Long-term modification of gut microbiota by broad-spectrum antibiotics improves stroke outcome in rats

Chang Liu,1,2 Xi Cheng,1 Shanshan Zhong,1 Zhouyang Liu,1 Fangxi Liu,1 Xinyu Lin,1 Yinan Zhao,1 Meiting Guan,1 Ting Xiao,3,4 Jukka Jolkkonen,5 Ying Wang,6 Chuansheng Zhao1,2

ABSTRACT

Background The brain-gut axis is a major regulator of the central nervous system. We investigated the effects of treatment with broad-spectrum antibiotics on gut and brain inflammation, infarct size and long-term behavioral outcome after cerebral ischemia in rats.

Methods Rats were treated with broad-spectrum antibiotics (ampicillin, vancomycin, ciprofloxacin, meropenem and metronidazole) for 4 weeks before the endothelin-1 induced ischemia. Treatment continued for 2 weeks until the end of behavioral testing, which included tapered ledged beam-walking, adhesive label test and cylinder test. Gut microbiome, short-chain fatty acids and cytokine levels were measured together with an assessment of infarct size, neuroinflammation and neurogenesis.

Results The results revealed that the antibiotics exerted a clear impact on the gut microbiota. This was associated with a decrease in systemic and brain cytokine levels, infarct size and apoptosis in the perilesional cortex and improved behavioral outcome.

Conclusion Our results highlighted the significant relationship between intestinal microbiota and beneficial neuro-recovery after ischemic stroke.

Key messages

What is already known on this topic ⇒ The modulation of the gut microbiota and brain resident immune cells could act as a major factor in the process of pathophysiology of stroke. How to modify the structure of the microbiota in intestine to ameliorate the outcome of ischaemic stroke has received extensive attention.

What this study adds ⇒ In our study, a combinatorial antibiotic is applied to modify the structure of the flora after stroke, thereby regulating the inflammatory reaction through the peripheral circulation and central nervous system and ultimately ameliorate the outcome of stroke.

How this study might affect research, practice or policy ⇒ This article may provide new avenues for further exploring mechanisms of brain-gut axis as well as promising therapeutic strategies for antibiotic application and to improve stroke recovery.
this effect possibly being mediated through the modification of the gut flora. However, extended administration of antibiotics may also lead to sepsis and severe bloody diarrhoea caused by reduced gut motility and increased permeability and eventually impaired neurological function. Nonetheless, exactly how modulation of the gut microbiota affects the evolution, development and recovery after a stroke is not completely understood.

Therefore, we aimed to explore whether an intestinal microbial shift caused by broad-spectrum antibiotics could alter brain pathology and stroke recovery. We hypothesised that the changes in the bacterial populations induced by antibiotics would trigger a peripheral immune reaction, which in turn would result in neuronal protection and functional restoration after a stroke. In these experiments, rats were divided into sham-operated (SHAM) group, ischaemic (ISC) group and ischaemic rats treated with a combination of antibiotics (ISCAB) group. We found that the combined broad-spectrum antibiotics selectively affected the abundance of the intestinal flora after stroke, which could lead to an increase in the intestinal content of short-chain fatty acids. In addition, antibiotics reduced the inflammatory response in the intestine and modulated the function of microglia resulting in decreased neuronal apoptosis and a reduced infarct volume consequently leading to an improved behavioural recovery.

RESULTS

The administration of antibiotics causes a shift in the gut microbiota after a stroke

We performed 16S rRNA sequencing of the intestinal contents collected from the experimental rats after sacrifice (n=24). The relative abundance of gut microbiota at the phylum level showed that Firmicutes had the highest relative abundance in the ISC rats (67.1%±3.3%) and antibiotics expanded the members of Proteobacteria, which occupied the dominant position of the gut microbiota in the ISCAB group (68.1%±4.8%) (figure 2A). The practice of clustering operational taxonomic units (OTUs) at 97% 16S sequence identity is intended to group together functionally diverse lineages. The results suggested that the abundance of microbiota colonies in the gut had been reduced by antibiotics (figure 2B). Subsequently, the Chao1 Index and Shannon Index were analysed based on the number of OTUs. These results showed that the microbial α-diversity in the ISCAB group was lower than other groups, indicating that the intervention with the antibiotics had indeed decreased the microbial α-diversity and richness (figure 2C and D). The principal coordinates analysis (PCoA) scatter-plot was performed to examine if there was any difference between the three groups based on the relative abundance of OTUs in terms of the collected ileal contents. The ISCAB group was clearly distant from the other groups indicating that the flora composition of ISCAB rats was different from the other two groups (Principal component 1 (PC1) explained 11.5% and Principal component 2 (PC2) explained 29.1% of the trend) (figure 2E).

Although the diversity in the ISCAB group had declined due to antibiotic treatment, the results obtained from hierarchical clustering and heat map analysis showed that absolute amount of probiotics such as Lactococcus, Coprococcus, Bacillus, Streptococcus, Enterococcus and Faecalibacterium had increased in the ISCAB rats (figure 2F and online supplemental figure 2A). The proportion of Desulfovibrio, which is a pathogenic bacterium, decreased in ISCAB rats and antibiotics also reduced the proportions of Ruminococcaceae,
Figure 2  Overall taxonomic analysis, microbial community diversity analysis and heatmap analysis. (A) Taxonomic composition and distribution map of flora were summarised at the phyla level. (B) OTUs in each sample that can be classified to the level of the phylum, the class, the order, the family, the genus and the species. (C) Measure of α-diversity species of samples by Chao1 Index based on the number of rare species. (D) α-diversity of samples indicated by Shannon’s diversity analysis. (E) β-diversity of principal component analysis. PCoA of gut microbiota indicated the microbial composition. (F) Hierarchically clustered heatmap analysis of the top 50 bacteria at the genus level in gut (n=8 per group). ISC, ischaemic; ISCAB, ischaemic rats treated with a combination of antibiotics; PCoA, principle coordinate analysis; SHAM, sham-operated.
Clostridium, Dorea, Lactobacillus, Roseburia, Oscillospira and Prevotella (online supplemental figure 2A). These reduced microbiota are commonly considered to produce butyrate in the ileum.7–13

Antibiotics alter the concentrations of SCFAs

Given that short-chain fatty acids (SCFAs) produced by bacterial fermentation are important metabolites in the intestine and many of them have immunomodulatory functions, we quantified the intestinal metabolites after the antibiotic intervention. Compared with ISC rats, the concentration of acetate increased (105.6±10.1 µg/g vs 58.9±5.0 µg/g, p<0.05), whereas that of butyrate decreased (0.1±0.03 µg/g vs 0.27±0.04 µg/g, p<0.05) after the antibiotics (online supplemental figure 2B).

Since acetate7 and butyrate15 are specifically produced by the microbiome, we correlated the changes in the microbiota with that of their metabolites. The results indicated that the levels of acetate were significantly correlated with Blautia and Turicibacter in SHAM rats, with Adlereceutia, Bacteroides, Butyrivimonas, Paraprevotella, Clostridium, Sutterella, Dethalobacterium, Oscillospira and Parabacteroides in ISC and with Ruminococcus both in ISC and ISCAB rats. The levels of propionate were significantly correlated with Christensenella and Ruminococcus in SHAM rats. The levels of butyrate were significantly correlated with Ruminococcus in ISC rats. The levels of caproate were significantly correlated with Cupriavidus in ISC (p<0.05) (online supplemental figure 3). Online supplemental figure 4 shows the Pearson correlation between the top 30 microbiota and behavioural tests. It is worth noting that in this analysis, Bacteroides, Butyrivimonas, Christensenella, Dethalobacterium, Oscillospira, Parabacteroides, Paraprevotella and Ruminococcus showed the negative correlations with behavioural indices of rat forelimbs. This is consistent with the previous results of acetic acid, suggesting that the changes of acetic acid produced by the gut microbiota might play an important role in the behavioural recovery. The combined data clearly revealed that a major effect of antibiotic treatment had been to alter the diversity and abundance of gut microbiota and their secretion of SCFAs.

Decreases in systemic inflammatory response by antibiotics

SCFAs have been recognised as negative regulators to the gut inflammation after cerebral ischaemia. Thus, the expression of proinflammatory (Toll-Like Receptor 4 (TLR4), Toll-Like Receptor 2 (TLR2), Nuclear factor kappa B (NF-kB), Interleukin 6 (IL-6) and tumour necrosis factor α (TNF-α)) and anti-inflammatory cytokines (IL-10 and Transforming growth factor beta (TGF-β)) mRNA was investigated in intestinal tissue. We found that in ISC rats, levels of all the above cytokines except TNF-α and TGF-β increased as compared with SHAM rats and the microbiota changed by antibiotics had opposite effects on the amounts of both anti-inflammatory and proinflammatory markers. However, although TNF-α showed the same tendency, there is no significant difference between the three groups (figure 3A). When TGF-β and IL-10 levels were measured in blood, we found that both markers had decreased after antibiotic treatment and TGF-β showed significant increase in ISC group compared with SHAM group (figure 3B).

The disruption of intestinal mucosa was specifically reflected in the decreased length of small intestinal villi, the reduced crypt foci and the decreased thickness of intestinal wall. In our experiments, we observed significant changes in the morphology of the small intestinal villi. The mean length of small intestinal villi was significantly reduced after the ischemic event (p<0.001), and when treated with a long course of antibiotics, the length of small intestinal villi was significantly restored compared with that in the ISC group (p<0.001, figure 3C), and we found that the ISCAB group showed a significant increase in intestinal permeability to FITC-dextran when compared with the ISC group (p<0.05, figure 3D), suggesting the recovery of mechanical barrier of intestinal mucosa after treatment.

Then we used Quantitative reverse transcription PCR (RT-qPCR) to examine if there were alterations of microglia-associated markers and cytokines in the perilesional cortex. There is a trend for the expression of Arginase 1 (Arg1), CD86 and Inducible nitric oxide synthase (iNOS) to increase in ISC rats and a further increase in the ISCAB group; however, only the increase of iNOS in the ISCAB group reached statistical significance (p<0.05, figure 3D). In ISC group, the mRNA levels of TLR4, TLR2, NF-κB, IL-6 and TGF-β showed significant increases as compared with the SHAM group. These increases tended to be reversed in the ISCAB group, although the change did not reach statistical significance. IL-10 and TNF-α also showed the tendency to increase in ISC but the change did not reach statistical significance. Nonetheless, in the ISCAB group, IL-10 mRNA level was significantly lower than that in the ISC group (p<0.05, figure 3E).

Improved behavioural outcome following stroke by antibiotics

Next, we aimed to evaluate how the antibiotic treatment affected neurological deficit in chronic post-stroke phase. Sensorimotor functions were measured on postoperative day 14 to assess behavioural outcomes in ischaemic rats. In the beam walking test, both forelimb and hindlimb slips were increased by ischaemia (8.7±2.0% vs 26.5±3.7%, p<0.001; 21.8±4.2% vs 49.6±2.8%, p<0.001, figure 4A) and the impaired hindlimb function was reversed in ISCAB group when compared with ISC group (17.0±2.3% vs 26.3±3.7%, p<0.05; 26.9±5.1% vs 49.6±2.8%, p<0.01, figure 4A). In the sticky label test, both the time taken to first touch the adhesive tape (14.3±1.6 s vs 19.2±1.5 s, p<0.05) and its removal time (44.4±5.1 s vs 75.1±9.6 s, p<0.05, figure 4A) were reduced in the ISCAB group as compared with the ISC group. Ischaemia impaired spontaneous forelimb use in cylinder test, and this was reversed by antibiotics (40.7±2.9% vs 20.1±4.7%, p<0.01, figure 4A). All the behavioural results together revealed that the sensorimotor functions of rats in the ISCAB group had undergone a significantly improved recovery after a stroke.
Antibiotics cause a decrease in infarct volumes and perilesional apoptosis

The endothelin-1 induced experimental stroke caused extensive damage to the cortex and striatum. To examine whether antibiotics decreased the extent of the ischaemic injury, infarct volumes were analysed from cresyl violet-stained sections. ISCAB rats had significantly smaller infarct volumes as compared with ISC rats (6.2±0.8% vs 11.8±1.4%, p<0.001, figure 4B).

Bax, as a marker of apoptosis, was significantly decreased in the ISCAB rats compared with their ISC counterparts (figure 4C). However, there was no difference in the expression of Bcl2 in the different experimental groups (figure 4C). NeuN can be used as a neuronal marker, which we used to identify the type of cells undergoing apoptosis. We found that there was a significant overall group effect in the number of the Tunel+/NeuN+ cells in the perilesional cortex (p<0.001). The number of Tunel+/NeuN+ cells in the ISC group was increased compared with that in the SHAM group (p<0.001, figure 4D). After 4-week administration of antibiotics, the number of Tunel+/NeuN+ cells was decreased compared with that in the ISC group (p<0.01, figure 4D). These results confirm that antibiotic can reduce apoptosis of neuronal cells.

Changes in glial and microglial cells after cerebral ischaemia

There was a significant overall group difference in the number of Iba-1+ cells in the perilesional cortex (figure 5A,B). In ISC animals, the expressions of Ionized calcium binding adaptor molecule 1 (Iba-1) and Glial fibrillar acidic protein (GFAP) were higher than in the SHAM rats (p<0.05); however, this effect was not reversed by antibiotics. Sholl analysis showed a reduction in the total branch number and length of microglia in the perilesional cortex after ischaemia (figure 5B). However, the ISCAB rats did not differ from their ISC counterparts (figure 5C).

DISCUSSION

Changes in the intestinal flora are reflected in SCFA metabolism

Previously, it has been demonstrated that rats develop an abnormal microbial composition after stroke and this in turn affects the prognosis of stroke. Oral administration of antibiotics have been reported to decrease the volume of...
cerebral infarct and reduce cerebral oedema by changing the intestinal flora.\(^4\)\(^\text{16}\) The absence of microbiota within 1–4 days after stroke did not affect infarct size and increased mortality within 5–7 days in mice, and this could be prevented by continuous antibiotic therapy or transplantation of gut microbiota obtained from Specific-pathogen-free (SPF) animals.\(^5\) In our study, the proportion of beneficial bacteria such as *Lactococcus*, *Coprococcus*, *Bacillus*, *Streptococcus* and *Faealibacterium* was significantly upregulated after treatment with antibiotics. After depletion of a large number of antibiotic-sensitive flora by the antibiotic cocktail, the other flora are no longer subjected to their competitive inhibitory effect, which can represent a growth advantage.\(^1\)\(^7\) Part of the intestinal flora was resistant to the antibiotic treatment. For example, *Enterococcus* is known to be resistant to ampicillin to a certain extent.\(^1\)\(^8\) Antibiotic resistance of *Bacillus* is another example.\(^1\)\(^9\) There is previous evidence that a probiotic intervention, including *Lactococcus* and *Coprococcus*, significantly ameliorated depression-like symptoms and improved cognitive functions of rats.\(^2\)\(^0\) The increased relative abundance of *Streptococcus* and *Faealibacterium* is known to attenuate the symptoms of colitis and suppress the immune response.\(^2\)\(^1\)

Short-chain fatty acids are important metabolites of intestinal flora. Acetate and butyrate are the most abundant SCFAs in the intestinal tract (≥95%).\(^2\)\(^2\) Acetate can serve as a carbon source for gut microbiota, and help to maintain the integrity of the epithelial barrier by regulating the properties of tight junction proteins. Acetate can be produced by most of the enteric bacteria such as *Lactobacillus* spp., *Prevotella* spp., *Ruminococcus* spp., *Streptococcus* spp and *Butaia* spp.\(^1\)\(^4\)\(^\text{23}\) whereas butyrate is produced by *Clostridium leptum*, *Faealibacterium*, *Oscillospira* and *Coprococcus*.\(^1\)\(^5\)\(^\text{24}\) Overall, our results

![Figure 4](http://svn.bmj.com/Stroke Vasc Neurol: first published as 10.1136/svn-2021-001231 on 16 May 2022. Downloaded from http://svn.bmj.com/)
revealed an increase in the proportions of the above microflora in conjunction with the changes of intestinal SCFAs.

**SCFAs participate in the inflammatory regulation that affects the CNS via the circulation**

Brain ischaemia leads to the breakdown of mucosal barriers and bacterial translocation. Microbial components, such as bacterial cell components or toxins, can enter the host’s blood circulation or lymphatic organs, further increasing the intestinal permeability and reducing the immune response in peripheral circulation, ultimately leading to bacterial translocation and infection. In experimental stroke, T and B cell numbers are significantly reduced in Peyer’s patches. Immune cells also can infiltrate across the leaky BBB into the parenchyma at the site of the ischaemic lesion.

SCFAs can repair the intestinal barrier. For example, acetate can increase cell membrane assembly, mucosal cell migration and the proliferation and differentiation of colonocytes as well as modulating the various biological responses of the host, for example, reducing inflammation and oxidative stress. In vitro, low concentrations (1–10 mM) of sodium butyrate significantly could improve the transepithelial resistance and permeability of the epithelium of colonic cells. Similarly, increases in the circulating levels of SCFAs may induce therapeutic effects during the chronic post-stroke recovery period. SCFAs are especially important for the human body in its defence against intestinal diseases such as Crohn’s disease, ulcerative colitis and colorectal cancer. In general, two different mechanisms are involved in these processes; the first is associated with a direct activation of certain G-protein-coupled receptors and the second is attributable to the direct inhibition of nuclear class I histone deacetylases (HDACs). The inhibition of HDACs is mainly associated with the anti-inflammatory immune phenotype, including declines in the concentrations of inflammation-associated cytokines (IL-6, IL-8 and TNF-α), reducing NF-kB activity and avoiding pathogen expansion. TNF-α expression was found to be downregulated after a diet containing 5% Plantago ovata seeds compared with control animals, while TNF-α and NF-kB expression in the intestine was downregulated after feeding with a diet containing 8% oligofructose-enriched inulin. In both studies, it was confirmed by two studies that the reduction of intestinal inflammatory factors was associated with higher concentrations of butyrate. This can be attributed to the anti-inflammatory effects of butyrate mediated by the inhibition of NF-kB signalling pathway and HDAC. As butyrate has been reported to bind and activate the nuclear transcription factor Peroxisome proliferator-activated receptor gamma (PPARγ), thereby antagonising NF-kB signalling. Our results revealed that the amounts of NF-kB, TLR2, TLR4, IL-6, TNF-a, IL-10 and TGF-β decreased in gut, which means that the extent of intestinal inflammation had been indeed significantly reduced by antibiotics. However, IL-10, as a recognised anti-inflammatory factor, is reduced significantly. This may result from the fact that SCFAs can inhibit Lipopolysaccharide-induced IL-10 production as previous.

![Figure 5](http://svn.bmj.com/) Gut inflammation response in the perilesional cortex. (A) Fluorescence images and quantification of Iba-1+cells and GFAP+cells in the perilesional cortex, n=6 per group. (B) Western blotting and quantification of Iba-1 and GFAP in the perilesional cortex, n=3 per group. (C) Quantification of the number of microglia branching, n=3 per group. Data are mean±SEM. Scale bar=50μm. Statistical significance: *p<0.05; ***p<0.001. ISC, ischaemic; ISCAB, ischaemic rats treated with a combination of antibiotics; SHAM, sham-operated; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride.
shown.\textsuperscript{34} Taken together, it seems that SCFAs exert a variety of beneficial functions in ischaemic stroke.

**Antibiotics promote a recovery of neurological function**

Our results show that the gut microbiota has an important impact on the behavioural recovery taking place after an experimental stroke. In addition, administration of these antibiotics affected the gut microbiome leading to neuroprotection and an improved functional recovery in ISC animals. Benakis \textit{et al} showed that infarct volumes were reduced by 60\%\textpm{}6\% in antibiotic-treated mice with an altered intestinal flora in comparison to antibiotic-resistant mice and this was associated with better sensorimotor functions.\textsuperscript{35} There is also evidence that apoptotic cell death may be associated with impaired survival of newly formed neurons.\textsuperscript{36} In line with these findings, our study showed that the animals drinking the antibiotic cocktail showed less evidence of apoptotic cell death in the perilesional brain. Although several mechanisms are likely involved in the bidirectional communications of the microbiota–gut–brain axis following a stroke, one important consideration is the initiation of the brain inflammatory response by the gut microbiota in conjunction with the cooperative role of microglia.\textsuperscript{35}

**Microglia activation by stroke and antibiotics**

Proinflammatory mediators released from microglia have been associated with the neuroinflammatory injury evident after a stroke. On the other hand, microglia contribute to neurogenesis and guidance of sprouting vessels through the production of neurotrophic factors, phagocytosing synapses and reshaping neuronal circuitries. Interestingly, microglia, unlike peripheral macrophages, may be more susceptible to changes in the microbiome.\textsuperscript{37} Erny \textit{et al} found that in comparison to SPF mice, germ-free (GF) microglia expressed reduced mRNA levels for several activation markers (eg, Il-1\textalpha, Stat1, Jak3 and B2m). In addition, the expressions of other genes controlling cell proliferation, cell cycle and apoptosis (eg, Cdk9, Ccnd3 and Bcl2) were substantially increased in GF mice. Accordingly, an immature morphology and also an increased microglial density were observed in different brain regions of GF mice. GF mice exhibited an upregulation of the surface markers CSF-1R, F4/80 and CD31, proteins which are known to be downregulated in mature adult microglia. Furthermore, GF microglia displayed a reduced capacity to respond to a viral infection challenge. A reduced complexity of microbiota also leads to microglial defects after antibiotic use. In contrast, the re-colonisation of the complex flora and SCFAs treatment restored the microglial characteristics.\textsuperscript{37}

Our immunofluorescence and RT-qPCR results in the cerebral cortex detected signs of upregulation of microglia-related genes after stroke. We noted that the usage of antibiotics did not significantly affect the number of Iba-1+cells but increased the number of proinflammation microglia, which means the ratio of the microglial phenotypes indeed changed. Sholl analysis showed that the alterations in microglial morphology induced by oral antibiotic therapy are indicative of the dynamic polarisation states of these cells. Activated microglia can retract their processes and enlarge their cell bodies in a manner like activated phagocytic cells, increasing the relative areas of Iba-1 staining after a stroke, while ameboid microglial demonstrate a protective state, attenuating the damage associated with the neuroinflammation associated with a stroke. The anti-inflammatory gene expression profile of microglia was associated with reduced mRNA expressions of TNF-\textalpha and IL-1\beta but increased expressions of TGF-\beta1 and IL-10.\textsuperscript{38} In our study, the levels of an activated microglia-related marker increased in parallel to the findings of reduced apoptosis. Although there was an increase in proinflammatory microglia, as indicated by the upregulation of the proinflammatory enzyme iNOS, there was also evidence of downregulation of both inflammatory and anti-inflammatory cytokines in the cortex at the same time, which hinted that the inhibition of the immune response may due to dysfunction and immaturity of microglial cells caused by the broad-spectrum antibiotic.\textsuperscript{37} This change in the properties of the microglia could lead to a reduction in the inflammatory response in the brain after stroke and promote stroke recovery.

**Limitations**

However, our study has some limitations. First, antibiotics may cross the leaky BBB. Metronidazole and meropenem are more likely to enter the CNS and affect the function of microglia and play a role in cerebral haemorrhage, brain trauma and other neurological diseases. Ampicillin has been reported to dose-dependently protect neurons against ischaemic brain injury, to attenuate the activity of matrix metalloproteinases and to reduce immunoreactivities of astrocytes and microglia by increasing the level of glutamate transporter-1.\textsuperscript{39} Ciprofloxacin also was claimed to inhibit the activation of the TLR4/NF-\kappaB signalling pathway.\textsuperscript{40} Thus, GF mice may have been a more ideal model in which to conduct these experiments. Second, the translational value of the present data is limited, since experimental animals live in a very controlled environment and are fed with a standard diet, whereas the human microbiome is influenced by a huge range of factors.

**Author affiliations**

1Department of Neurology, The First Hospital of China Medical University, Shenyang, Liaoning, China
2The Stroke Center, The First Hospital of China Medical University, Shenyang, Liaoning, China
3Department of Dermatology, The First Hospital of China Medical University, Shenyang, Liaoning, China
4Key Laboratory of Immunodermatology, Ministry of Health, Ministry of Education, Shenyang, Liaoning, China
5A.I. Virtanen Institute for Molecular Sciences, University of Eastern Finland, Kuopio, Finland
6Department of Neurology, First Affiliated Hospital of Dalian Medical University, Dalian, China

**Contributors** XL performed immunofluorescence staining, FL performed proof assistance, ZL and YZ performed behavioural tests, CL and MG performed data analysis, XC, SZ and TX provided expert technical assistance, CL and CZ conceived and designed experiments, CL and JJ wrote and edited the manuscript, CZ corrected and approved the final version of the manuscript, and is responsible for the overall content.
REFERENCES


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 amplified 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-GGTTACCTTGTTACGACTT-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°C (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°C for 2 min, followed by 40 cycles of 95°C prolongation for 15 s and 60°C 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: TTGAACCAACCCGGCAATCTAC, R: CCAAGGAGTTGCTCCGGTATA
- IL-6 F: GAGACTTCACCCAGCAGTTGCC, R: TGAAGTCTCCTCTCCGGACTT
- TGF-beta F: TGAGACTTTTCCGCTGCTACT, R: TGTCTGGAGTCTCAGGTCC
- TNF-alpha F: ATTGTGGCTCTGGGTCCAAC, R: AGCGTCTCGTGTGTTTCTGA
- TLR4:F: GAGGACAATGCTCTGGGGAG, R: ATGGGTTTTTAGGCGCAGAGT
- TLR2:F: CATCTGCTCCTGTGAACCTC, R: CTGGTGACACTCCAAGCTGA
- NF-kB:F: GCTATAACTCGCCTGGTGACA, R: CCGCAATGGAGGAGAAGTCT
- GAPDHE:F: ACAGCAACAGGGTGTTGGGAC, R: TTTGAGGGTGCAGCGAACTT
- iNOS F: GTCTCTATCTCTCTCCTACTA, R: CTTCGCCATATTGACACAGA
- CD86 F: GGCCTCTAGCCTCATCAAT, R: ATAGGCTGATGGGAGACACT
- 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.