DNA Extraction

The DNeasy PowerSoil Kit (QIAGEN, Inc., Netherlands) was used to extract total genomic DNA samples, in accordance with the manufacturer's instruction stored at -20 °C prior to 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.

16S rRNA sequencing

Polymerase chain reaction (PCR) was used for V3-V4 hypervariable regions of 16S rRNA gene amplification with the primers of 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. The PCR components contained 5μl of buffer (5×), 0.25μl of Fast pfu DNA Polymerase (5 U/μl), 2μl (2.5 mM) of dNTPs, 1μl (10uM) of each Forward and Reverse primer, 1μl of DNA Template, and 14.75μl 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 45s, 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 Illlumina MiSeq platform with MiSeq Reagent Kit v3 at Shanghai Personal Biotechnology Co., Ltd (Shanghai, China).

Metabolite Analysis and Identification

Ultraperformance liquid chromatography to quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS) approach was applied to identify the fecal metabolites. All experiments were performed on ice. Sample treated method: 100 mg feces were put into the homogenization tube, added three 1.5 mm zirconium beads and 500μL precooled extract (methanol: ultrapure water = 1:1). The mixtures were

homogenized three times (6000 rpm, 30 s each time, and 10 s apart), and centrifuged at 12,000 rpm at 4 ℃ for 15 min. The supernatant was collected and added 500μL precooled extract to repeat extraction once. The combined the supernatant was centrifuged and transferred into liquid phase vial.

We took 10μL from each sample as quality control (QC) sample. Before sample testing, QC sample was continuously tested for 10 times and analyzed. If the instrument had good repeatability and high stability, the samples were conducted to be tested. The QC sample was tested every 10 target samples during the analysis process.

The screening process of differential fecal metabolites was as follows: First, the metabolites with fold change > 2 or < 0.5 were screened. Then, metabolites with variable importance in projection (VIP) > 2 were identified to be analyzed. Finally, Wilcoxon rank-sum test was used to compare the fold change between two groups, and Kruskal-Wallis was implemented to compare the fold changes between groups to find the significantly changed features. A two tailed $p < 0.05$ was regarded as statistical difference.

To identify potential biomarkers, Human Metabolome Database (HMDB) (http://hmdb.ca/), Metlin (http://metlin.scripps.edu/index.php) and ChemSpider (http://cssp.chemspider.com/) was searched. The search criteria were average m/z value. Metabolite set enrichment and pathway analyses were based on MetaboAnalyst (www.meta-boanalyst.ca).