Materials and methods

1. *DNA extraction and 16S rRNA Gene sequencing of intestinal microorganisms*

(1) **DNA Extraction**: DNA Extraction Total genomic DNA samples were extracted using the OMEGA Soil DNA Kit (M5635-02) (Omega Bio-Tek, Norcross, GA, USA), following the manufacturer's instructions and stored at -20°C before further analysis. The quantity and quality of extracted DNAs were measured using a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively.

(2) **16S rRNA Gene Amplicon Sequencing**: 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'). Sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. The PCR components contained 5 μL of buffer (5×), 0.25 μL of Fast pfu DNA Polymerase (5 U μL^{-1}), 2 μL (2.5 mM) of dNTPs, 1 μL (10 μM) of each Forward and Reverse primer, 1 μL of DNA Template, and 14.75 μL of ddH_2O. Thermal cycling consisted of initial denaturation at 98°C for 5 min, followed by 25 cycles consisting of denaturation at 98°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 45 s, with a final extension of 5 min at 72°C. 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 Illumina MiSeq platform with MiSeq Reagent Kit V3 at Shanghai Personal Biotechnology Co., Ltd (Shanghai, China).

For sequence analysis and bioinformatics and statistical analysis, the specific analysis process could be found in the supplementary material.

2. Detection methods of metabolites in intestine (LC-MS/MS analysis)

(1) **Sample pretreatment**: Samples were slowly thawed at 4°C, 1 mL precooling methyl alcohol/acetonitrile/water (2:2:1, v/v/v) was added and adequately vortexed. After ultrasonic decomposition at low temperature for 30 min, the samples are incubated for 10 min at -20°C to precipitate the protein and then centrifuged (14000 g, 4°C, 20 min). The supernatants were collected and dried under vacuum and then stored at -80°C standby. Redissolve the sample in 100 μL acetonitrile/water (1:1, v/v), adequately vortexed, and centrifuged (14000 rpm, 4°C, 15 min). The supernatants were collected for LC-MS/MS analysis.

(2) **LC-MS/MS analysis**: Sample separation was performed using an UHPLC (1290 Infinity LC, Agilent Technologies) HILIC and RPLC. The column temperature was 25°C and flow rate was 300 μL min^{-1}. 2 μL sample was loaded. The samples were placed in an autosampler at 4°C. Samples were analyzed randomly to avoid the influence caused by instrument detecting signal fluctuation. QC samples were inserted into the analysis queue to evaluate the system stability and data reliability during the experimental process. The mobile phase of chromatography was composed of buffer A (Water +25 mM ammonium acetate +25 mM ammonium hydroxide) and buffer B

(Acetonitrile). The gradient was 95% B for 1 min and was linearly reduced to 65% in 13 min, and then was reduced to 40% in 2 min and kept at 40% for 2 min, and then increased to 95% in 0.1 min, with a 5 min re-equilibration period employed. Samples were detected in both ESI positive and negative modes. Analyses were performed using an UHPLC coupled to a quadrupole time-of-flight (AB SCIEX TripleTOF 6600). The ESI source conditions following HILIC separation were set as follows: Ion Source Gas1 (Gas1) as 60, Ion Source Gas2 (Gas2) as 60, curtain gas (CUR) as 30, source temperature: 600℃, Ion Spray Voltage Floating (ISVF) 5500 V in positive mode, and -5500 V in negative mode. In MS only acquisition, the instrument was set to acquire over the m/z range 60-1000 Da, product ion scan m/z range 25-1000 Da, TOF MS scan accumulation time 0.20 s/spectra, product ion scan accumulation time 0.05 s/spectra. The product ion scan is acquired using information dependent acquisition (IDA) with high sensitivity mode selected. The collision energy (CE) was fixed at 35 eV with ± 15 eV. Declustering potential (DP) was set as ± 60 V. IDA was set as follows: Exclude isotopes within 4 Da, Candidate ions to monitor per cycle.

(3) **Data process**: The raw MS data (wiff.scan files) were converted to MzXML files using ProteoWizard MS Convert and processed using XCMS for feature detection, retention time correction and alignment. The metabolites were identified by accuracy mass (< 25 ppm) and MS/MS data matched with our standards database. In the extracted ion features, only the variables having more than 50% of the nonzero measurement values in at least one group were kept. SIMCA-P 14.1 (Umetrics, Umea, Sweden) was used for principal component analysis (PCA) and partial least squares-

discriminant analysis (PLS-DA). Single dimensional statistical analysis includes Student's t-test and fold change. The volcano plot was obtained by R software (4.2.0)

3. Sequence Analysis:

Microbiome bioinformatics were performed with QIIME2 2019.4 with slight modification according to the official tutorials (https://docs.qiime2.org/2019.4/tutorials/). Briefly, raw sequence data were demultiplexed using the demux plugin following by primers cutting with cutadapt plugin. Sequences were then quality filtered, denoised, merged and chimera removed using the DADA2 plugin. Non-singleton amplicon sequence variants (ASVs) were aligned with mafft and used to construct a phylogeny with fasttree2. Alpha-diversity metrics, observed species, Shannon, Simpson, Faith's PD, Pielou's evenness and Good's coverage, beta diversity metrics (weighted UniFrac), unweighted UniFrac, Jaccard distance, and Bray-Curtis dissimilarity) were estimated using the diversity plugin with samples were rarefied to 741 sequences per sample. Taxonomy was assigned to ASVs using the classify-sklearn naïve Bayes taxonomy classifier in feature-classifier plugin against the SILVA Release Database.

4. Bioinformatics and Statistical Analysis:

Bioinformatics and Statistical Analysis Sequence data analyses were mainly performed using QIIME2 and R packages (v3.2.0). ASV-level alpha diversity indices, such as Chao1 richness estimator, observed species, Shannon diversity index, Simpson index, Faith's PD, Pielou's evenness and Good's coverage were calculated using the ASV table in QIIME2, and visualized as box plots. ASV-level ranked

abundance curves were generated to compare the richness and evenness of ASVs among samples. Beta diversity analysis was performed to investigate the structural variation of microbial communities across samples using Jaccard metrics (Jaccard, 1908), Bray-Curtis metrics and UniFrac distance metrics and visualized via principal coordinate analysis (PCoA), nonmetric multidimensional scaling (NMDS) and unweighted pair-group method with arithmetic means (UPGMA) hierarchical clustering. Principal component analysis (PCA) was also conducted based on the genus-level compositional profiles. The significance of differentiation of microbiota structure among groups was assessed by PERMANOVA (Permutational multivariate analysis of variance), ANOSIM (Analysis of similarities), Permdisp using QIIME2. The taxonomy compositions and abundances were visualized using MEGAN and GraPhlAn. Venn diagram was generated to visualize the shared and unique ASVs among samples or groups using R package “VennDiagram”, based on the occurrence of ASVs across samples/groups regardless of their relative abundance. Taxa abundances at the ASV levels were statistically compared among samples or groups by MetagenomeSeq , and visualized as Manhattan plots. LEfSe (Linear discriminant analysis effect size) was performed to detect differentially abundant taxa across groups using the default parameters. OPLS-DA (Orthogonal Partial Least Squares Discriminant Analysis) was also introduced as a supervised model to reveal the microbiota variation among groups, using the R package “muma”. Random forest analysis was applied to discriminating the samples from different groups using QIIME2 with default settings. Nested stratified k-fold cross validation was used for

automated hyperparameter optimization and sample prediction. The number of k-fold cross-validations was set to cv = 1 Co-occurrence network analysis was performed by SparCC analysis. The pseudocount value in SparCC was set to 10-6. The cutoff of correlation coefficients was determined as 70 through random matrix theory-based methods as implemented in R package RMThreshold. Based on the correlation coefficients, we constructed co-occurrence network with nodes representing ASVs and edges representing correlations between these ASVs. Network was visualized using R package igraph and ggraph. Microbial functions were predicted by PICRUSt2 (Phylogenetic investigation of communities by reconstruction of unobserved states) (Gavin M. Douglas, et al., preprint) upon MetaCyc (https://metacyc.org/) and KEGG (https://www.kegg.jp/) databases.

Table S1. Primer sequences used for real-time PCR analysis

Gene	F/R	Primer sequence (5′-3′)	TM (℃)	Accession number
Hepcidin-1	F	CATTCACCGGGGTGCAA	56.14	EU502754.1
	R	CCTGATGTGATTTGGCATCATC	53.94	
Hepcidin-2	F	TCAGTGGAATCCTGGAAGATGC	56.98	EU502755.1
	R	ACAGCAAAAGCGACATTTAATGC	54.28	
IL-1β	F	CGTGACTGACAGCAAAAAGAGG	56.49	RNA-seq by Ref. (Sun et al., 2020)
	R	GATGCCCAGAGCCACAGTTC	59.24	
IL-15	F	GTATGCTGCTTCTGTGCCTGG	59.21	RNA-seq by Ref. (Sun et al., 2020)
	R	AGCGTCAGATTTCTCAATGGTGT	56.33	
IL-10	F	CGGCACAGAAATCCCAGAGC	59.46	RNA-seq by Ref. (Sun et al., 2020)
	R	CAGCAGGCTCACAAAATAAACATCT	55.42	
TGF-β1	F	GCTCAAAGAGAGCGAGGATG	55.92	RNA-seq by Ref. (Sun et al., 2020)
	R	TCCTCTACCATTCGCAATCC	54.28	
Caspase-3	F	GCTTCATTCGTCTGTGTTC	51.13	RNA-seq by Ref. (Sun et al., 2020)
	R	CGAAAAAGTGATGTGAGGTA	49.2	
Caspase-8	F	GAGACAGACAGCAGACAACCA	57.08	RNA-seq by Ref. (Sun et al., 2020)
	R	TTCCATTTCAGCAAACACATC	50.77	
Caspase-9	F	CTGGAATGCCTTCAGGAGACGGG	62.89	RNA-seq by Ref. (Sun et al., 2020)
	R	GGGAGGGGCAAGACAACAGGGTG	65.87	
SOD1	F	CCCCACAACAAGAATCATGC	54.37	MK614709.1
	R	TCTCAGCCTTCTCGTGGA	55.47	

SOD2	F	CTGACCTACGACTATGGTGC	55.11	MK614710.1
	R	CGTCACATCTCCCTTCGCTA	56.82	
SOD3a	F	ATCCATCAGTACGGAGACCT	54.68	MK614711.1
	R	TTCCAATCGCAGACATCCCT	56.31	
β-actin	F	AAAGGGAAATCGTGCGTGAC	55.91	XM020651307.1
	R	AAGGAAGGCTGGAAGAGGG	57.22	