithromycin in COVID-19 patients, whereas some of the other studies showed negative results with HCQ therapy along with the risk of adverse reactions. The contradictory clinical effect of HCQ may be attributed to the methodological limitations in clinical study, as well as the complex pharmacokinetics and various dosage of HCQ [45]. Stronger evidence from well-designed robust randomized clinical trials is required to determine the role of HCQ in the treatment of COVID-19.

5. Conclusion

Considering the gut microbiota of COVID-19 patients were characterized by elevated abundance of opportunistic pathogens and decreased of beneficial commensals [11], together with our finding that short-term high dose HCQ might induce gut microbiota dysbiosis in mice, we suggested that more clinical safety evaluations of HCQ on intestinal microecology are urgently needed.

CRediT authorship contribution statement

Zhi-Yuan Pan: Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Yu-Xiao Chang:** Investigation. **Ni Han:** Investigation. **Feng-Yi Hou:** Investigation. **B.J. Yang Lee:** Conceptualization. **Fa-Chao Zhi:** Formal analysis. **Rui-Fu Yang:** Conceptualization, Writing - original draft, Writing - review & editing. **Yu-Jing Bi:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2020.118450.

References

[1] K.D. Rainsford, A.L. Parke, M. Clifford-Rashotte, W.F. Kean, Therapy and pharmacological properties of hydroxychloroquine and chloroquine in treatment of systemic lupus erythematosus, rheumatoid arthritis and related diseases, Inflammopharmacology 23 (2015) 231–269, https://doi.org/10.1007/s10787-015-0239-y.
[2] E. Schrezenmeier, T. Dorner, Mechanisms of action of hydroxychloroquine and chloroquine: implications for rheumatology, Nat. Rev. Rheumatol. 16 (2020) 155–166, https://doi.org/10.1038/s41584-020-0372-x.
[3] P. Colson, J.M. Rolain, J.C. Lagier, P. Brouqui, D. Raoult, Chloroquine and hydroxychloroquine as available weapons to fight COVID-19, Int. J. Antimicrob. Agents 55 (4) (2020) 105932, https://doi.org/10.1016/j.ijantimicag.2020.105932.
[4] X. Yao, F. Ye, M. Zhang, C. Cui, B. Huang, P. Niu, et al., In vitro antiviral activity and projection of optimized dosing design of hydroxychloroquine for the treatment of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), Clin. Infect. Dis. 71 (15) (2020) 732–739, https://doi.org/10.1093/cid/ciaa237.
[5] W.J. Wiersinga, A. Rhodes, A.C. Cheng, S.J. Peacock, H.C. Prescott, Pathophysiology, transmission, diagnosis, and treatment of Coronavirus Disease 2019 (COVID-19): a review, JAMA 324 (8) (2020) 782–793, https://doi.org/10.1001/jama.2020.12839.
[6] G. Ruiz-Irastorza, M. Ramos-Casals, P. Brito-Zeron, M.A. Khamashta, Clinical efficacy and side effects of antimalarials in systemic lupus erythematosus: a systematic review, Ann. Rheum. Dis. 69 (1) (2010) 20–28, https://doi.org/10.1136/ard.2008.101766.
[7] C. Ponticelli, G. Moroni, Hydroxychloroquine in systemic lupus erythematosus (SLE), Expert Opin. Drug Saf. 16 (3) (2017) 411–419, https://doi.org/10.1080/14740338.2017.1269168.
[8] K.S. Cheung, I.F.N. Hung, P.P.Y. Chan, K.C. Lung, E. Tso, R. Liu, et al., Gastrointestinal manifestations of SARS-CoV-2 infection and virus load in fecal samples from a Hong Kong cohort: systematic review and meta-analysis, Gastroenterology 159 (1) (2020) 81–95, https://doi.org/10.1053/j.gastro.2020.03.065.
[9] S.H. Wong, R.N. Lui, J.J. Sung, Covid-19 and the digestive system, J. Gastroenterol. Hepatol. 35 (5) (2020) 744–748, https://doi.org/10.1111/jgh.15047.
[10] T. Zuo, Q. Liu, F. Zhang, G.C. Lui, E.Y. Tso, Y.K. Yeoh, et al., Depicting SARS-CoV-2 faecal viral activity in association with gut microbiota composition in patients with COVID-19, Gut (2020), https://doi.org/10.1136/gutjnl-2020-322294 In press.
[11] T. Zuo, F. Zhang, G.C.Y. Lui, Y.K. Yeoh, A.Y.L. Li, H. Zhan, et al., Alterations in gut microbiota of patients with COVID-19 during time of hospitalization, Gastroenterology 159 (3) (2020) 944–955, https://doi.org/10.1053/j.gastro.2020.05.048.
[12] E. Angelakis, M. Million, S. Kankoe, J.C. Lagier, F. Armougom, R. Giorgi, et al., Abnormal weight gain and gut microbiota modifications are side effects of long-term doxycycline and hydroxychloroquine treatment, Antimicrob. Agents Chemother. 58 (6) (2014) 3342–3347, https://doi.org/10.1128/AAC.02437-14.
[13] T. Magoc, S.L. Salzberg, FLASH: fast length adjustment of short reads to improve genome assemblies, Bioinformatics 27 (21) (2011) 2957–2963, https://doi.org/10.1093/bioinformatics/btr507.
[14] J.G. Caporaso, J. Kuczynski, J. Stombaugh, K. Bittinger, F.D. Bushman, E.K. Costello, et al., QIIME allows analysis of high-throughput community sequencing data, Nat. Methods 7 (5) (2010) 335–336, https://doi.org/10.1038/nmeth.f.303.
[15] R.C. Edgar, UPARSE: highly accurate OTU sequences from microbial amplicon reads, Nat. Methods 10 (10) (2013) 996–998, https://doi.org/10.1038/nmeth.2604.
[16] C. Quast, E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, et al., The SILVA ribosomal RNA gene database project: improved data processing and web-based tools, Nucleic Acids Res. 41 (2013) D590–D596, https://doi.org/10.1093/nar/gks1219.
[17] Q. Wang, G.M. Garrity, J.M. Tiedje, J.R. Cole, Naive Bayesian classifier for rapid

assignment of rRNA sequences into the new bacterial taxonomy, Appl. Environ. Microbiol. 73 (16) (2007) 5261–5267, https://doi.org/10.1128/AEM.00062-07.
[18] R.C. Edgar, MUSCLE: multiple sequence alignment with high accuracy and high throughput, Nucleic Acids Res. 32 (5) (2004) 1792–1797, https://doi.org/10.1093/nar/gkh340.
[19] J.R. White, N. Nagarajan, M. Pop, Statistical methods for detecting differentially abundant features in clinical metagenomic samples, PLoS Comput. Biol. 5 (4) (2009) e1000352, , https://doi.org/10.1371/journal.pcbi.1000352.
[20] S. Achuthan, J. Ahluwalia, N. Shafiq, A. Bhalla, A. Pareek, N. Chandurkar, et al., Hydroxychloroquine's efficacy as an antiplatelet agent study in healthy volunteers: a proof of concept study, J. Cardiovasc. Pharmacol. Ther. 20 (2) (2015) 174–180, https://doi.org/10.1177/1074248414546324.
[21] C.Y. Tao, J. Shang, T. Chen, D. Yu, Y.M. Jiang, D. Liu, et al., Impact of antimalarial (AM) on serum lipids in systemic lupus erythematosus (SLE) patients: a systematic review and meta-analysis, Medicine (Baltimore) 98 (14) (2019) e15030, , https://doi.org/10.1097/MD.0000000000015030.
[22] D. Liu, X. Li, Y. Zhang, J.S. Kwong, L. Li, Y. Zhang, et al., Chloroquine and hydroxychloroquine are associated with reduced cardiovascular risk: a systematic review and meta-analysis, Drug Des. Dev. Ther 12 (2018) 1685–1695, https://doi.org/10.2147/DDDT.S166893.
[23] K.A. Verbeke, A.R. Boobis, A. Chiodini, C.A. Edwards, A. Franck, M. Kleerebezem, et al., Towards microbial fermentation metabolites as markers for health benefits of prebiotics, Nutr. Res. Rev. 28 (1) (2015) 42–66, https://doi.org/10.1017/S0954422415000037.
[24] M.G. Rooks, W.S. Garrett, Gut microbiota, metabolites and host immunity, Nat. Rev. Immunol 16 (6) (2016) 341–352, https://doi.org/10.1038/nri.2016.42.
[25] J.B. Lynch, E.Y. Hsiao, Microbiomes as sources of emergent host phenotypes, Science. 365 (2019) 1405–1409.
[26] L. Lin, J. Zhang, Role of intestinal microbiota and metabolites on gut homeostasis and human diseases, BMC Immunol. 18 (1) (2017) 2, https://doi.org/10.1186/s12865-016-0187-3.
[27] J.P. Karl, A.M. Hatch, S.M. Arcidiacono, S.C. Pearce, I.G. Pantoja-Feliciano, L.A. Doherty, et al., Effects of psychological, environmental and physical stressors on the gut microbiota, Front. Microbiol. 9 (2018) 2013, https://doi.org/10.3389/fmicb.2018.02013.
[28] G. Ianiro, H. Tilg, A. Gasbarrini, Antibiotics as deep modulators of gut microbiota: between good and evil, Gut 65 (11) (2016) 1906–1915, https://doi.org/10.1136/gutjnl-2016-312297.
[29] B.E. van den Borne, B.A. Dijkmans, H.H. de Rooij, S. le Cessie, C.L. Verweij, Chloroquine and hydroxychloroquine equally affect tumor necrosis factor-alpha, interleukin 6, and interferon-gamma production by peripheral blood mononuclear cells, J. Rheumatol. 24 (1) (1997) 55–60.
[30] K. Sperber, H. Quraishi, T.H. Kalb, A. Panja, V. Stecher, L. Mayer, Selective regulation of cytokine secretion by hydroxychloroquine: inhibition of interleukin 1 alpha (IL-1-alpha) and IL-6 in human monocytes and T cells, J. Rheumatol. 20 (5) (1993) 803–808.
[31] K. Hjorton, N. Hagberg, E. Israelsson, L. Jinton, O. Berggren, J.K. Sandling, et al., Cytokine production by activated plasmacytoid dendritic cells and natural killer cells is suppressed by an IRAK4 inhibitor, Arthr. Res. Ther 20 (1) (2018) 238, https://doi.org/10.1186/s13075-018-1702-0.
[32] S.M. Monzavi, A. Alirezaei, Z. Shariati-Sarabi, J. Tavakol Afshari, M. Mahmoudi, B. Dormanesh, et al., Efficacy analysis of hydroxychloroquine therapy in systemic lupus erythematosus: a study on disease activity and immunological biomarkers, Inflammopharmacology 26 (5) (2018) 1175–1182, https://doi.org/10.1007/s10787-018-0512-y.
[33] R. Willis, A.M. Seif, G. McGwin Jr., L.A. Martinez-Martinez, E.B. Gonzalez, N. Dang, et al., Effect of hydroxychloroquine treatment on pro-inflammatory cytokines and disease activity in SLE patients: data from LUMINA (LXXV), a multiethnic US cohort, Lupus 21 (8) (2012) 830–835, https://doi.org/10.1177/0961203312437270.
[34] A. Wozniacka, A. Lesiak, J. Boncela, K. Smolarczyk, D.P. McCauliffe, A. Sysa-Jedrzejowska, The influence of antimalarial treatment on IL-1beta, IL-6 and TNF-alpha mRNA expression on UVB-irradiated skin in systemic lupus erythematosus, Br. J. Dermatol. 159 (5) (2008) 1124–1130, https://doi.org/10.1111/j.1365-2133.2008.08804.x.
[35] I. Karres, J.P. Kremer, I. Dietl, U. Steckholzer, M. Jochum, W. Ertel, Chloroquine inhibits proinflammatory cytokine release into human whole blood, Am. J. Phys. 274 (4) (1998) R1058–R1064, https://doi.org/10.1152/ajpregu.1998.274.4.R1058.
[36] S. Otani, C.M. Coopersmith, Gut integrity in critical illness, J. Intensive Care 7 (17) (2019) 17, https://doi.org/10.1186/s40560-019-0372-6.
[37] A.V. Hartstra, K.E. Bouter, F. Backhed, M. Nieuwdorp, Insights into the role of the microbiome in obesity and type 2 diabetes, Diabetes Care 38 (1) (2015) 159–165, https://doi.org/10.2337/dc14-0769.
[38] S.D. Viana, S. Nunes, F. Reis, ACE2 imbalance as a key player for the poor outcomes in COVID-19 patients with age-related comorbidities — role of gut microbiota dysbiosis, Ageing Res. Rev. 62 (2020) 101123, , https://doi.org/10.1016/j.arr.2020.101123.
[39] S. Hanada, M. Pirzadeh, K.Y. Carver, J.C. Deng, Respiratory viral infection-induced microbiome alterations and secondary bacterial pneumonia, Front. Immunol. 9 (2018) 2640, https://doi.org/10.3389/fimmu.2018.02640.
[40] S. Tims, C. Derom, D.M. Jonkers, R. Vlietinck, W.H. Saris, M. Kleerebezem, et al., Microbiota conservation and BMI signatures in adult monozygotic twins, ISME J 7 (4) (2013) 707–717, https://doi.org/10.1038/ismej.2012.146.
[41] U. Gophna, T. Konikoff, H.B. Nielsen, Oscillospira and related bacteria — from metagenomic species to metabolic features, Environ. Microbiol. 19 (3) (2017) 835–841, https://doi.org/10.1111/1462-2920.13658.
[42] M. Hernandez, M. de Frutos, D. Rodriguez-Lazaro, L. Lopez-Urrutia, N.M. Quijada, J.M. Eiros, Fecal microbiota of toxigenic Clostridioides difficile-associated diarrhea, Front. Microbiol. 9 (2018) 3331, https://doi.org/10.3389/fmicb.2018.03331.
[43] L.R. Lopetuso, V. Petito, C. Graziani, E. Schiavoni, F. Paroni Sterbini, A. Poscia, et al., Gut microbiota in health, diverticular disease, irritable bowel syndrome, and inflammatory bowel diseases: time for microbial marker of gastrointestinal disorders, Dig. Dis. 36 (1) (2018) 56–65, https://doi.org/10.1159/000477205.
[44] N.O. Kaakoush, Insights into the role of Erysipelotrichaceae in the human host, Front. Cell. Infect. Microbiol. 5 (2015) 84, https://doi.org/10.3389/fcimb.2015.00084.
[45] S. Das, S. Bhowmick, S. Tiwari, S. Sen, An updated systematic review of the therapeutic role of hydroxychloroquine in Coronavirus Disease-19 (COVID-19), Clin. Drug Investig 40 (7) (2020) 591–601, https://doi.org/10.1007/s40261-020-00927-1.