Supporting Information

CuO nanoparticles elicit intestinal immunotoxicity in zebrafish based on intestinal microbiota dysbiosis

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Supporting Methods

Targeted Metabolomics (Short-chain fatty acids (SCFAs) profiling)

A certain number of samples was extracted in 50 μL of 15% phosphoric acid with 100 μL of 125 μg/mL 4-methylvaleric acid solution as internal standard and 400 μL ether by ultrasonic method in an ice bath for 10 min. Subsequently, the samples were centrifuged at 4 °C for 10 min at 12000 rpm and the supernatant was transferred into the vial prior to GC-MS analysis. The GC analysis was performed on trace 1300 gas chromatograph (Thermo Fisher Scientific, USA). The GC was fitted with a capillary column Agilent HP-INNOWAX (30 m × 0.25 mm ID × 0.25 μm) and helium was used as the carrier gas at 1 mL/min. Injection was made in split mode at 10:1 with an injection volume of 1 μL and an injector temperature of 250 °C. The temperature of the ion source and interface were 300 °C and 250 °C, respectively. The column temperature was programmed to increase from an initial temperature of 90°C, followed by an increase to 120 °C at 10 °C/min, and to 150 °C at 5 °C/min, and finally to 250 °C at 25 °C/min which was maintained for 2 min (total run-time of 15 min). Mass spectrometric detection of metabolites was performed on ISQ 7000 (Thermo Fisher Scientific, USA) with electron impact ionization mode. Single ion monitoring (SIM) mode was used with the electron energy of 70 eV. Acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, 4-methylvaleric acid and caproic acid were applied as standards.

16S rRNA sequencing of intestinal flora

Total genomic DNA samples were extracted using the OMEGA Soil DNA Kit

(M5636-02) (OmegaBio-Tek, Norcross, GA, USA), and stored at -20 °C prior to further analysis. The concentrations and quantity of extracted DNAs were measured using a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively. PCR amplification of the bacterial 16S rRNA genes V3–V4 region was performed using the forward primer 338F (5'-ACTCCTACGGGAGGCAGCA-3') and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'). PCR amplicons were purified with Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). After the individual quantification step, amplicons were pooled in equal amounts, and pair-end 2×250 bp sequencing was performed using the Illlumina NovaSeq platform with NovaSeq 6000 SP Reagent Kit (500 cycles) at Shanghai Personal Biotechnology Co., Ltd (Shanghai, China). The Silva Database (Release132) was used to annotate taxonomic information based on the sequence data analyses, which were mainly performed using QIIME2 and R packages (v3.2.0). Principal component analysis (PCA) was also conducted based on the genus-level compositional profiles. Alpha diversity analysis was performed to identify the complexity of species diversity of each sample utilizing QIIME2 software. Beta diversity analysis was performed to investigate the structural variation of microbial communities among samples. One-way analysis of variance was used to compare bacterial abundance and diversity. LEfSe (Linear discriminant analysis effect size) was performed to detect differentially abundant taxa across groups using the default parameters. Random forest analysis was applied to

discriminating the samples from different groups using QIIME2 with default settings.

ICP-MS

After weighing the animal tissue samples, the digestion solution was added and pre-digested on an electric heating plate. The pre-digested samples were digested by microwave. Microwave digestion was that take a certain sample in the microwave digestion tank with 2 mL nitric acid. After 120 ℃ of pre-digestion for half an hour, the procedure became: 130 ℃ of warming time 5 min, stable time 3 min; 150 ℃ of heating time 3 min, stable time 10 min; 180 ℃ of heating time 3 min, stable time 30min. Finally, the sample was cooled to 40 ℃, and transferred to 10 mL volume bottle with deionized water to 10 mL. After microwave digestion, Agilent 7800 was used to detection the sample solution under the instrument setting: Pump Rate: 20 r/min, Nebulizer Flow: 1.00 L/min, Auxiliary Gas: 1.00 L/min, Sample Flush Time: 40 s, RF Power: 1550 w. The inductively coupled plasma detection is calibrated by internal standard, and the detection sensitivity is high. The matrix interference of elements is corrected effectively.

Supporting Table

Table S1: Primer information used in qRT-PCR. All sequences are shown 5'-3'.

Gene	Forward primer	Reverse primer
β- actin F	CCGTGACATCAAGGAGAAGC	TACCGCAAGATTCCATACCC
myd88 F	CAGTGGTGGACAGTTGTGGAC	GAAAGCATCAAAGGTCTCAGGTG
Trif F	CGCAACGTTCTCCGAAGACTTTG	CGGAATAACGGAGTTCAGCTTGTG
nf-kB F	GGCAGAGTTCGTCAAAGC	AGACGCACAGCCTCCATA
IL-10 F	TCACGTCATGAACGAGATCC	CCTCTTGCATTTCACCATATCC
IFN F	GCCTGGGGAGTATGTTTGCT	CAGGAAGATGGGGTGTGCAT
IgM F	CGGTTTTCGCCTAAACAATACGAG	TGCTTCCATGTTTCTGCGTTG
Gpr84 F	CGTTACTTTGGTGTGCTCTTGGGAT	ATAGGAGGTCAGCAAAGGCAAGGTT
GPR108 F	TGCATACAGACACACACTCAC	ACCTGACACACACACACATAC
SIN3-HDAC F	AGGACGAGGATGATGGAGAA	CGTCCACTTCCTGCTCTATTT

Supporting Figure

Figure S1. Characterization of CuO NPs. (A) TEM image of CuO NPs; (B) Zeta potentials of CuO NPs; (C) EDS analysis of CuO NPs; (D) The XRD of CuO NPs.

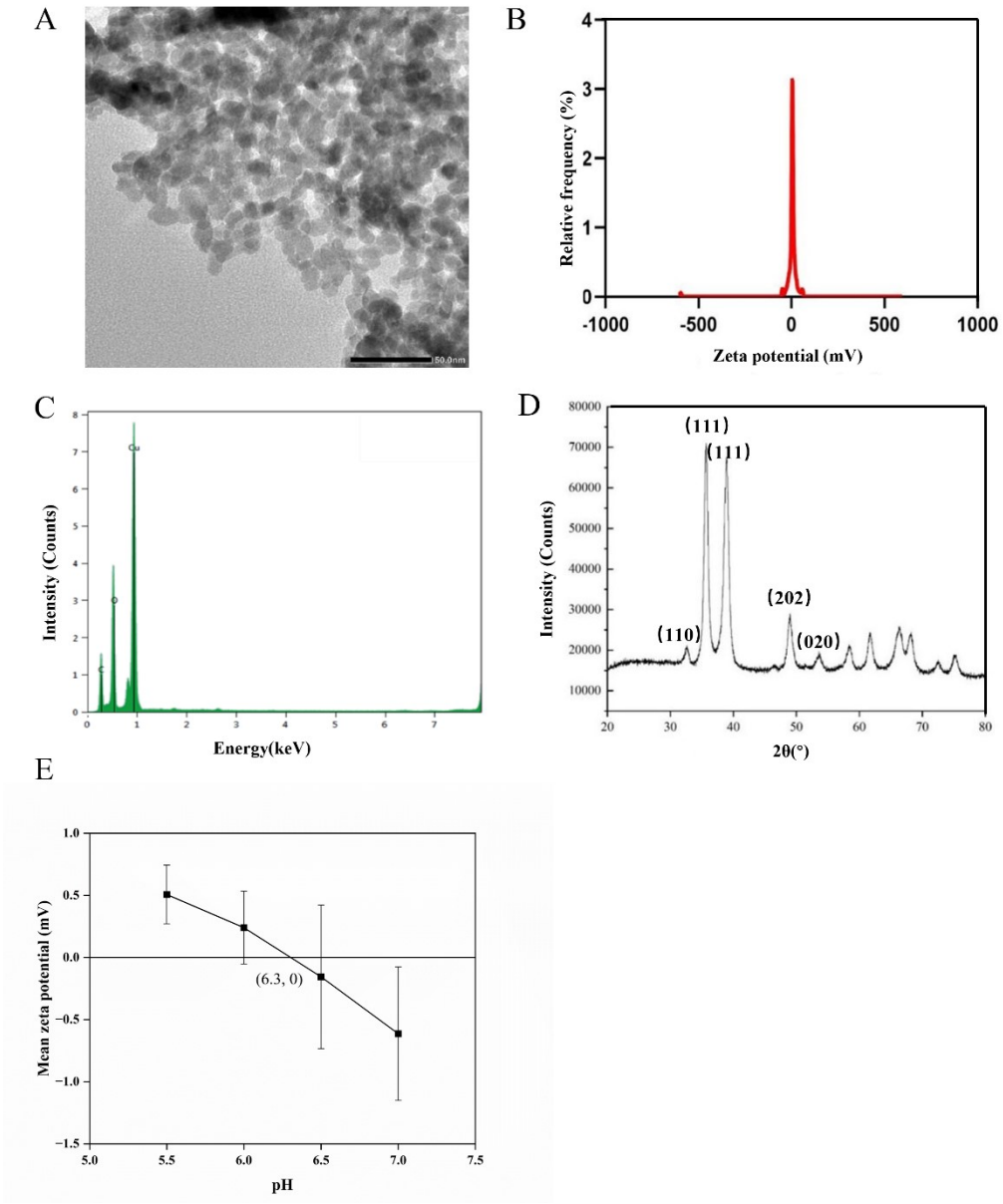


Figure S2. The residue of CuO NPs in treated zebrafish. (*n*=4)

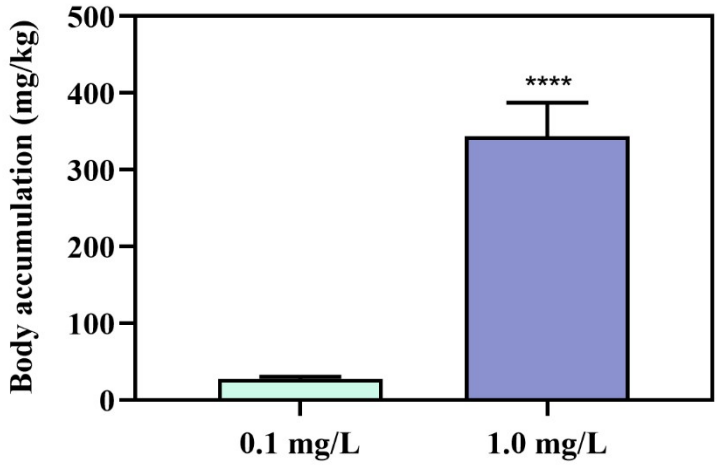


Figure S3. Effects on development of zebrafish under different concentrations of CuO NPs exposure (0, 0.1 and 1 mg/L) for 21 days. (A) Weight; (B) Length; (C) ISI: ISI =intestine weight × 100/total weight; (D) Survival rate.

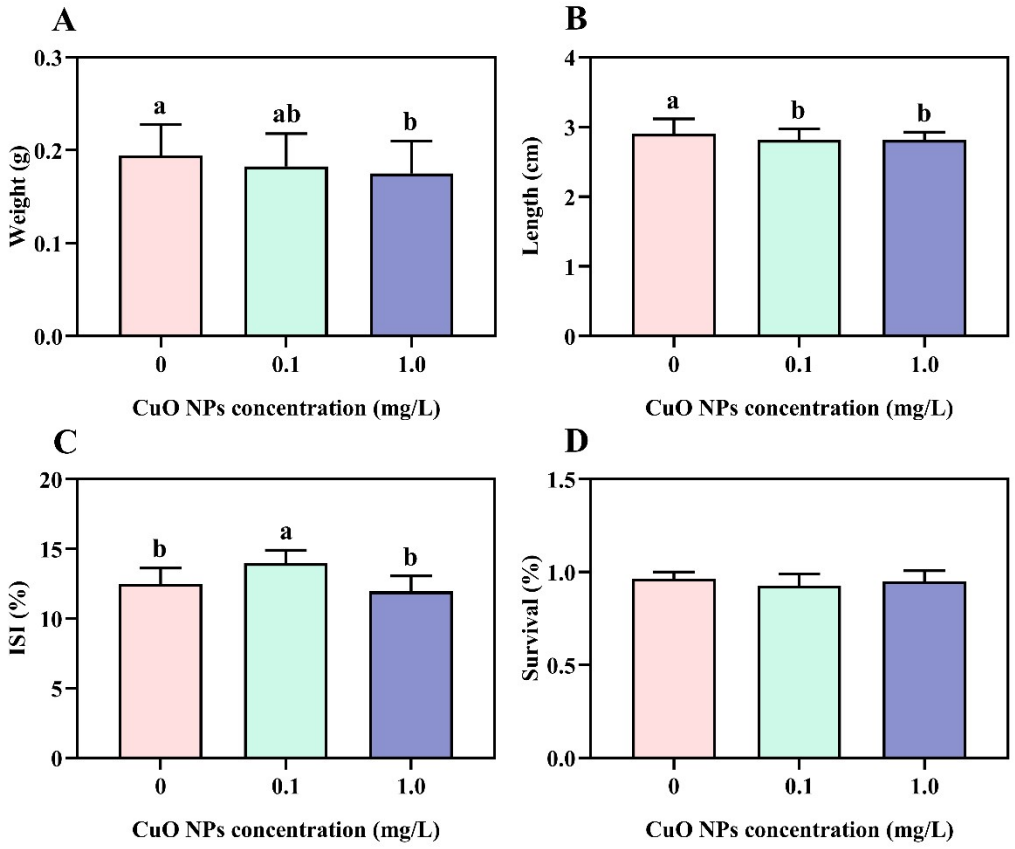


Figure S4. The dilution curve of the sample in 16S rRNA sequencing of intestinal flora.

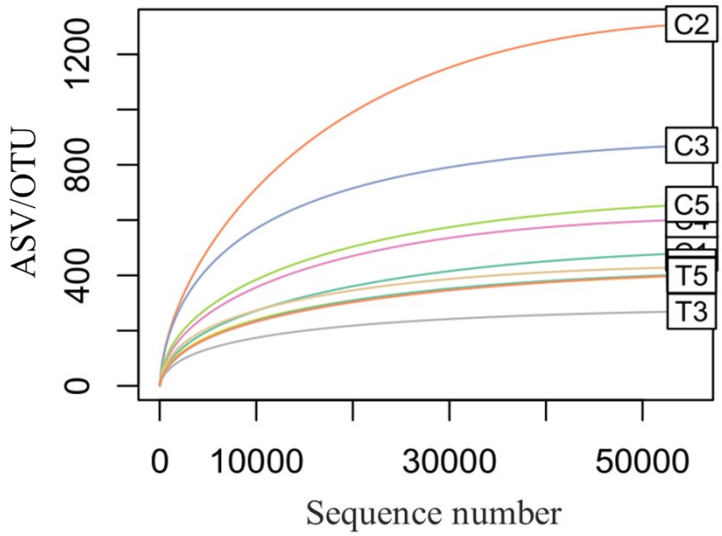


Figure S5. The influence of CuO NPs exposure on the gut microbiota composition of zebrafish at a genus level.

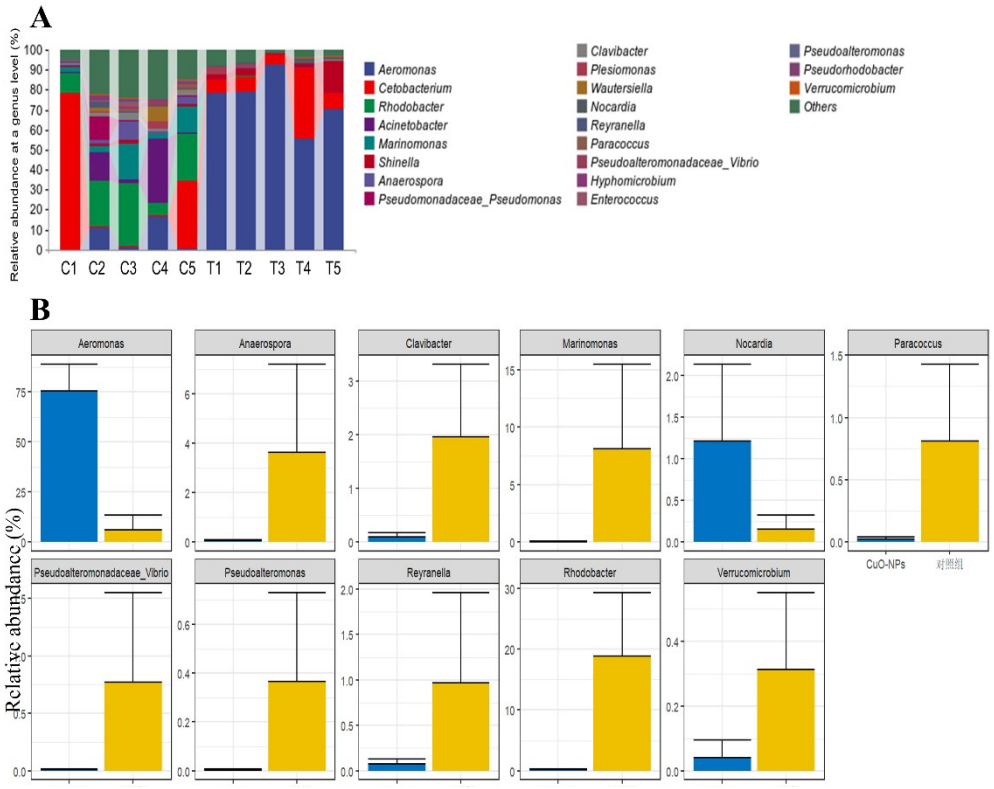
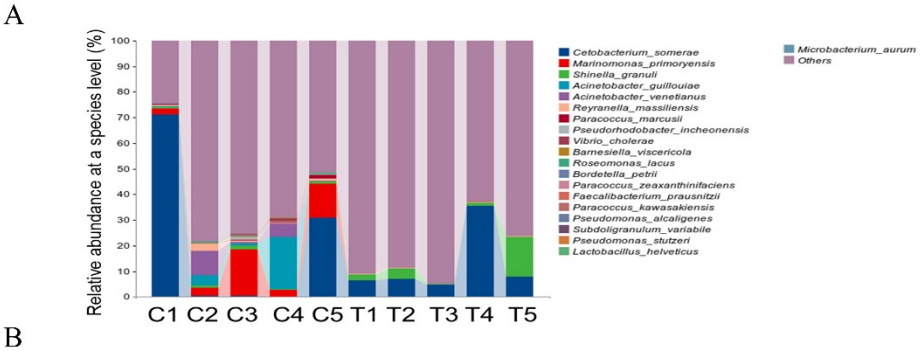


Figure S6. The influence of CuO NPs exposure on the gut microbiota composition of zebrafish at a species level.

A



B

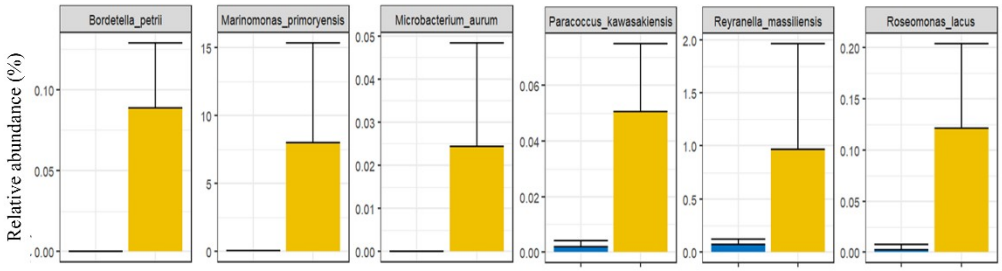


Figure S7. Pearson correlation analysis between gut microbiota and SCFAs.

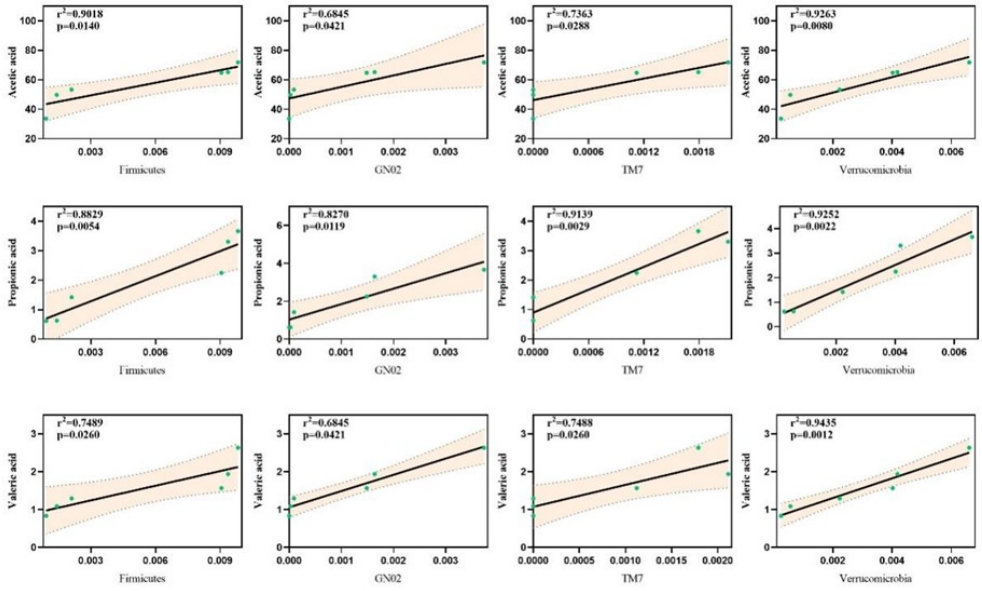


Figure S8. Pearson correlation analysis between SCFAs and immune factors.

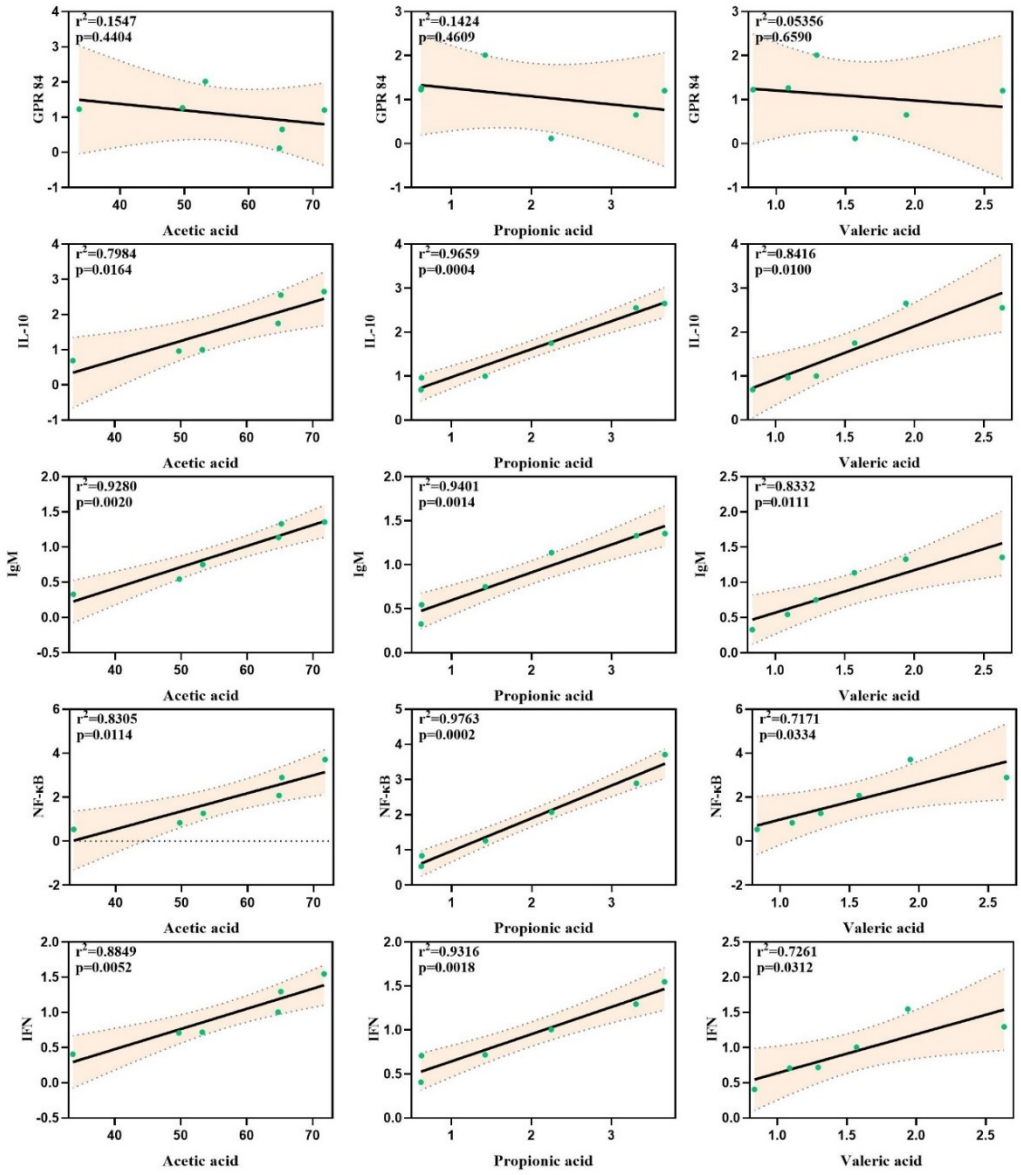


Figure S9. Pearson correlation analysis of gut microbiota with immune factors