Gut microbiota regulates hepatic ischemia-reperfusion injury-induced cognitive dysfunction via the HDAC2-ACSS2 axis in mice

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Abstract

Hepatic inflow occlusion is a common procedure in liver surgery aimed at reducing intraoperative bleeding and improving surgical visualization. However, as a complication, hepatic ischemia-reperfusion injury (HIRI) resulting from this procedure is inevitable. Research has confirmed that cognitive dysfunction induced by HIRI is closely related to dysbiosis of the gut microbiota.

To investigate the mechanisms underlying this complication, gut microbiota transplantation, HDAC2-ACSS2 axis detection, and LC/MS short-chain fatty acid detection were employed. Results showed a significant decrease in ACSS2 expression in the hippocampus of mice with hepatic ischemia-reperfusion injury, highlighting impaired acetate metabolism in this region. Moreover, both the phenotype of cognitive impairment and the dysregulation of the HDAC2-ACSS2 axis could be transferred to germ-free mice through fecal microbial transplantation. Enzyme-linked immunosorbent assay also revealed reduced Acetyl-coenzyme A (acetyl-CoA) levels in the hippocampus. These findings suggest that acetate metabolism is impaired in the hippocampus of HIRI-induced cognitive impairment mice and related to dysbiosis, leading to compromised histone acetylation.

Keywords： hepatic ischemia, reperfusion injury, cognitive dysfunction, gut microbiota**,** HDAC2-ACSS2 axis

Introduction

Hepatic inflow occlusion is a common procedure in liver surgery[1-3]. Studies have shown that occluding the hepatic portal can significantly reduce intraoperative bleeding and improve surgical visualization[4-6]. However, hepatic ischemia-reperfusion injury, an inevitable consequence of such therapy, may have adverse effects on patient prognosis, contributing to detrimental effects such as early liver failure, tissue damage, and even liver transplant failure[7-10].

Previous studies have confirmed that dysbiosis of the gut microbiota is involved in the development of various diseases, such as Crohn's disease, diabetes, and hypertension[11-13]. In recent years, the gut microbiota and gut-brain axis have been hot topics in various disease pathophysiology studies[14-17]. There is evidence suggesting that dysbiosis of the gut microbiota can cause cognitive impairment through several pathways, such as inducing and exacerbating inflammation in the central nervous system, changing microbial signals, and altering intestinal barrier permeability[16, 18, 19].

As is well known, histones are one of the fundamental components of DNA packaging[20]. In recent years, post-translational modifications of histones, an important part of epigenetics, have been shown to play a crucial role in life regulation[21, 22]. Histone post-translational modifications include methylation, phosphorylation, ubiquitination, etc[23, 24]. Among them, histone acetylation/ deacetylation has been shown to be closely related to cognitive function[25, 26]. Histone deacetylase 2 (HDAC2) and Acetyl-CoA synthetase 2 (ACSS2) are key enzymes that regulate this balance. ACSS2 uses acetate as a substrate to synthesize Acetyl-CoA, directly promoting histone acetylation, which has an impact on learning and memory[27, 28] . Previous studies have reported that mice with reduced ACSS2 expression exhibit impaired cognitive function, possibly related to the mechanism mentioned below[29].

Previous research, including our team's previous work, has confirmed that hepatic ischemia-reperfusion injury can induce cognitive dysfunction in mice[30-32]. The occurrence of this cognitive impairment is closely related to gut microbiota and hippocampal lipid metabolism. To further explore the mechanisms underlying hepatic ischemia-reperfusion-induced cognitive dysfunction, our research group employed techniques including gut microbiota transplantation and LC/MS short-chain fatty acid detection to investigate changes in the hippocampus of mice subjected to hepatic ischemia-reperfusion injury. This provides a preliminary theoretical basis for clinical practices aimed at treating cognitive dysfunction induced by hepatic ischemia-reperfusion injury.

Materials and Methods

Animals

Male-specific pathogen-free (SPF) C57BL/6 mice, aged 6-8 weeks, were obtained from the Animal Centre of Tongji Hospital and housed in a temperature-controlled colony room under standard 12-hour light/dark conditions (8:00, light on; 20:00, light off), with ad libitum access to food and water. Hepatic ischemia-reperfusion injury surgeries were performed at Zeitgeber Time 12 (ZT12, 20:00). All experimental procedures were approved by the Institutional Animal Care and Use Committee at Tongji Hospital, Huazhong University of Science and Technology.

The mouse model of hepatic ischemia-reperfusion injury

All procedures were performed during nighttime. As mentioned in previous studies[31, 33], briefly, after anesthesia with 1% pentobarbital sodium (50 mg/kg, intraperitoneal), mice were placed on an operating table, and a median incision made to expose the liver area after disinfecting the abdominal skin. The hepatic ischemia-reperfusion injury group (HIRI group) was subjected to previously described procedures. Briefly, ischemia was induced by occluding the left hepatic artery and portal vein for 90 minutes using an artery clamp. The sham group (Sham group) underwent dissection of the hepatic artery and hepatic vein without occlusion. During the operation, the mice were kept warm, and the surgical incision was treated with lidocaine after the procedure. Behavioral tests were conducted 72 hours after reperfusion, and feces, liver tissue, serum, and hippocampal tissue were collected for subsequent experiments and analysis.

Behavioral Test

Behavioral experiments for the HIRI and control groups were conducted 72 hours after reperfusion. For the pseudo-germ-free mice group, behavioral experiments were performed on the day following the completion of the microbiota transfer. The open field test was used to analyze locomotion, anxiety, and stereotypical behaviors. The Y-maze test was employed to assess spatial working and reference memory, and the novel object preference test was conducted to evaluate non-spatial visual learning memory. The test apparatus was wiped with 75% ethanol after each experiment to eliminate odor. Behavioral data were automatically recorded and analyzed through intelligent video tracking software.

Open Field Test (OFT)

As previously described[25, 34, 35], the mice were placed into the center of a black open-field chamber (L × W × H: 40 cm × 40 cm × 40 cm) after habituation. They moved freely under dim light conditions (300 lux) for 5 minutes, and the total distance traveled, and time spent in the center area were analyzed.

Novel Object Recognize Test (NORT)

The NORT test was implemented as previously described[36, 37]. Briefly, two identical objects were placed at two corners, 6 cm from each border, as previously described[25]. In the training stage, the animal was allowed to explore freely for 5 minutes after accommodation without objects, and the total exploration time around each object was recorded. After 2 hours, the mice were placed again in the same apparatus and allowed to freely explore for 5 minutes, with one of the identical objects being replaced with a novel object (test session). The exploration time around the novel (NT) and familiar objects (FT) was recorded and used to calculate the recognition index.

Y-Maze Test

As previously described[35], the Y-maze was performed in a Y-shaped compartment with three identical arms, each at an angle of 120° (L × W × H: 30 cm × 8 cm × 15 cm). The start arm (animal entry) and the common arm were always kept open, and the new arm was blocked in the first stage. Then, the mice explored the two open arms for 5 minutes. After 2 hours, all the arms were opened, and the mice had free access to the three arms for 5 minutes (test trial). We recorded the time spent within each arm, the number of mice entering the new arm, and the total distance traveled by the mice for analysis.

Pseudo germ-free mice and fecal microbial transplantation

Microbiota depletion and fecal microbial transplantation (FMT) were conducted following previously established protocols[38]. Pseudo germ-free mice were subjected to intragastric administration of vancomycin (100 mg/kg), neomycin sulfate (200 mg/kg), metronidazole (200 mg/kg), and ampicillin (200 mg/kg) once daily for 4 days to deplete their gut microbiota. For fecal microbial transplantation, feces from donor mice belonging to the Sham and HIRI groups were collected and resuspended in phosphate-buffered saline (PBS) to achieve a concentration of 0.125 g/mL. This suspension was orally administered to mice through gavage (0.15 mL) daily for 3 days. Pseudo germ-free mice receiving FMT were divided into four groups: F-Sham and F-HIRI. Behavioral experiments were performed on the following day after FMT.

16S rRNA microbiome sequencing

After the completion of behavioral tests, fecal samples were collected, rapidly frozen, and stored at -80°C following the established protocols[29]. The microbiota's 16S rRNA sequencing was conducted at the OE biotech Co., Ltd. (Shanghai, China). The fecal samples underwent DNA extraction, amplification, library construction, and sequencing using an Illumina Inc. platform (San Diego, CA) and the services provided by OE Biotech Company (Shanghai, China) to generate raw data in FASTQ format. The company provided the software and platform (https://cloud.oebiotech.cn/task/) utilized for further bioinformatic analysis of the raw data.

Western Blotting

After deep anesthesia with sodium pentobarbital, the hippocampus was collected and homogenized in lysis buffer. Following centrifugation, the protein concentration of the supernatant was determined using the bicinchoninic acid kit (Boster, Wuhan). Subsequently, 20 μg of each nuclear protein sample was separated on 10% SDS-PAGE gels and transferred onto a polyvinylidene fluoride membrane. The membrane was then blocked with 5% BSA for 1 hour at room temperature and incubated overnight at 4 °C with specific primary antibodies. After three washes, the blots were incubated with the appropriate secondary antibody for 1 hour at room temperature. Immuno-reactivity was quantified by densitometry using enhanced chemiluminescence.

The primary antibodies used were as follows: anti-GAPDH (1:1000, A19056, ABclonal); anti-ACSS2 (1:1000, A12334, ABclonal); anti-HDAC2 (1:1000, A19626, ABclonal).

Enzyme-linked immunosorbent assay (ELISA)

The levels of Acetyl-CoA in the hippocampus of mice were quantified using the corresponding ELISA kit (Bio-Swamp Co., Ltd.). The entire procedure was conducted following the manufacturer's protocol.

Determination of short-chain fatty acids

In brief, 30 mg of accurately weighed samples were subjected to crushing and homogenization, followed by treatment with methanol and L-2-chlorophenylalanine. Metabolic profiling in both electrospray ionization (ESI) positive and ESI negative ion modes was performed using the ACQUITY UPLC I-Class system (Waters Corporation, Milford, CT, USA) and VION IMS QTOF Mass spectrometer (Waters Corporation, Milford, CT, USA). The original LC-MS data were processed using Progenesis QI V2.3 software (Non-linear Dynamics, Newcastle, UK) to perform

baseline filtering, peak identification, integration, retention time correction, peak alignment, and normalization. Subsequently, principal component analysis and orthogonal partial least-squares-discriminant analysis were conducted using R to explore the correlation between the two groups.

Statistical analysis

All quantification data were expressed as means ± SEM, and error bars represented SEM. The normality distribution assessment was evaluated using the Kolmogorov-Smirnov test, and differences among groups were assessed through one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. A p-value less than 0.05 was considered statistically significant. Statistical analyses and graphs (except for some data from 16S rRNA microbiome sequencing analyses and LC-MS) were performed using GraphPad Prism 6.0 and Adobe Photoshop 22.1.1.

Results

Hepatic ischemia-reperfusion injury induced cognitive impairment and dysbiosis of gut microbiota in mice.

After a 3-day reperfusion period, both groups of mice underwent cognitive function tests (Figure 1). Consistent with previous research findings, mice subjected to hepatic ischemia during the night (HIRI group) exhibited significant cognitive dysfunction compared to the Sham group, indicating the evident impact of this surgery on the central nervous system. We further performed 16S rRNA analysis on the fecal samples of the two groups (Figure 2). The results indicate that 63 bacteria in fecal samples differed between the two groups at all levels, suggesting a noticeable dysbiosis of gut microbiota in the HIRI group, particularly with altered levels of Blautia, Akkermansia, and Anaerofustis.

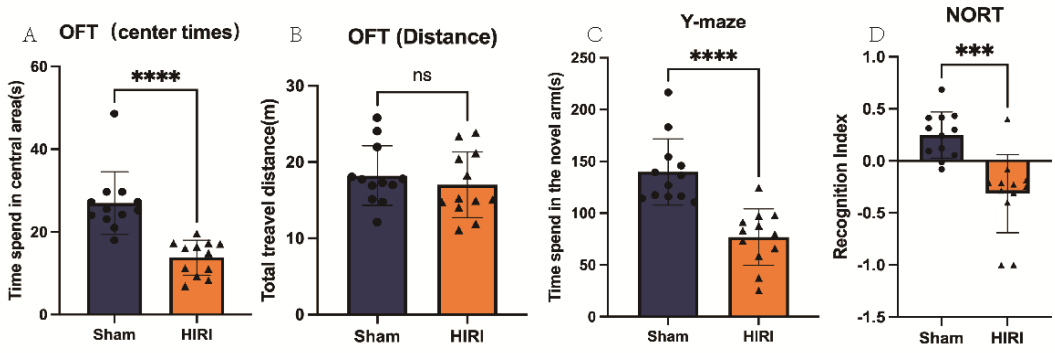

Figure 1. Cognitive impairment in the mouse model of hepatic ischemia-reperfusion injury

under ZT12. (A) Time spent in the central area of OFT. (B) Total travel distance of open field test (OFT). (C) Time spent in the novel arm of the Y-maze test. (D) Recognition index of novel object recognition test (NORT). Data are presented as the mean ± SEM. ***P < 0.001, ****P < 0.0001; ns, not significant.

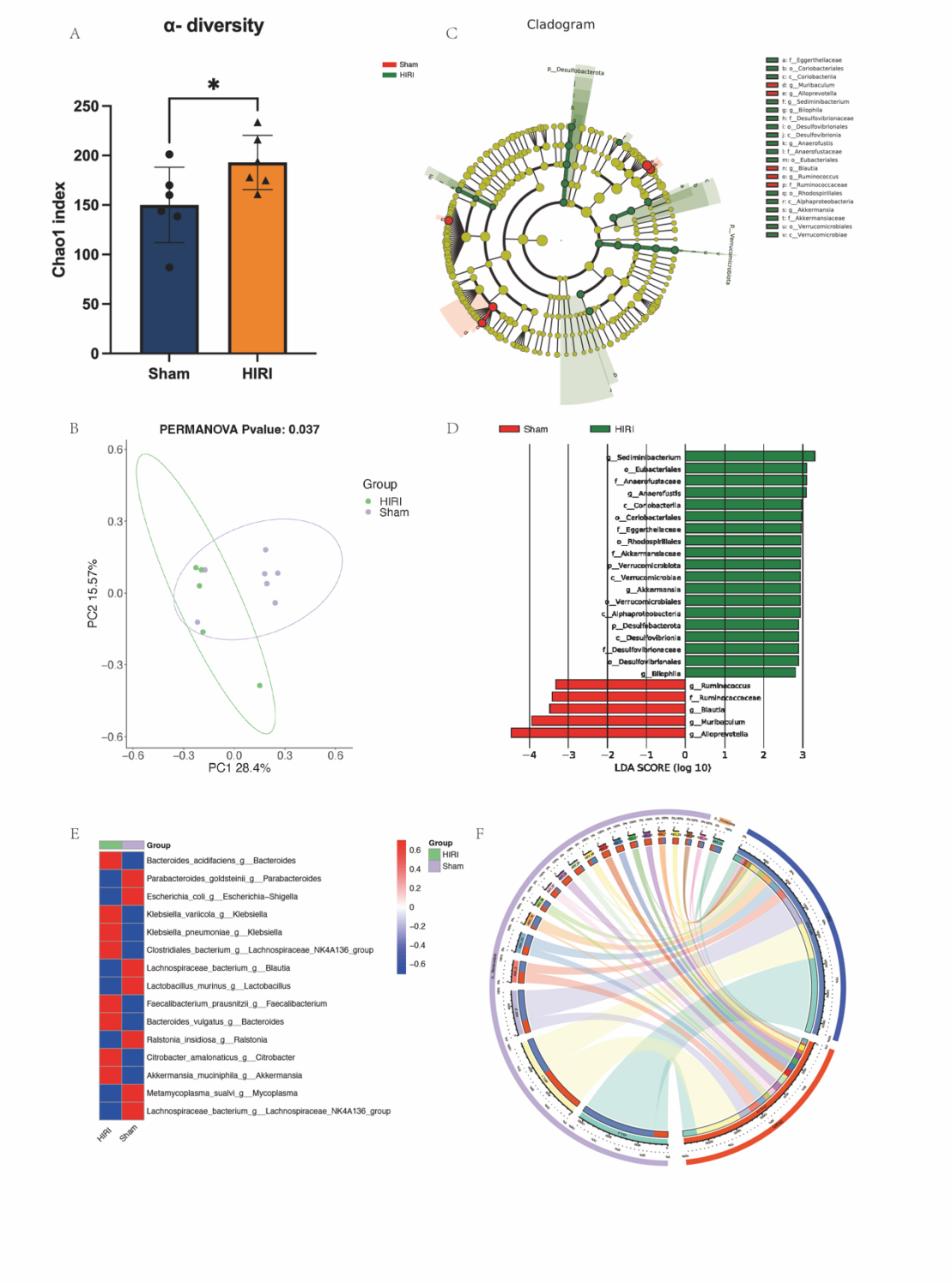

Figure 2. HIRI procedure induced gut microbiota alternation in mice. (A) α-diversity Chao1 index. (B) Principal Co-Ordinates Analysis (PCoA). (C-D) Linear discriminant analysis Effect Size (LEfSe) analysis. (E) Top 15 different species between the two groups. Data are presented as the mean ± SEM. ∗P < 0.05.

Hepatic ischemia-reperfusion injury led to dysregulation of the HDAC2-ACSS2 axis in the hippocampus of mice.

We focused on HDAC2 and ACSS2, two key enzymes highly associated with histone acetylation/deacetylation in the hippocampus of mice. Using western blotting experiments, we assessed the expression of these two proteins (Figure 3). The results showed a significant decrease in ACSS2 expression in the hippocampus of mice in the HIRI group, while HDAC2 expression remained relatively stable. This suggests that hepatic ischemia impairs acetate metabolism in the hippocampal region.

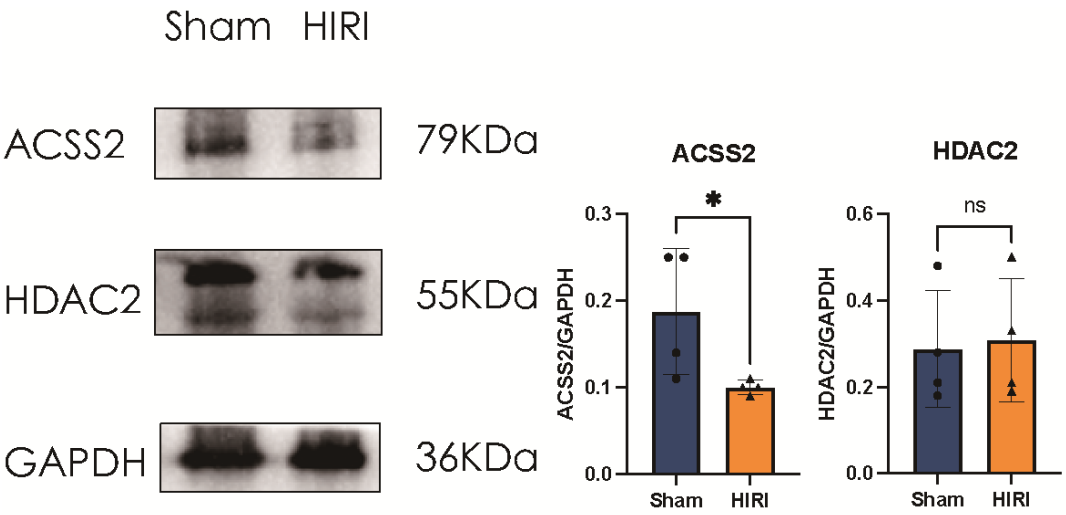

Figure 3. Dysregulation of HDAC2-ACSS2 axis in HIRI-induced cognitive impairment mice. Data are presented as the mean ± SEM. ∗P < 0.05; ns, not significant.

Microbiota transplantation induces cognitive impairment and gut microbiota dysbiosis in germ-free mice.

To further validate the relationship between gut microbiota and cognitive impairment induced by HIRI, we transplanted fecal samples from the two groups of mice into germ-free mice. Similar to

previous experimental results, gut microbiota dysbiosis and cognitive dysfunction were effectively transmitted to germ-free mice through microbiota transplantation (Figure 4).

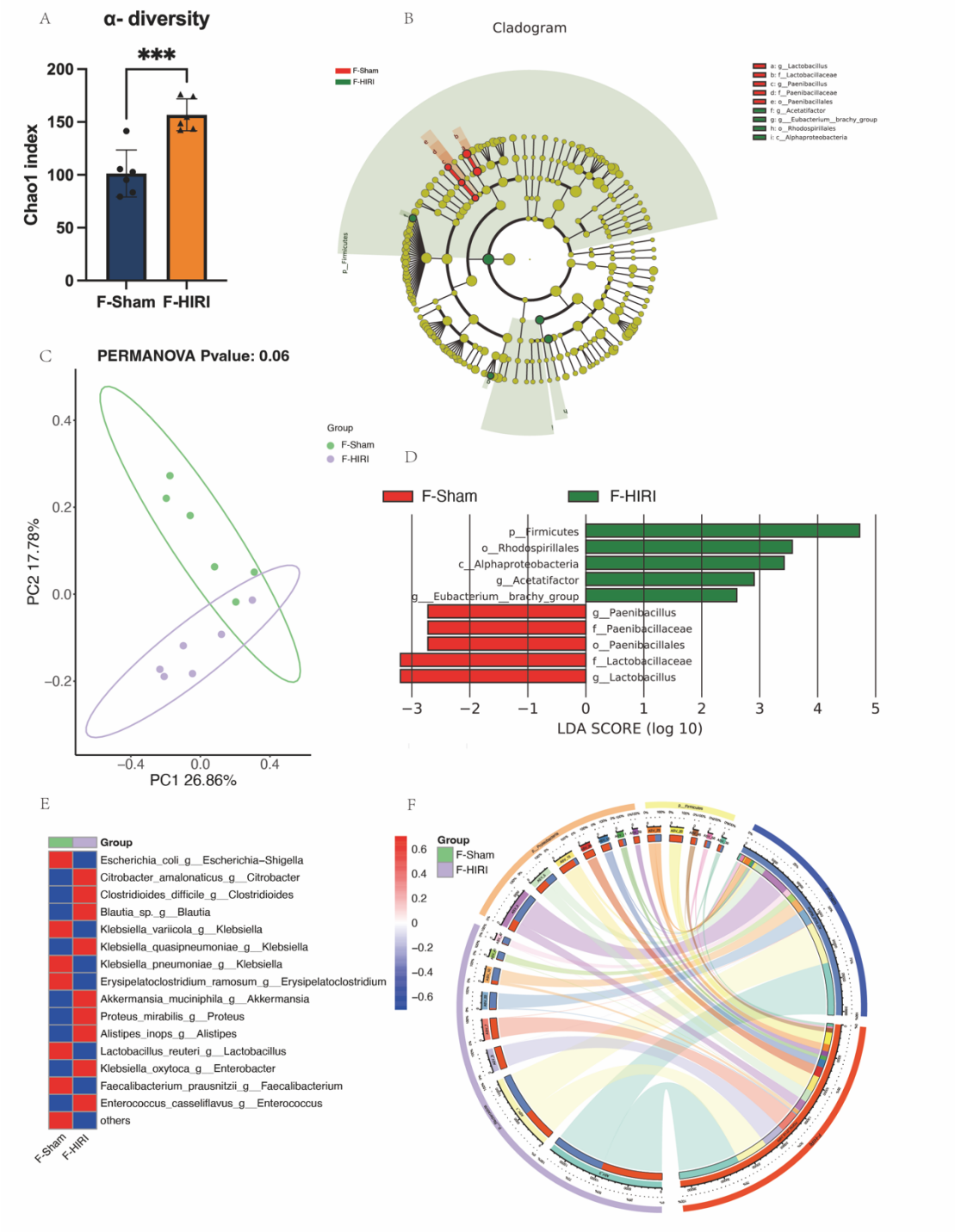

Figure 4. Feces microbial transplantation induced gut microbiota alternation in Pseudo germ-free mice. (A) α-diversity Chao1 index. (B) Principal Co-Ordinates Analysis (PCoA). (C-D) Linear discriminant analysis Effect Size (LEfSe) analysis. (E) Top 15 different species between the two groups. Data are presented as the mean ± SEM. ***P < 0.001.

Microbiota transplantation induced dysregulation of the HDAC2-ACSS2 axis and abnormal short-chain fatty acid metabolism in the hippocampus of germ-free mice.

Surprisingly, the phenomenon of HDAC2-ACSS2 axis dysregulation was also successfully transmitted to germ-free mice through microbiota transplantation (Figure 5). To understand the metabolism of short-chain fatty acids in the hippocampus of germ-free mice, we further measured the levels of various common short-chain fatty acids and acetyl-CoA in the hippocampus of both groups (Figure 6). The results revealed that mice receiving microbiota transplantation from the HIRI group had significantly higher acetate levels in the hippocampus compared to mice receiving transplantation from the Sham group. At the same time, the concentration of acetyl-CoA in the hippocampus of F-HIRI group mice was notably reduced, suggesting the presence of disturbances in acetate utilization, acetyl-CoA synthesis, and histone acetylation in the hippocampus of F-HIRI group mice (Figure 7).

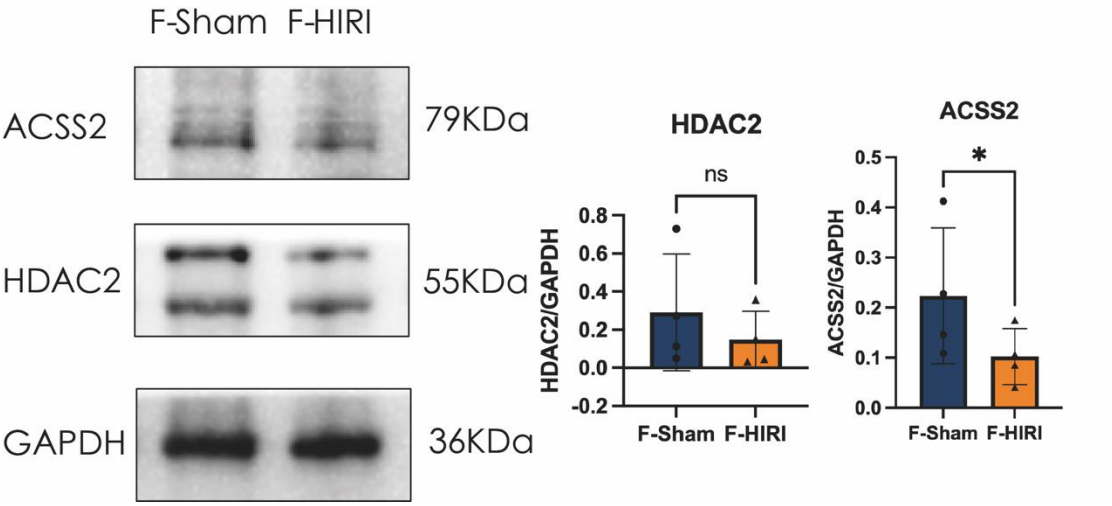

Figure 5. Dysregulation of HDAC2-ACSS2 axis in FMT mice. Data are presented as the mean ± SEM. ∗P < 0.05; ns, not significant.

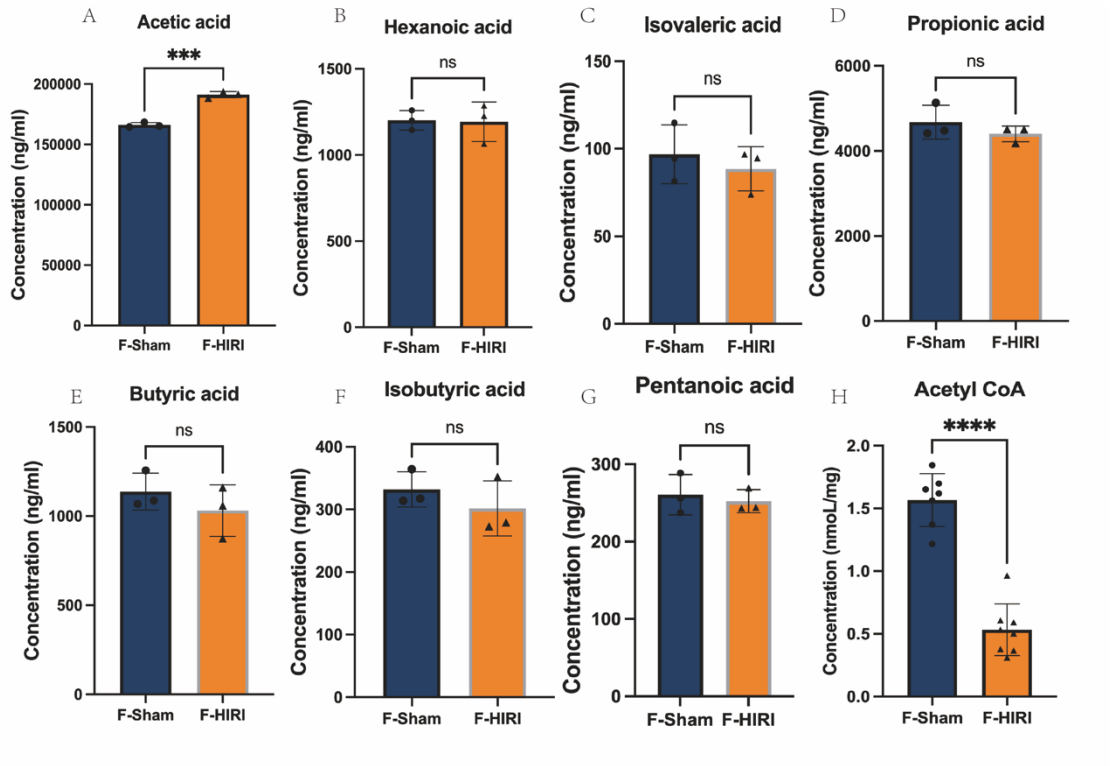

Figure 6. The levels of short-chain fatty acids (SCFAs) and acetyl CoA in the hippocampus in FMT mice. (A-G) Comparison of the SCFA levels in the hippocampus between F-Sham and F-HIRI. (H) Comparison of the acetyl CoA levels in the hippocampus between F-Sham and F-HIRI. Data are presented as the mean ± SEM. ***P < 0.001; ns, not significant.

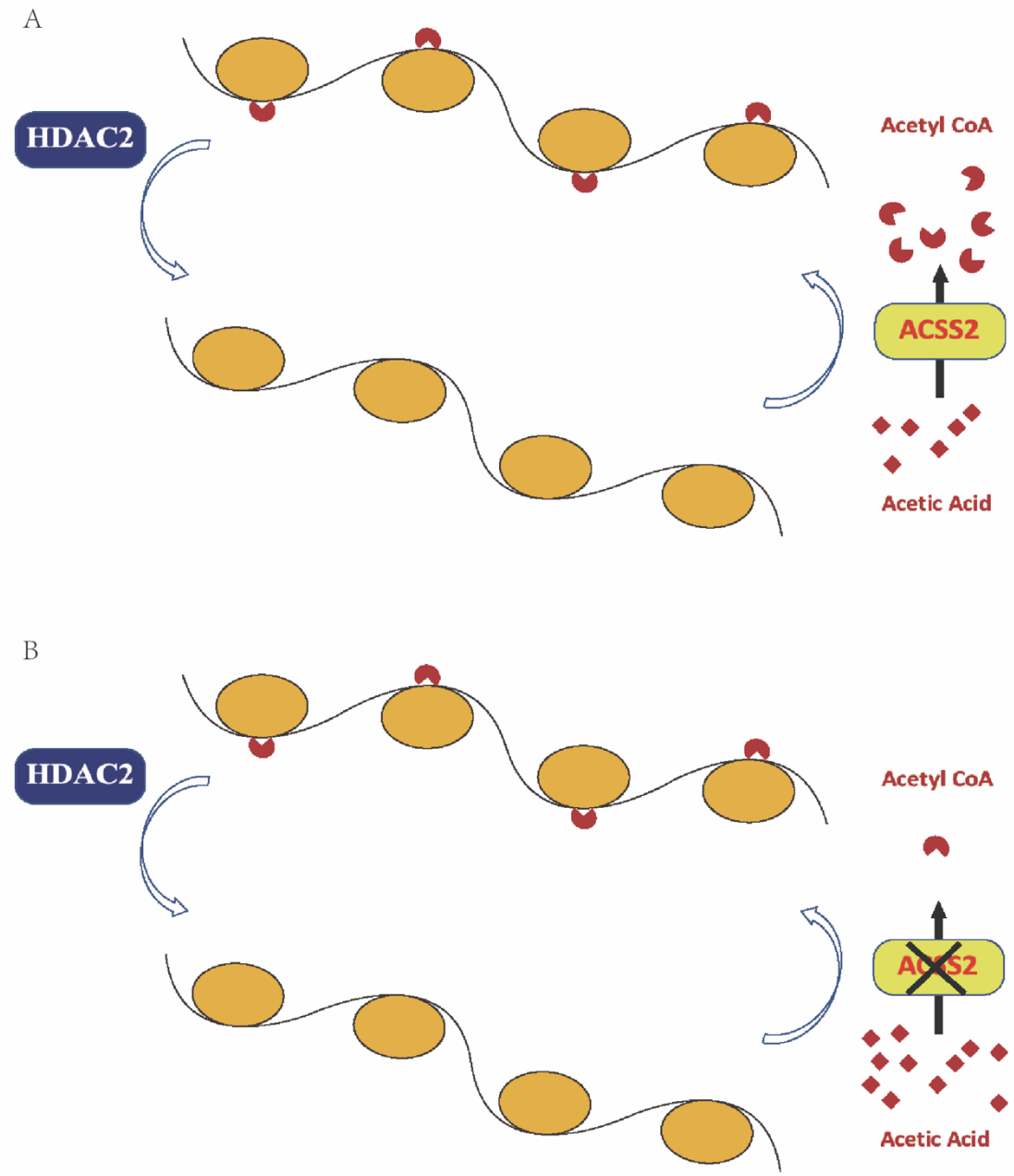

Figure 7. A hypothetical mechanism for disturbances in acetate utilization, acetyl-CoA synthesis, and histone acetylation in the hippocampus of HIRI-induced cognitive impairment mice. (A) Normal mice. (B) HIRI-induced cognitive dysfunction mice and FMT mice.

Discussion

The present study utilized multiple techniques, including gut microbiota analysis, behavioral tests, and LC/MS short-chain fatty acid profiling to demonstrate the occurrence of postoperative cognitive impairment in mice subjected to hepatic ischemia-reperfusion injury during nighttime. Additionally, western blotting (WB) analysis revealed a significant decrease in ACSS2 expression in the

hippocampus of mice with hepatic ischemia-reperfusion injury, indicating impaired ACSS2 function in this region. Moreover, both the phenotype of cognitive impairment and the reduced ACSS2 expression could be transferred to germ-free mice through fecal microbial transplantation. Subsequent analysis of short-chain fatty acid levels in the hippocampus of transplanted germ-free mice highlighted a significant reduction in acetate levels in mice receiving cognitive impairment-associated gut microbiota, accompanied by a marked decrease in acetyl-coenzyme A (acetyl-CoA) levels. These findings suggest impaired acetate metabolism in the hippocampus of cognitive impairment mice, that can lead to compromised histone acetylation.

Furthermore, 16S rRNA analysis revealed significant differences in several short-chain fatty acid metabolism and inflammatory response-related gut microbial taxa, such as Anaerofustis, Blautia, Akkermansia muciniphila, and Alistipes, between the HIRI and Sham groups, as well as the F-HIRI and F-Sham groups. These changes may be correlated with cognitive impairment and hippocampal acetate metabolic dysregulation, possibly contributing to the development of cognitive impairment following fecal microbial transplantation[39-45]. However, this study did not elucidate the specific underlying mechanisms, and further investigations are warranted to address this issue.

Nevertheless, the current study has some limitations. Firstly, the precise reasons behind the gut microbiota-induced HDAC2-ACSS2 axis imbalance were not extensively explored. Previous studies have implicated alterations in hippocampal short-chain fatty acid levels (e.g., significantly reduced acetate levels in the experimental group compared to the control group) in cognitive impairment models induced by various factors, which is related to the gut microbial short-chain fatty acid metabolism[34, 46, 47]. The results of this study differ somewhat from the aforementioned findings, suggesting that the impact of gut microbial metabolites, such as short-chain fatty acids, on cognitive function may vary under different conditions. Therefore, further in-depth research is needed. Secondly, due to experimental constraints, we were unable to confirm our conclusions by directly measuring histone acetylation levels.

Hepatic ischemia-reperfusion injury, as one of the most common complications in liver surgery, has been a focus of research in the academic community. The current understanding of the etiology of hepatic ischemia-reperfusion injury mainly attributes it to the damage caused by oxygen-free radicals upon blood reperfusion, resulting in cell death due to calcium overload, and immune system-related actions. Similarly, these damages can exacerbate inflammation in the central nervous

system and promote the development of cognitive impairment. The present study provides a novel research perspective on the development of cognitive impairment caused by hepatic ischemia-reperfusion injury, offering a theoretical basis for subsequent clinical treatments.

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