Supplementary Material

1 Supplemental Methods

Endothelin-1 (ET-1) Stroke Model

To induce focal cerebral ischemia, the vasoconstrictive peptide endothelin-1 (ET-1) (Sigma, USA) was injected at the following coordinates: (1) AP +0.7 mm, ML +2.2 mm, DV −2.0 mm; (2) AP +2.3 mm, ML +2.5 mm, DV −2.3 mm; and (3) AP +0.7 mm, ML +3.8 mm, DV −5.8 mm (16). The ET-1 dose was 0.5 μg/μl (2 μl) and with an injection speed of 0.5μl/min. The needle was left in situ for 3min post-injection before being slowly removed to minimize backflow. Sham-operated animals underwent the same surgery except that saline was injected instead of ET-1. Anesthesia was induced by 4% isoflurane and then maintained with 1.5-2% isoflurane. Ketorolac (3mg/kg, i.m.) was given after the operation. Rats with no lesion or with severe complications were excluded from the study. Rats from different groups were randomly subjected to the surgery.

Extraction of ileum and content

After sacrifice, a 3 cm ileal segment was cut off. The ileum and its content were collected into sterile tubes for microbiological analysis and stored at -80℃.

Gut Microbiome 16S rRNA Gene Sequencing and Analysis

Samples of ileal contents were collected and stored at -80℃. DNA was extracted from 8 samples per group and its quality was measured by 0.8% agarose gel electrophoresis, and the DNA was quantified by ultraviolet spectrophotometer. PCR amplificated within variable regions and specific gene fragments of rRNA genes. The bacterial 16S rDNA genes were amplified (35 cycles) using the degenerate forward primer 5-AGRGTTTGATCMTGGCTCAG-3 and the non-degenerate reverse primer 5-GGTTACCTTG TTACGACTT-3. The PCR amplification products were detected by 2% agarose gel electrophoresis, and the target fragments were cut and recovered, and the gel recovery kit of AXYGEN was used for recovery. Referring to the preliminary quantitative results of electrophoresis, the PCR amplification products were subjected to fluorescence quantification (Quant-iT PicoGreen dsDNA Assay Kit) by a Microplate reader (BioTek, FLx800). According to the fluorescence quantitative results, the sequencing volume requirements of each sample were determined and the samples were handled with the following protocol 1) First, sequence end repair was performed on the above-mentioned amplified product. The End Repair Mix2 in the kit was used to remove the overhanging base at the 5'end of the DNA sequence, and a phosphate group added to fill in the missing base at the 3'end; 2) An A base was added to the 3'end of the DNA sequence to prevent self-linking of DNA fragments, while ensuring that the target sequence could be connected to the sequencing adapter; 3) A sequencing adapter containing a library-specific tag at the 5'end of the sequence was added so that the DNA molecule was immobilized on the Flow Cell; 4) BECKMAN AMPure XP Beads were used to remove the self-linked fragments of the adaptor through magnetic bead screening, and the library system was purified after adding the adaptor; 5) PCR amplification was performed on the above-mentioned DNA fragments connected to the adapters to enrich the sequencing library template, and BECKMAN AMPure XP Beads used again to purify the library enriched products; 6) The final fragment selection was performed by purification of the library by 2%

agarose gel electrophoresis. 7) Before sequencing on the computer, a quality inspection was conducted on the library on the Agilent Bioanalyzer, using the Agilent High Sensitivity DNA Kit. 8) After that, the Quant-iT PicoGreen dsDNA Assay Kit was used to quantify the library on the Promega QuantiFluor fluorescence quantification system. 9) After serially diluting each qualified sequencing library, it was mixed according to the required sequencing volume in the corresponding proportions, and then denatured to single-stranded by NaOH prior to sequencing; 10) The MiSeq sequencer was used to perform 2×300bp pair-end sequencing, the corresponding reagent was MiSeq Reagent Kit V3 (600 cycles). Figures and index were drawn and analyzed with the use of the R software. Please contact the corresponding author for data requests.

SCFA Analyses

SCFAs were analyzed by GC-MS. Briefly, 300 mg gut samples were homogenized in 1.2 mL dH2O in a ball mill for 4 min at 45 Hz and then ultrasound treated for 5 min. After centrifugation for 20 min at 5000 rpm, 4°C, 0.15 mL 50% H2SO4 was added to the supernatant (0.6 ml) with 0.8 mL of 2-methylvaleric acid (50 μg/mL stock in ethyl ether) as internal standard. After centrifugation (10 min at 12000 rpm, 4°C), the supernatant was used for analysis.

Histology and Immunofluorescence Staining

Rats were transcardially perfused with 4% paraformaldehyde on postoperative day 14. The brains were removed, post-fixed for 24 h in 4% paraformaldehyde and cryoprotected in 30% sucrose. After embedding in OCT medium, brain tissue through the infarct was cut into 8μm-thick sections (Thermo Electron, Waltham, MA, USA) for immunofluorescence staining and 25μm-thick for cresyl violet.

The ileum slice were collected, then we used Hematoxylin and Eosin Staining Kit (Beyotime, China) for hematoxylin–eosin (HE) staining. Coronal sections (25μm thick) were collected at 1mm intervals for staining with 1% cresyl violet acetate (Sigma) at 37 °C for 10 min. The infarct area of each slice was measured with Fiji NIH. The respective volumes were obtained by multiplying the area of infarction by the distance between sections. The infarct volume ratio was measured using the following formula: (the volume of the contralateral hemisphere – the volume of the infarcted hemisphere)/the volume of the contralateral hemisphere*100%.

Brain sections were stained with primary antibody rabbit anti-neuronal nuclei antigen (NeuN)(1:200, Millipore, USA), rabbit anti-glial fibrillary acidic protein (GFAP) (1:200, Abcam), or goat anti-ionized calcium binding adaptor molecule1 (Iba1) (1:500, Abcam) followed by incubation with secondary antibodies Alexa Fluor 488 (1:500, Invitrogen, USA) for GFAP, Alexa Fluor 594 IgG goat anti-rabbit (1:200, Abcam) for Iba1 and NeuN. Iba1+ and GFAP+ cells were acquired with a 40× objective (every sixth section between bregma levels +0.96 mm and -0.24 mm, five sections per rat). The intensity of Iba1 and GFAP staining was estimated as the mean integrated optical density (IOD). To assess microglia morphology by Sholl analysis, we used FIJI software (Fiji, NIH) and IMARIS software (IMARIS BITPLANE v.9.0). The results reflected the number of dendritic intersections at various distances from the cell body.

To investigate cell apoptosis, we performed a TdT-mediated dUTP-biotin nickend labeling (TUNEL) assay according to the manufacturer's instructions (In situ cell death detection kit; Roche). Briefly, slides were washed with PBS three times, fixed with 4% paraformaldehyde for 30 min, incubated in 0.1% Triton X-100 for 2 min, and then in a TUNEL reaction mixture for 60 min at 37 °C (17). Finally, sections were observed with inverted fluorescence microscope (Olympus FV-1000, Japan). The

immunofluorescence images of Tunel+/NeuN+ were acquired with a 20X objective. Results are presented as the number of Tunel+/NeuN+ cells per section. Positive cells were counted in each section by an experimenter blinded to the treatment conditions.

ELISA

Plasma was collected for ELISA in EDTA-coated tubes. Concentrations of TGF-β1 and IL-10 were measured by using ELISA kits (MEIMIAN, China) after appropriate dilution according to the manufacturer's instructions. The minimum detectable dose of IL-10 is typically less than 0.1 pg/mL and the minimum detectable dose of TGF-β1 is typically less than 0.1 ng/mL. The final detection concentration was higher than the minimum detection concentration.

Intestinal permeability

Rats were gavaged with phosphate buffered saline containing 600 mg/kg body weight FITC-dextran (Sigma, USA). After 6 hours, blood samples were collected in heparin anticoagulation tubes and centrifuged at 3750 r/min for 15 min in a centrifuge to preserve the serum from light for measurement. Fluorescence intensity was measured using a fluorospectrophotometer (excitation wavelength 480 nm and emission wavelength 520 nm; Perkin-Elmer, Waltham, MA).

Real-time qPCR

In each experimental group, mRNA was isolated from cortex striatum and ileum with the miRNeasy Mini Kit (Qiagen, Germany) and stored at -80℃ (three rats for each group). According to the manufacturer's instructions, the concentration was quantified with a Nanodrop spectrophotometer (ND-1000, Nanodrop, USA). Reverse transcription was performed to synthesize complementary DNA (cDNA) by utilizing 1μg mRNA extracted as templates with the GoScript Reverse Transcription Kit (Promega) according to the protocols supplied. Primer sequences for markers: IL-10, IL-6, NF-KB1, TNF-a, TGF-β, iNOS, Arg-1, CD86, TLR2, TLR4 and GAPDH were designed using Primer 6.0 software. Sequences can be found in Data Supplement. Expression levels of mRNA were quantified utilizing RT2 SYBR Green qPCR Mastermix (Promega, USA) and detected with the 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). The rt-qPCR mix contained 0.4μl of each primer, 5μl of 2X qPCR Master Mix and 1μl of cDNA. Nuclease-free water was added to achieve a final reaction volume of 10μl. The rt-qPCR reaction condition was set to 95℃ for 2 min, followed by 40 cycles of 95℃ prolongation for 15 s and 60℃ for 1 min. A melting curve was then calculated for each PCR product to confirm the synthesis specificity. The sequences for RT-qPCR were as follows(5' to 3'):

IL-10 F: TTGAACCACCCGGCATCTAC, R: CCAAGGAGTTGCTCCCGTTA
IL-6 F: GAGACTTCCAGCCAGTTGCC, R: TGAAGTCTCCTCTCCGGACTT
TGF-beta F: TGAGACTTTTCCGCTGCTACT, R: TGTCTGGAGTCCTCAGGTCC
TNF-alpha F: ATTGTGGCTCTGGGTCCAAC, R: AGCGTCTCGTGTGTTTCTGA
TLR4:F:GAGGACAATGCTCTGGGGAG, R: ATGGGTTTTAGGCGCAGAGT
TLR2:F: CATCTGCTCCTGTGAACTCCT,R: CTGGTGACACTCCAAGACTGA
NF-kB:F: GCTATAACTCGCCTGGTGACA, R: CCGCAATGGAGGAGAAGTCT
GAPDH:F: ACAGCAACAGGGTGGTGGAC, R: TTTGAGGGTGCAGCGAACTT
iNOS F: GTCCTATCTCCATTCTACTACT, R: CTTCCGCATTAGCACAGA
CD86 F: GGCTCTACGACTTCACAAT, R: ATAGGCTGATGGAGACACT
Arg-1 F:CAATGACTGAAGTGGACAAG, R:GTCTCTGGCTTATGATTACCT

Western Blotting

Samples were homogenized in RIPA lysis buffer supplemented with 1mM proteinase and the 1mM phosphatase inhibitor cocktail (key GEN BioTECH, China), and further centrifuged at 15,000 rpm at 4°C for 20 min. The protein concentration was measured with the BCA protein assay kit (Beyotime, China). Twenty μg protein were boiled at 95°C before loading onto 8%–12% SDS-PAGE gels. After being electrophoresed and being transferred to polyvinylidene difluoride (PVDF) membranes, the membranes were blocked with QuickBlock Blocking Buffer for Western Blot (Beyotime, China) and incubated with primary antibodies overnight at 4℃ as follows: Bax (1:1000, Abcam), Bcl2 (1:1000, Abcam). The membranes were incubated with secondary antibody goat anti-rabbit IgG H&L (HRP) (1:5000, Abcam) at room temperature for 2 h. The chemiluminescence signal was detected by the MF-ChemiBIS imaging system (DNR, Israel). Digital images were quantified using software measuring densitometry (Fiji, NIH). The quantity of GAPDH was used to normalize each lane. The relative optic density (ROD) was obtained by comparison with the SHAM group.

Behavioral Outcome Measures

The tapered ledged beam-walking test was used to evaluate the sensorimotor function of the impaired limbs (contralateral to the lesion). The performance of the rats was recorded by a video camera and later analyzed by calculating the slip ratio of the impaired forelimb or hindlimb to total steps.

The adhesive tape removal test was used to assess the sensorimotor function of rats. Two round sticky labels (diameter 10 mm) were placed on the paws of both forelimbs. The time that the rats needed to touch and remove the sticky label was recorded.

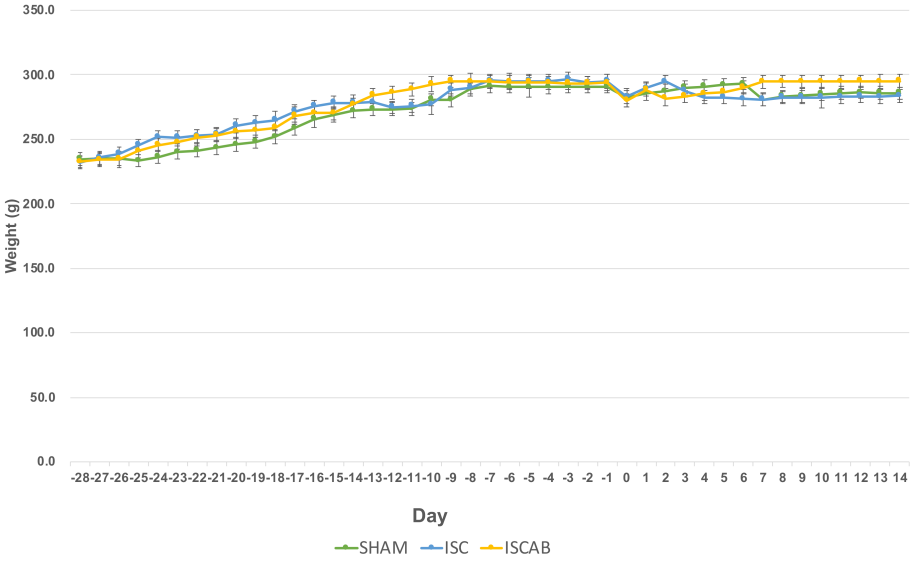
The cylinder test was used to evaluate spontaneous use of the forelimbs. A clear Plexiglas cylinder (20 cm in diameter, 45 cm high) was used to allow the researchers to videotape the rats. A person blind to the experimental groups counted the number of contacts that each rat made with the cylinder walls by its forelimbs. The percentage of impaired (contralateral to the lesion) forelimb use was calculated according to the following formula: impaired forelimb contact+0.5*both forelimb contacts/(impaired+ unimpaired + both forelimb contacts) × 100%.

Statistics

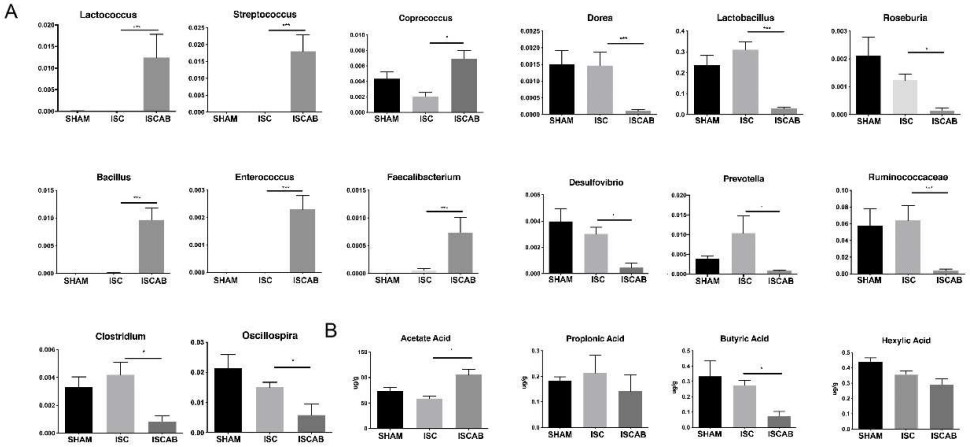
Experimental data analyses were performed using GraphPad Prism and SPSS22.0 software. The data are presented as mean ± SEM and significance levels are as follows: *P < 0.05, **P < 0.01, ***P < 0.001. Analyses were performed using either a Student's t-test (two-tailed) when comparing two groups or one-way analysis of variance (ANOVA) for more than two groups followed by the least significant difference (LSD) post hoc test. The calculations conducted by PASS 11 software for the number of animals needed for statistical analysis of PCR data:

TLR4, effect size=1.660, α=0.1, sample size=3,
TLR2, effect size=1.391, α=0.1, sample size =3,
INOS, effect size=1.310, α=0.1, sample size=3,
NF-kb, effect size=1.414, α=0.1, sample size =3,
IL-6, effect size=1.620, α=0.1, sample size=3,
IL-10, effect size=1.350, α=0.1, sample size=3,
TGF-b, effect size=1.464, α=0.1, sample size=3,

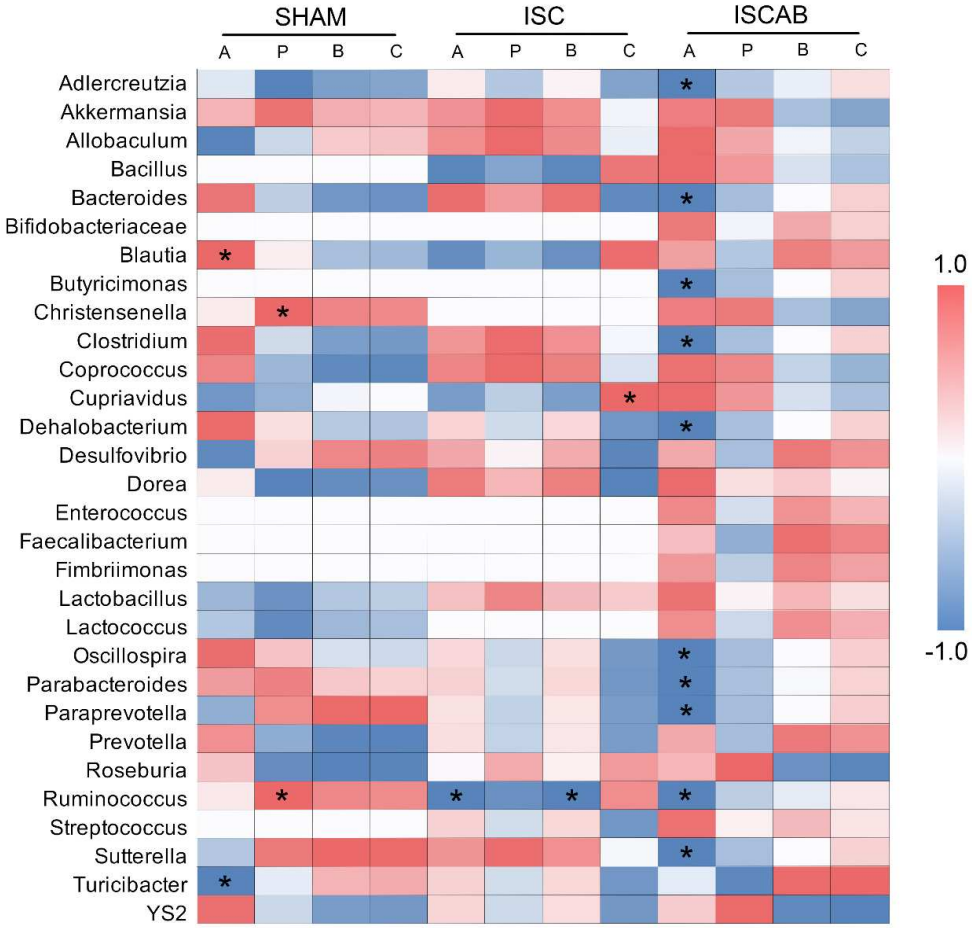
2 Supplemental Figures



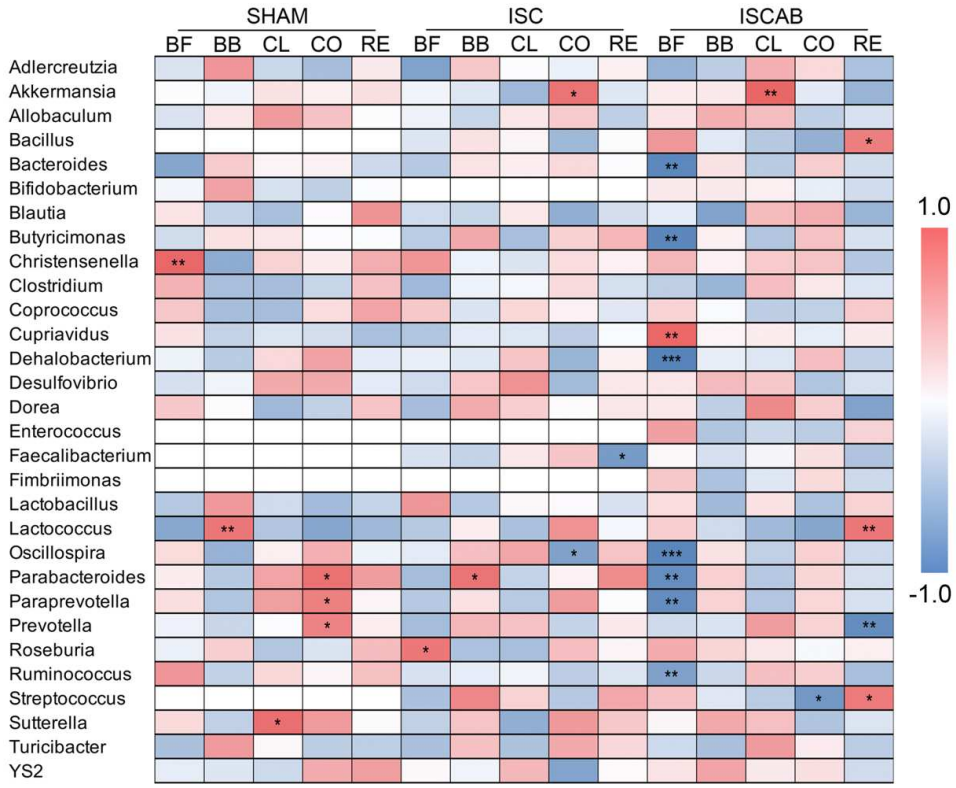
Supplementary Figure 1. The growth and weight of rats were monitored every day during this experiment.



Supplementary Figure 2. Gut microbiota and SCFAs. **A**, The relative abundance of the main bacterial species in ISCAB group comparison to the ISC and SHAM group (n=8 per group). **B**, The levels of SCFAs in ISCAB group comparison to the ISC and SHAM group. Statistical significance: * P<0.05, ** P<0.01, *** P<0.001, n=3 per group. Data are mean ± SEM.



Supplementary Figure 3. Pearson correlations between microbiota with SCFAs concentrations at the genus level (A, acetate; P, propionate; B, butyrate; C, caproate). Statistical significance: * P<0.05.



Supplementary Figure 4. Pearson correlations between microbiota with behavioral test at the genus level (BF, forelimb score in beam test; BB, hindlimb score in beam test; CL, cylinder test; CO, contact time in the sticky label test; RE, removal time in the sticky label test). Statistical significance: * P<0.05, ** P<0.01, *** P<0.001.