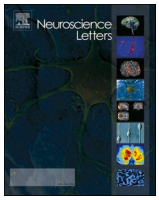
Neuroscience Letters 826 (2024) 137714

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet



Social isolation induces intestinal barrier disorder and imbalances gut microbiota in mice

Yue Wang [a,1], Hidayat Ullah [a,1], Ting Deng [a], Xinxiu Ren [a], Zinan Zhao [a], Yi Xin [a], Juanjuan Qiu [b,*]

[a] Department of Biotechnology, Dalian Medical University, Dalian, China
[b] Central Lab, The First Affiliated Hospital of Dalian Medical University, Dalian, China

ARTICLE INFO

Keywords:
Social isolation
Gut microbiota
Intestinal barrier

ABSTRACT

Social isolation, a known stressor, can have detrimental effects on both physical and mental health. Recent scientific attention has been drawn to the gut-brain axis, a bidirectional communication system between the central nervous system and gut microbiota, suggesting that gut microbes may influence brain function. This study aimed to explore the impact of social isolation on the intestinal barrier and gut microbiota. 40 male BALB/c mice were either individually housed or kept in groups for 8 and 15 weeks. Socially isolated mice exhibited increased anxiety-like behavior, with significant differences between the 8-week and 15-week isolation groups ($P < 0.05$). After 8 weeks of isolation, there was a reduction in tight junction protein expression in the intestinal mechanical barrier. Furthermore, after 15 weeks of isolation, both tight junction protein and mucin expression, key components of the intestinal chemical barrier, decreased. This was accompanied by a substantial increase in inflammatory cytokines (IL-6 mRNA, IL-10, and TNF-α) in colon tissue in the 15-week isolated group ($P < 0.05$). Additionally, Illumina MiSequencing revealed significant alterations in the gut microbiota of socially isolated mice, including reduced *Firmicutes* and *Bacteroides* compared to the control group. *Lactobacillus* levels also decreased in the socially isolated mice.

1. Introduction

Social isolation is when people lack or nearly lack social connections. It's known to greatly affect health, causing loneliness, depression, anxiety, and declining mental well-being [16,21]. Studies consistently show a strong connection between social isolation and psychiatric symptoms like depression and anxiety. This underscores how vital social interaction is for individuals. Moreover, research indicates that strong social connections protect health, while weak ties are linked to various health issues [30,21]. Social isolation has been identified as a contributing factor to a range of physical and mental health ailments [12].

Earlier studies have demonstrated that germ-free mice exhibited stress reactivity, hyper-grooming behavior, and impaired social interaction, which were subsequently ameliorated upon colonization with gut microbiota [52,13]. Scientific evidence suggests a connection between gut microbiota and symptoms of autism spectrum disorder (ASD), possibly mediated by their impact on metabolism and immune system function [1,39]. Transplantation of microbiota from anxious animals into normal animals has been observed to induce anxiety-like behavior [9]. In animal models, certain species of Lactobacillus and Bifidobacterium have been linked to the regulation and improvement of depression and stress-related behaviors [31,10,46].

The fecal microbiota composition of adult rats underwent significant alterations following maternal separation compared to the non-separated group [50]. In another study, Bendtsen et al. demonstrated that restraint stressors could substantially disturb microbiota structure [5]. Bravo et al. [10] found that *Lactobacillus rhamnosus* (JB-1) regulated depression-like behavior by altering GABAB1b mRNA expression in the brain. Stress reduced Firmicutes and Bacteroides abundance but increased Clostridium [3].

The link between gut microbiota and the brain isn't fully understood but is under discussion. Stress-induced "leaky gut" is believed to potentially play a significant role in this connection [19]. Anxiety and depression can affect intestinal permeability and the expression of tight

* Corresponding author.
E-mail address: qiujuanjuan123wo@126.com (J. Qiu).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.neulet.2024.137714
Received 2 August 2023; Received in revised form 1 March 2024; Accepted 6 March 2024
Available online 11 March 2024

junction proteins in the intestinal mucosa [57]. Specifically, the increased translocations of bacterial products through a leaky gut have been linked to the activation of the immune system [33,34]. Furthermore, research has shown that stress-induced alterations in microbiota composition can elevate levels of inflammatory cytokines, notably IL-6 and MCP. Stress also disrupts the microbiota balance in rodents, leading to increased pro-inflammatory cytokines [3,19,20].

The existing evidence supports a bidirectional relationship between the gut and the brain, facilitated by mechanisms like the gut-brain axis and the influence of gut microbiota on brain function. Stress-induced "leaky gut" and changes in microbiota composition further emphasize this intricate interplay. In our study, we hypothesized that exposing mice to different durations of social isolation would disrupt gut microflora balance, weaken intestinal barrier integrity, and trigger increased inflammatory responses. We also anticipated that these changes would correlate with altered behavior, including depressive-like and impaired social behavior. Our experimental design aimed to investigate how social isolation affects the gut-brain axis in mice, impacting gut microbiota composition, intestinal barrier function, and behavioral outcomes.

2. Methodology

2.1. Experimental design and animal housing

Male BALB/c mice, aged 4–5 weeks and raised in specific pathogen-free conditions, were obtained from Liaoning Changsheng Biotechnology Co., Ltd., with approval from the ethical committee of Dalian Medical University for experimental use. After a week of acclimation in a controlled environment (22 ± 3℃, 55 ± 5 % humidity, 12–12-hour light/dark cycle), with free access to food and water, the mice were divided into two groups: normal control (NC, n = 20) and socially isolated (SI, n = 20). In the NC group, mice were housed in groups of five per cage, while the SI group was individually housed without any sensory communication with other mice. Additionally, based on the duration of social isolation, the NC and SI groups were subdivided into 8-week isolation (NCd and SId, n = 10) and 15-week isolation (NCc and SIc, n = 10) groups. Various assessments and analyses were conducted at different time points throughout the study, as depicted in Fig. 1.

2.2. Body Weight, Food, and water intake determination

Throughout the study, the body weight of the mice was recorded every week. Additionally, food and water intake measurements were obtained once a week throughout the experimental period.

2.3. Open field test (OFT)

At specific time points, namely 6 weeks and 13 weeks, the mice underwent assessment using the open field test to evaluate anxiety-like behavior. Each mouse was placed in the center of a 50 × 50 × 40 cm black box with a 5 × 5 grid overlay. Their behavior was recorded for 15 min using video tracking software (Noldus, Ethovision XT 11.5) on a computer system. To minimize potential interference, only personnel not involved in the experiment were allowed in the testing area. During the test, the ambulatory distance, time spent in the center of the grid, and number of fecal boli produced were recorded for each mouse. Before testing each mouse, the testing area was cleaned with 75 % ethanol and thoroughly dried to eliminate any odor cues that could influence subsequent behavior.

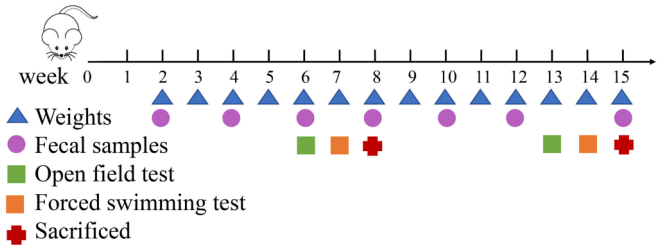


Fig. 1. Diagrammatic representation of the study design. Experimental subjects were exposed to different tests and parameters on different days.

2.4. Forced swimming test (FST)

A forced swimming test was performed at weeks 7 and 14. The mice were placed in a cylinder (29 × 11 cm) filled with water at a temperature of 25 ± 2.5 ℃ to 18 cm. The testing phase lasted for 6 min during this time frame the immobility duration of the mice was recorded on a computer using a video camera for the final 5 min (Noldus, Ethovision XT 11.5). During this testing period, two main behaviors were measured: immobility and manic behaviors. Immobility was operationally defined as the absence of any additional movements beyond those necessary to keep the animal's head above water. Manic behaviors were defined as any observable actions other than immobility, including swimming, struggling, or any active movements exhibited by the mice. After the test, the mice were promptly dried and warmed under a heating lamp.

2.5. Histopathological examination of the colon

Following approved ethical guidelines, mice were euthanized via isoflurane anesthesia and cervical dislocation. Colon samples were washed with cold PBS to remove gut content. For histological examination, colon tissue was fixed in 4 % paraformaldehyde, dehydrated, cleared, and embedded in paraffin. Sections were stained with HE and underwent immunohistochemistry for VIPR1 and MUC-2 expression. Images were captured at 200 × and 400 × magnification under a Leica microscope.

Immunofluorescent staining was employed to assess the expression levels of Zonula occludin (ZO-1), Occludin, and Claudin in colon tissue. Paraffin-embedded colon tissue sections (5 μm) were placed on positively charged slides. After deparaffinization and rehydration, antigen retrieval was conducted in a citrate buffer in a microwave oven. Slides were then blocked with 3 % BSA for one hour and incubated overnight at 4 °C with primary antibodies against ZO-1, Occludin, and Claudin. All antibodies used in IHC, and Immunofluorescence, along with their dilutions and sources, are detailed in (Table 1) Following washing, slides were incubated with Alexa-conjugated secondary antibodies, and DAPI was used for nuclear staining. Confocal microscopy was utilized to capture images.

2.6. Measurement of cytokine level by ELISA in colon tissue

To assess the expression levels of IL-6, IL-10, and TNF-α in a colon tissue sample of different experimental groups of mice, the colon tissue was homogenized on PBS using homogenizers, the mixture was subjected to centrifugation at 3500 rpm for 20 min, the supernatant was collected and keep in −80℃ for analysis. To quantify the levels of IL-6, IL-10, and TNF-α in the colon tissue ELISA kit (Shanghai Lengton Bioscience Co., LTD, China) was employed following manufacturer

Table 1
Antibodies used in IHC and IF.

Antibody target	Antibody type	Antibody dilution	Company
Claudin-1	Polyclonal	1:1000	Proteintech, USA
Occluden	Monoclonal	1:800	Proteintech, USA
Zo-1	Polyclonal	1:200	BIOSS USA
Mucin-2	Polyclonal	1:1000	Proteintech, USA

instructions.

2.7. Determination of intestinal mRNA

IL-6, IL-10, and TNF-alpha mRNA levels were measured by using RT qPCR. RNA-iso PLUS (Takara, Japan) was used to extract total Colon RNA, the total RNA was stored at −80 ∘C., which was reverse transcribed into cDNA using a reverse transcriptase kit, and RT-qPCR was performed using the SYBR Green RT-qPCR kit (Takara, Japan). RT qPCR was run at 95 ℃ for five minutes, followed by 45 cycles at 95℃ for 20 s. The primer annealing temperature was 60℃ for 30 sec and extension at 72℃. Beta-actin was used as a reference gene. Each sample was tested three times, and system software gene 9660 was used to calculate and analyze the relative expression of the gene, and GraphPad prism was used to analyze change between groups.

2.8. Microbiota 16S rRNA pyrosequencing

All stored mouse feces samples were used to extract stool DNA using a stool DNA isolation kit (stool/soi) by MoBio Laboratories (Carlsbad, CA, USA). Nano-drop was used to measure DNA concentration. The 16S bacterial rRNA gene's V4 variable region was amplified using Forward primer 515F(5GTGCCAGCMGCCGCGGTAA-3′)andreverseprimer806R (5′GGACTACHVGGGTWTCTAAT). GUHE Info Technology Co., Ltd (Hangzhou, China). Samples sequencing was analyzed using the Illumina Hiseq4000 sequencer (2 × 150 bp paired-end).

2.9. Evolutionary computation and statistical analysis

All Data results were expressed as Mean ± Standard Error of Mean (SEM). Statistical analysis utilized GraphPad Prism, employing Repeated Measures ANOVA followed by Tukey's multiple comparison tests to assess differences between 8-week and 15-week isolated mice and controls (NCc and NCd groups). Sequence data were analyzed with QIIME and R programs (v3.2.0), determining alpha and beta diversity. Ecological function, prokaryotic clades, and high-level phenotype were analyzed using STAMP (v2.1.3), FAPROTAX, and Bug Base.

3. Results

3.1. Social isolation effect on the body Weight, Food, and water intake

The results revealed no significant difference in body weight among groups throughout the Study. All groups exhibited a relatively stable body weight throughout the experimental period (Fig. 2A). Interestingly, the data indicated no significant differences in food and water intake. This finding suggests that social isolation did not significantly impact the amount of food and water consumed during the study period.

3.2. Social isolation led to anxiety and behavior disorders (Open field test Performance)

In the open field test, mice from the social isolation group exhibited reduced ambulatory distance compared to the standard control group. Specifically, the 8-week and 15-week socially isolated mice showed less ambulatory distance ($p < 0.05$). The open-field test results also indicated that socially isolated mice spent less time in the center than the normal control ($p < 0.05$). Moreover, these findings were further supported by the observation of fecal boli formation in the mice. Eight weeks of socially isolated mice produced significantly more fecal boli than the normal control mice ($p < 0.05$). In comparison, the 15-week isolated group showed more fecal boli formation than their normal control $p < 0.01$, as depicted in (Fig. 2D), which suggests the anxiety-like behavior associated with the effects of social isolation.

3.3. Forced swimming test

During the force swimming test, mice from the social isolation group exhibited decreased immobility time and increased manic behavior compared to the normal control group ($P < 0.05$, Fig. 2E, F). This difference was significant in the 8-week socially isolated group. However, in the 15-week socially isolated group, the difference was insignificant compared to the normal control.

3.4. Social isolation caused gut barrier dysfunction

Immunofluorescent labeling of tight junction proteins, including ZO-1, Occluding, and Claudin, revealed the dysfunction of the gut barrier. Compared to the normal control group, the socially isolated group exhibited considerably low intensity of these tight junction proteins, indicating reduced production. After social isolation, the production and expression of all these three tight junction proteins in colon tissue significantly decreased (Fig. 3A, B, C, D, E, F).

3.5. Social isolation-induced inflammation in colon

To assess the impact of social isolation on colonic inflammation and injury, we performed Hematoxylin Eosin (HE) on the colonic tissue

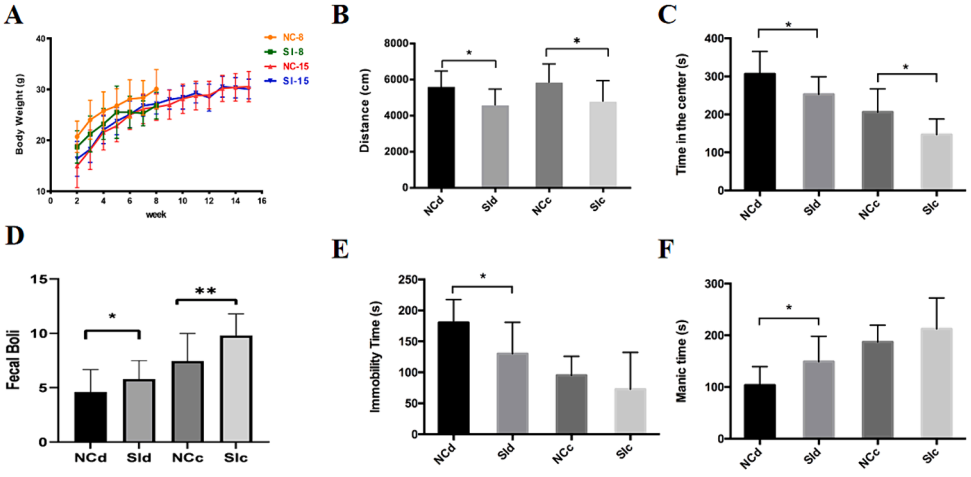


Fig. 2. Social isolation induces despair behavior. (A) Change in body weight from post-natal day 21 to 8 and 15 weeks. Comparison of (B) distance traveled, (C) time spent (s) in the center, (D) some fecal boli expelled in NC and SI mice recorded over 15 min in the open field test. (E) The immobility time (s) and (F) Comparison of manic time. Data mean ± SEM, *$p < 0.05$.

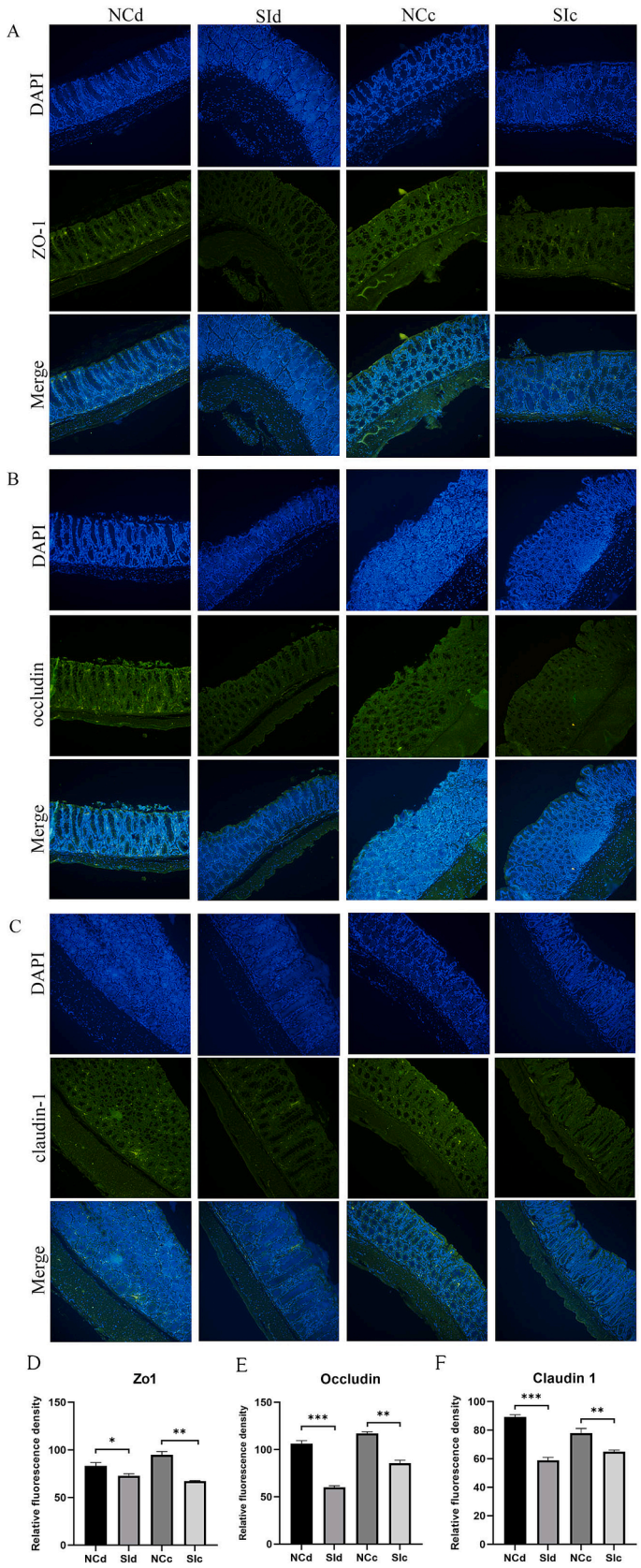


Fig. 3. Expression of tight junction protein in colon tissue through immunofluorescence. (A) expression of ZO-1 (B) occludin (C) expression of claudin-1. All pictures were captured at 200 ×. (D, E, F) the bar graph shows the quantitative representation of different tight junction proteins.

sections from the socially isolated and normal control groups. Strikingly, the HE results from the colonic tissue of the SIc group revealed a significant increase in inflammatory cell infiltration compared to the normal control (NCc). The inflammatory cell infiltrate observed in the SIc group indicated a pronounced inflammatory response within the colonic tissue, suggesting an elevated immune response due to social isolation (Fig. 4A). Conversely, the colonic tissue of the NCd and SId mice exhibited no evidence of inflammatory infiltration. The SId group showed no signs of colonic injury, consistent with healthy colonic tissue like normal control (NCd), indicating a lack of inflammation in the Colon in 8 weeks of isolated mice.

3.6. Social isolation increased cytokines levels in the colon

Remarkably, the socially isolated group exhibited significantly higher relative expression for IL-6 compared to normal control. However, considering isolation duration, there was higher expression noted in the SIc group ($p < 0.05$), while no significant difference was observed in the 8-week isolated group ($p > 0.05$). Results also unveiled a notable increase in the IL-10 in the SI group; the difference in IL-10 expression was statistically significant ($p < 0.05$ Fig. 4C). SIc groups displayed substantially higher levels of TNF-α expression in colon tissue ($p < 0.05$). While the SId group was found to show no significant difference figure (Fig. 4G).

3.7. Immuno-histochemical localization

To investigate the potential impact of social isolation on the Gut barrier, VIPR1 expression levels in the colon tissue were examined by utilizing Immunohistochemical staining to assess the levels of VIPR1 in different experimental groups. Surprisingly, the NCd and SId (10 weeks isolated) groups displayed similar VIPR1 staining patterns in the colon tissue. The intensity of VIPR1 staining appeared comparable between these groups, indicating that the protein expression of VIPR1 remained consistent in response to short-term isolation (Fig. 5A). Contrarily, the NCd and SId groups exhibited a significant increase in the VIPR1 expression in the colon tissue compared to normal control. The immunohistochemistry results revealed a visibly higher intensity of VIPR1 in the SIc group, indicating an upregulation of VIPR1 levels due to social isolation (Fig. 5A, C).

3.8. MUC2 expression in the colon

Goblet cells produce MUC-2, a crucial compound protective mucous layer that shields the gut lumen from harmful flora and maintains integrity. To assess the potential influence of social isolation on Goblet cell-derived MUC-2 expression in the Colon, we utilized IHC staining to analyze the level of the Muc-2 in different experimental groups. Strikingly, the socially isolated mice for 15 weeks (SIc) displayed s significantly lower number of MUC-2 expressions than normal control (NCc). The results analysis revealed the intensity of MUC-2 staining in the SIc group, Indicating a decrease in MUC-2 production due to long-term social isolation. In contrast, the NCd and SId (8 weeks) socially isolated mice exhibited similar levels of MUC-2 expression in Colon, suggesting that the expression of MUC-2 remained consistent in response to 8 weeks of isolation (Fig. 5B, D).

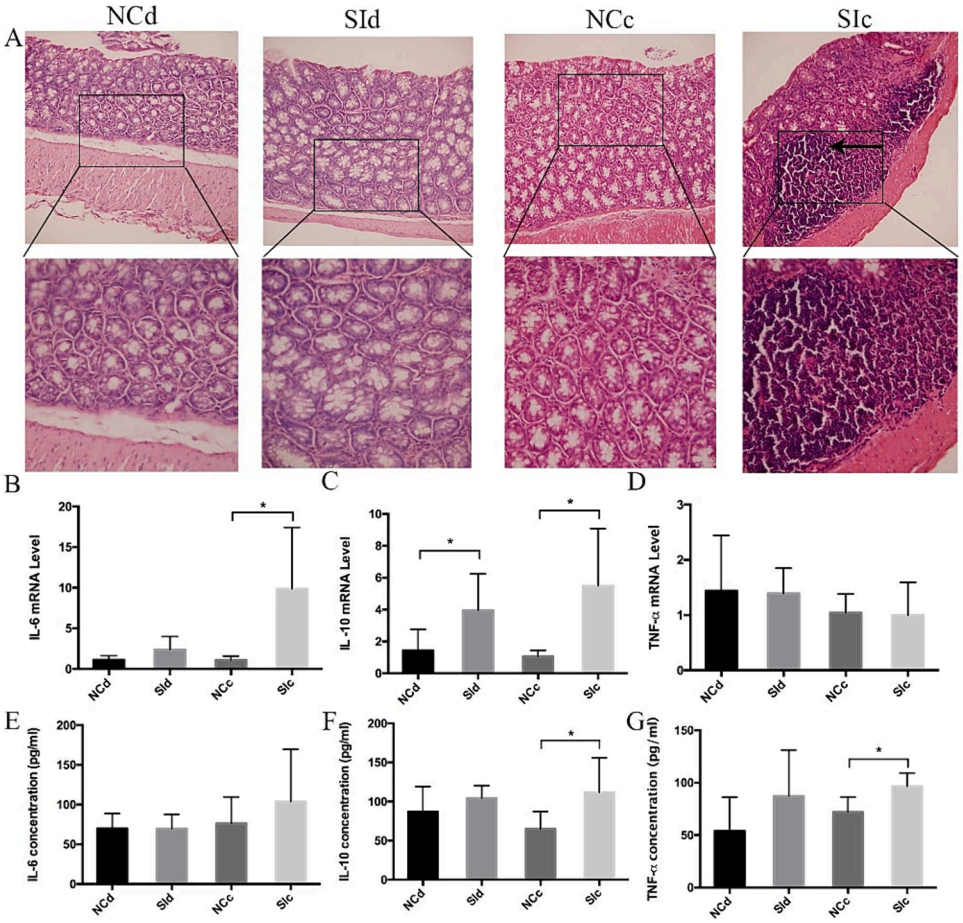


Fig. 4. Histological changes (200× and 400×) and social isolation increase the level of cytokines in mice's Colon. (A) H&E staining of the Colon in four groups. Arrow marks pointed to inflammatory infiltration. The mRNA level of (B) IL-6, (C) IL-10, and (D) TNF-α. In colon tissue inflammatory cytokines (E) IL-6 (F) TNF-α and anti-inflammatory cytokine (G) IL-10 levels in the colon tissue were analyzed by ELISA kit.

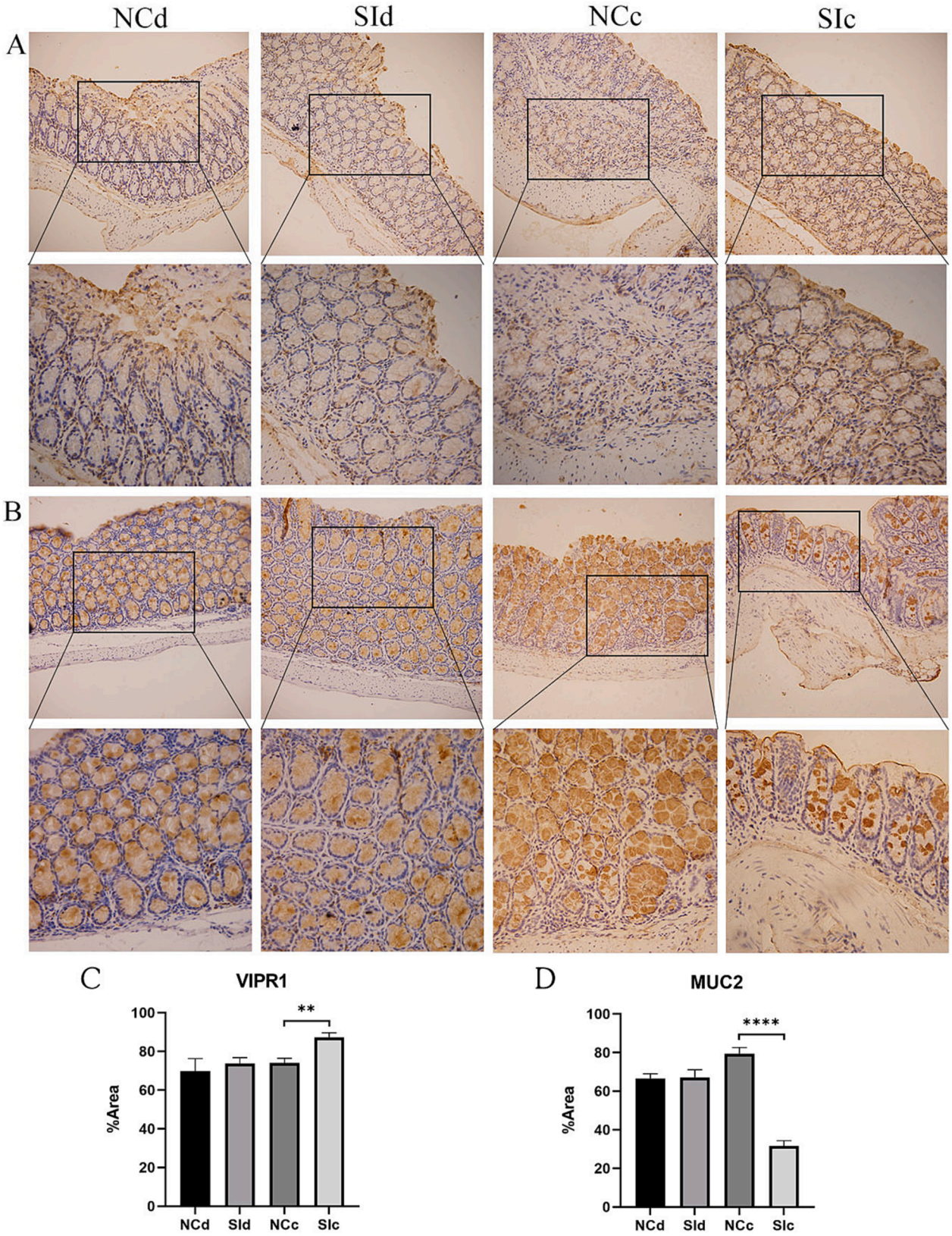


Fig. 5. Immunohistochemistry staining for expression of VIPR1 and MUC2 proteins in the Colon (200 × and 400 ×). (A) The expression of VIPR1 in four groups of the Colon. (B) The expression of MUC2 in four groups of colons. (C) show the quantitative expression for VIPR1 (D) quantitative expression for mucin.

3.9. Social isolation alters gut microbiota composition

Social isolation led to significant changes in gut microbiota, analyzed via Illumina MiSeq genome sequencing. Operational taxonomic units (OTUs) were employed for bacterial taxonomy, with a sequence identity above 97 % considered equivalent to a species. Species richness and alpha diversity were assessed using a dilution curve, where each color represented a distinct sample. The curve trended upward when sequencing coverage was insufficient but flattened when adequate data represented most microbial species (Fig. 6A). Shannon and Simpson indices, measuring gut microbiota alpha diversity, were higher in the SIc group compared to the NCc group ($P < 0.05$; $P < 0.001$, respectively), with no difference between NCd and SId ($P > 0.05$, Fig. 6B). After 15 weeks of social isolation, isolated mice exhibited a greater diversity of gut microbiota species than group-housed mice. Principal Coordinate Analysis (PCoA) and Principal Component Analysis (PCA) depicted differences in gut microbiota beta diversity among all groups (Fig. 6C, D).

Significant differences were observed between groups in the taxonomic composition of microbial populations, with Firmicutes and Bacteroidetes dominating the intestinal microbiome (Fig. 7A). Compared to NCd, SId exhibited lower Firmicutes abundance ($P = 0.0816 > 0.05$), while SIc had substantially less Firmicutes compared to NCc ($P = 0.0023 < 0.01$, Fig. 7B). The SI group showed a higher relative abundance of Bacteroidetes than the NC group, with significance observed only in the 15-week group ($P = 0.0046 < 0.01$), while the 8-week group showed no significant alterations ($P = 0.0616 > 0.05$, Fig. 7C). Proteobacteria and Actinobacteria abundances were similar across all groups ($P > 0.05$, Fig. 7A). Family and genus differences were also noted, with Lactobacillaceae, S24-7, Lachnospiraceae, and Bacteriodeaceae being dominant (Fig. 7D). The 15-week socially isolated group exhibited significantly greater S24-7 abundance than NC mice ($P = 0.0126 < 0.05$), whereas the 8-week socially isolated group did not reach statistical significance ($P = 0.0823 > 0.05$, Fig. 7E). Lactobacillus and Bacteroides were the most dominant genera. In the 15-week socially isolated group, SI mice had lower Lactobacillus compared to NC mice ($P = 0.0371 < 0.05$), while the 8-week socially isolated group did not show significant change ($P = 0.7231 > 0.05$, Fig. 7F).

Bacteroides were increased in SI mice than NC mice in the 15-week socially isolated group ($P = 0.0084 < 0.01$) but in the 8-week socially isolated group, it did not reach statistical significance ($P = 0.1716 > 0.05$, Fig. 7G). SId has fewer *Odoribacter* than NCd ($P = 0.0530 > 0.05$). SIc had less *Odoribacter* than NCc ($P = 0.0119 < 0.05$, Fig. 7H).

Hierarchical cluster analysis based on prominent genera divided microbial communities into four groups, revealing greater distinctiveness of SI compared to NC (Fig. 8A). LEfSe analysis identified different dominating bacterial groups in the four groups (Fig. 8B, C). The NCc group exhibited Odoribacteroidaceae, Lactobacillaceae, Methylobacteriaceae, and Oceanospirillaceae as dominant flora, whereas the SIc group showed Bacteroidaceae, S24-7, Deferribacteraceae, Mogibacteriaceae, Alcligenaceae, and Comamonadaceae (Fig. 9A). Furthermore, Study using the LEfSe biomarker finding tool showed a substantial rise in *Bacteroides* after 15 weeks of social isolation. Phylum *Firmicutes* and genus Lactobacillus were found most abundant in normal control (Fig. 9B).

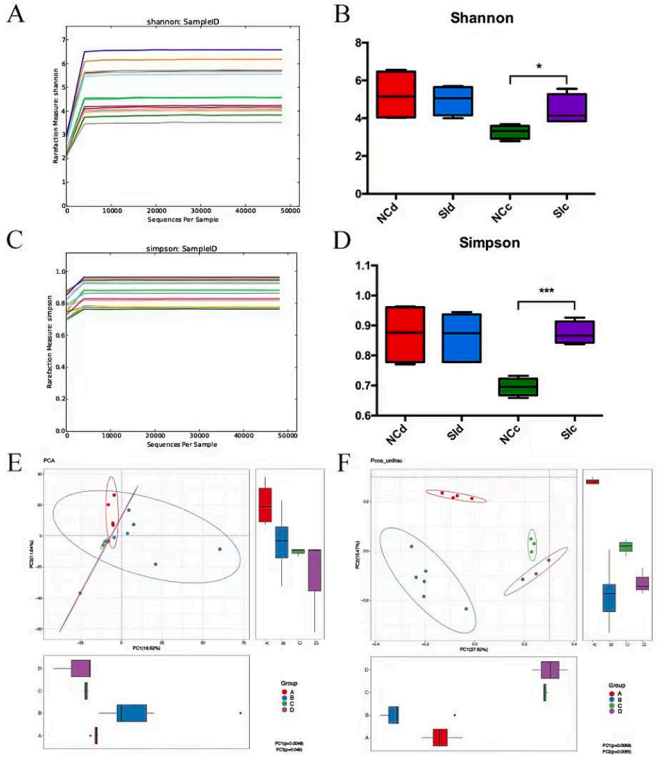


Fig. 6. Alpha and beta diversity index. (A) In the dilution curve of the sample species richness Shannon index, the x-axis represents the number of selected reads, and the y-axis represents the value of the corresponding alpha diversity index. (B) Shannon. (C) and (D) show principal coordinate analysis (PCoA) plot with Bray–Curtis dissimilarity. Legend in Figure C and Figure D the legend A represents NC-8, B is group SI-8, C is group NC-15, and D is group SI-1.

4. Discussion

Social relationships, vital to human well-being, profoundly impact mental and physical health [56]. Positive connections offer health benefits, while weak relationships are associated with adverse health effects [54]. The gut-brain axis, influenced by the intestinal microbiota, plays a crucial role in the bidirectional interaction between the central nervous system and the enteric nervous system. The gut-brain axis, influenced by the intestinal microbiota, plays a crucial role in the bidirectional interaction between the central nervous system and the enteric nervous system [11]. Changes in the gut microbiome are linked to CNS disorders like anxiety, depression, and ASD [60,17,41]. The brain-gut microbiome has been studied extensively, but its mechanism is unknown. Current work found that social isolation anxiety increases intestinal permeability, mucosal barrier degradation, inflammatory response, and gut microbiota imbalance.

In our study, we examined the effect of social isolation on body weight, food, and water intake in mice. However, we found no significant changes in these parameters due to social isolation. Contrary to our findings, a previous study reported weight gain and other physical and behavioral changes associated with social isolation in singly-housed mice [7]. These findings imply that the influence of social isolation on weight regulation can differ based on specific experimental conditions, including the duration of isolation, housing conditions, or the genetic background of the mice.

A study showed that socially isolated mice spent less time in the center of the maze compared to non-socially isolated mice [48]. This behavior is indicative of anxiety-like behaviors, as mice showing higher levels of anxiety tend to stay closer to the maze, or outer wall and exhibit reduced defecations [24]. In the open field test SI mice subjected to stress showed activation of the sympathetic nervous system, resulting in decreased defecation. Social isolation during rearing has been shown to induce hyperactivity in mice, which is a common symptom of “isolation syndrome [28]. The immobility observed in the FSR test stems from rodents feeling unable to escape stress, resulting in a passive coping style. Initially, rodents actively attempt to escape through swimming, struggling, and climbing. However, they eventually transition to immobility to conserve energy until a new escape option arisesIn the present study, socially isolated (SId) and depressed mice demonstrated increased activity and reduced immobility times compared to non-isolated (NCd) mice. This suggests higher levels of hyperactivity and

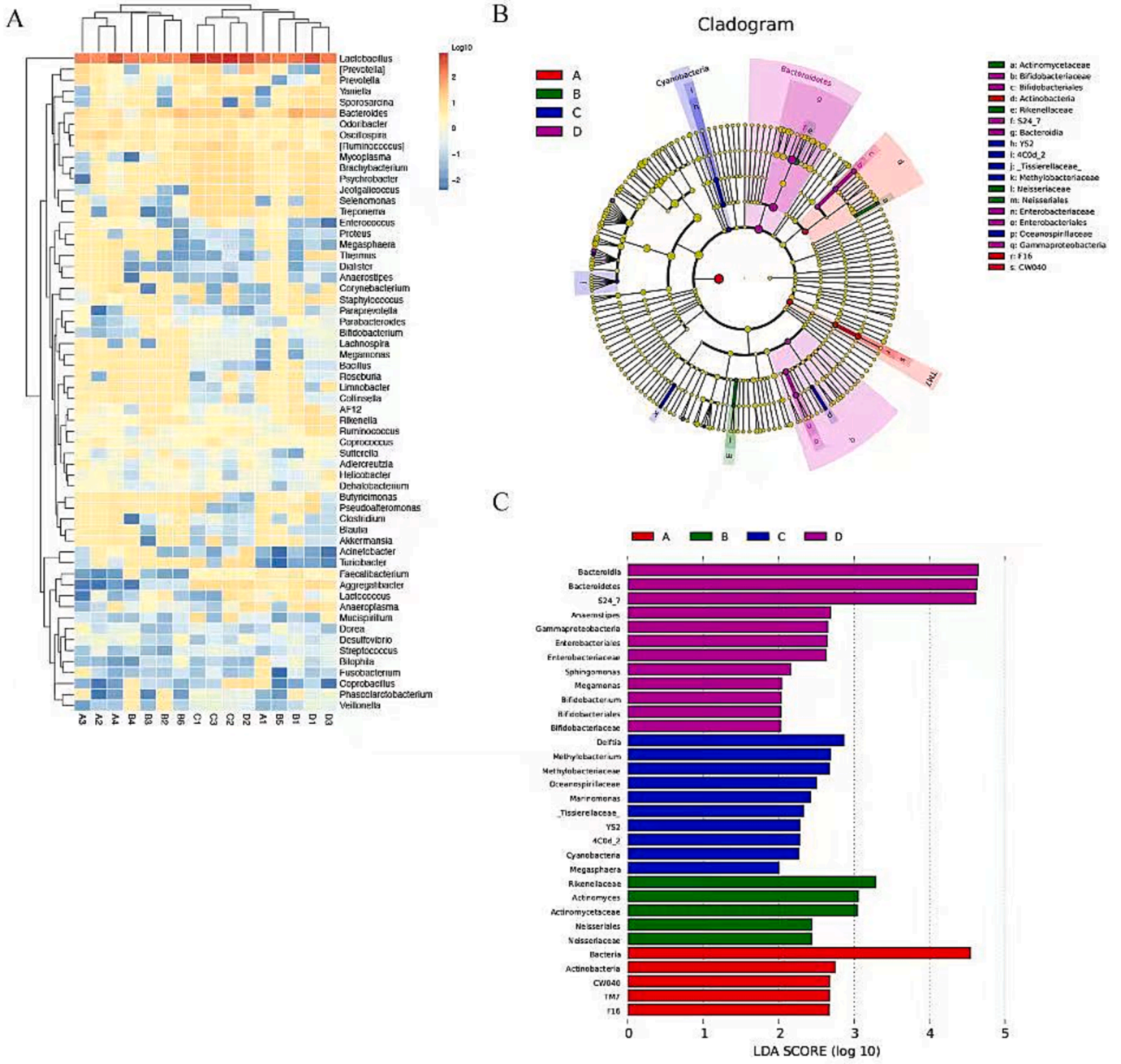


Fig. 7. Alterations in the gut microbiota**.** (A) Relative abundance of major phylum in four groups. Relative abundance at phylum level of (C) *Firmicutes* and (D) *Bacteroidetes.* (B) Relative abundance of major families in four groups. Relative abundance at the family level of (E) *s24-7*. Relative abundance at genus level of (F) *Lactobacillus*, (G) *Bacteroides*, (H) *Odoribacter*. Data are mean ± SEM, *p < 0.05, **p < 0.01.

decreased depressive-like behaviors in SId mice, possibly linked to heightened anxiety and fear. Variations in stress reactivity and coping strategies among mouse strains may influence their response to social isolation. Additionally, factors such as age and previous experiences can impact behavioral outcomes. The duration and intensity of social isolation may also contribute to observed differences. Future studies should systematically compare different strains and age groups under standardized conditions to better understand these influences.

Tight junction proteins (TJs), including ZO-1, occludin, and claudin-1, prevent intestinal mucosal permeability and intestinal barrier integrity. These proteins control tight junction construction and intestinal epithelial integrity [25]. Reducing TJ expression may increase intestinal epithelium permeability. Some studies showed chronic stress affects intestinal barrier function [14]. Interestingly, after 8 and 15 weeks of social isolation, our experiment found that the expression of tight junction proteins of ZO-1, occludin, and claudin-1 in the Colon decreases, destroying the intestinal mechanical barrier.

Many cells secrete IL-6, a pro-inflammatory cytokine associated with acute or chronic inflammation. IL-10, an anti-inflammatory cytokine, is partly stimulated by IL-6 and other factors. TNF-α, a cytokine directly related to inflammation, is also secret [51,27,36,29]. In socially isolated mice, H&E staining indicated SIc animals had more colonic inflammation than NCc mice. After 15 weeks of social isolation, colon cytokines IL-6, IL-10, and TNF-α raise considerably, matching histological data. After eight weeks of social exclusion, colons showed no inflammatory infiltration or increased cytokines. Thus, long-term (15 weeks) social

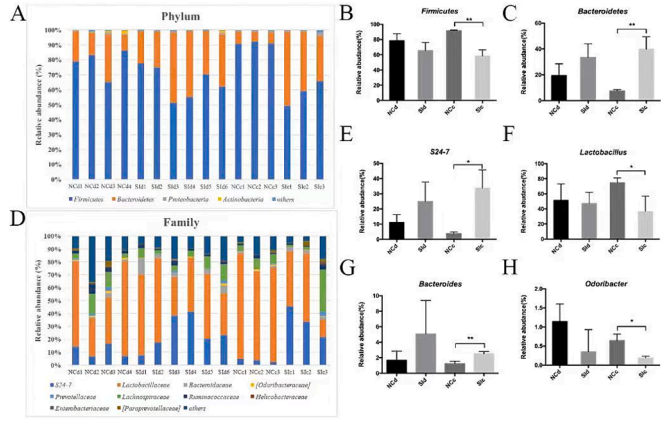


Fig. 8. Social isolation's impact on gut microbiota is depicted through heat map analysis of predominant genera in the study (A), highlighting biomarkers distinguishing different groups. Cladogram representation (B) illustrates taxonomic hierarchy of identified phylotype biomarkers, with colors indicating group representation. Phylotypes' effect sizes in each group are ranked (C), aiding comparison. NCd, SId, NCc, and SIc denote different experimental groups (A represents the NCd group, B represents the SId group, C represents the NCc group, and D represents the SIc group).

isolation generated a chronic gut inflammatory response, but short-term (8 weeks) social isolation did not impair the immunological barrier.

Physiological mechanisms regulate intestinal barrier function. Vasoactive intestinal peptide (VIP) is a neuropeptide and small molecule immune-active peptide in numerous immune cells and enteric neurons of the GI system (Keita, Carlsson et al.). VIP regulates intestinal permeability in several studies. IBS patients express higher VIP receptor 1(VIPR1) than healthy persons. When inflamed, immune cells release VIP [6]. We found that the intestinal inflammatory response promoted VIPR1 expression in the Colon of mice after 15 weeks of social isolation but not after eight weeks.

The intestinal mucus layer regulates growth, immunological modulation, physical and chemical protection, and homeostasis [53]. Mucin, a protein on intestinal epithelial cells, inhibits bacterial translocation and pathogenic invasion, reducing inflammation [22]. MUC2, released by goblet cells, makes up the Colon's double-layer mucosal structure [38]. The outer layer houses symbiotic bacteria, whereas the inner layer is impermeable to bacteria and attached to the epithelium [44]. SI group, after 15 weeks of social isolation, MUC2 expression reduced dramatically, indicating a gut chemical barrier rupture. Social isolation of 8 weeks failed to influence intestine MUC2 expression, maybe because of short time isolation.

The intricate interplay between the gut microbiota and brain, known as the gut-brain axis, relies on the pivotal role of the intestinal microbiota in facilitating bidirectional interactions [11]. Recent studies indicated the impact of stress on the composition of gut microbiota, suggesting a significant relationship between psychological well-being and microbial inhabitants of our digestive system [19]. In the present study, at 15 weeks, socially isolated mice had remarkably high gut microbial diversity (measured by Shannon and Simpson indices) compared to NC animals. At eight weeks, there was no significant difference. Bacterial variety may benefit human health, although its impact on CNS function is uncertain. Feces from autistic children and depressive individuals showed higher gut microbial variety and richness [15,23]. Thus, the effects of social isolation-induced bacterial diversity in mice remain unclear. SI and NC mice have different microbiota compositions, indicating the difference in gut microbiota structure.

Microbiota analysis revealed significant differences between socially isolated and group-housed mice after 15 weeks but not after 8 weeks. Bacteroidetes and Firmicutes phyla varied notably in socially isolated mice (SIc), with a notable increase in the Bacteroides genus within the Bacteroidaceae family, similar to findings in IBS-D and depression

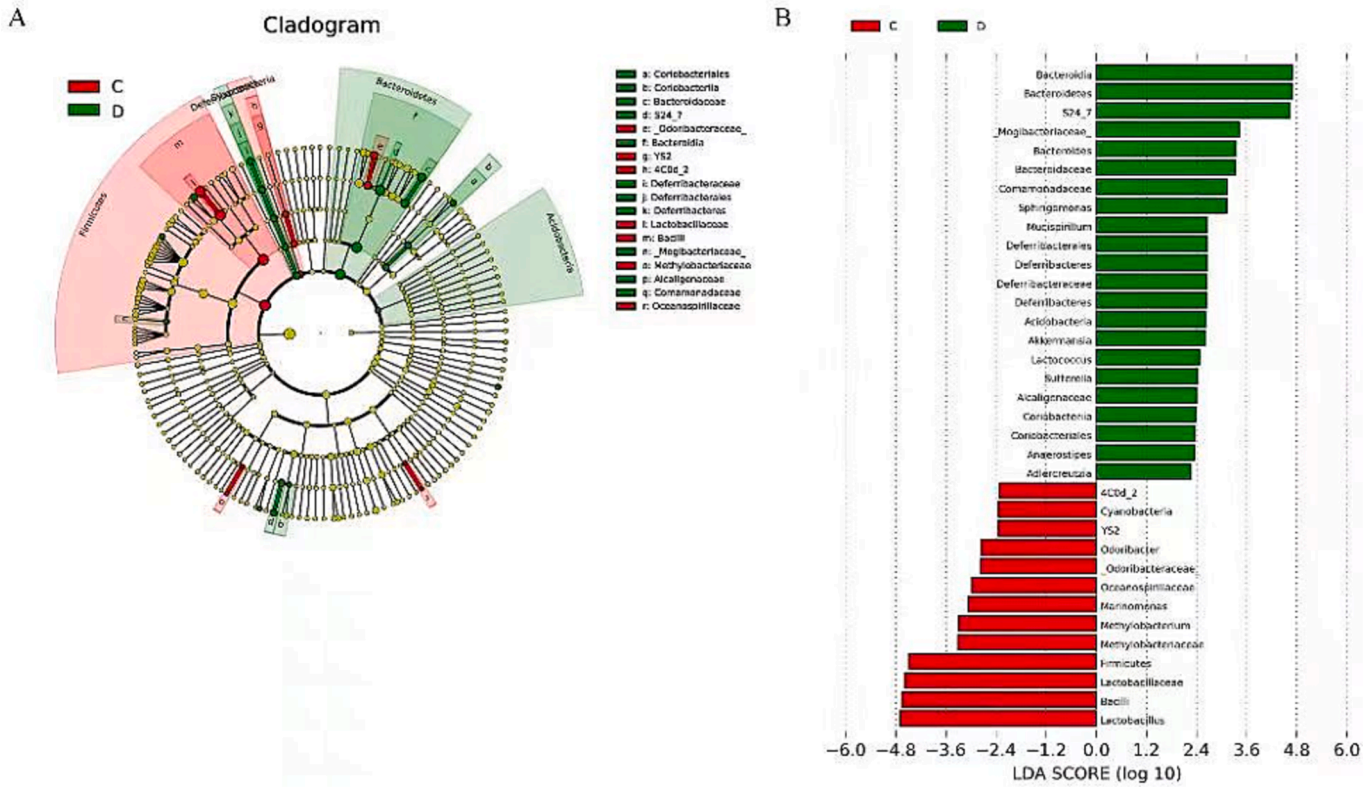


Fig. 9. Different gut microbiota in NCc and SIc groups. (**A**) The Cladogram is in two groups. (**B**) The LDA score is in two groups. (C represents the NCc group, and D represents the SIc group).

studies by [32]. IBS is the most common functional gastrointestinal disorder, and IBS combined with psychological distress is common [8]. Social isolation could promote Bacteroides growth, resembling IBS-like symptoms, while reducing Lactobacillus levels, potentially exacerbating metabolic and behavioral issues associated with long-term stress 35].

Most studies utilized *Lactobacillus (Lactobacillus helveticus* and *Rhamnobacter rhamnsus)* at dosages of 10^9 and 10^{10} colony-forming units for two weeks in animals and four weeks in humans. These probiotics improve anxiety, depression, autism spectrum disorder (ASD), obsessive–compulsive disorder, and memory (both spatial and non-spatial) [58]. According to previous studies, after 15 weeks of social isolation, the mice showed obvious anxiety behavior and a significant decrease of *Lactobacillus* in gut microbiota composition. We hypothesized that long-term social isolation destroyed the intestinal tract's probiotic balance.

The current study showed that the genus *Odoribacter,* a member of the *Odoribacteraceae* family, was considerably reduced in SIc group. Healthy persons have more *Odoribacter* than metabolic syndrome patients. Additionally, *Odoribacter* improved host metabolism [40]. Social isolation decreases *Odoribacter,* which may be linked to metabolism and central nervous system illnesses. Wang et al. showed that Crohn's disease faeces contain much less *Odoribacter,* a short-chain fatty acid (SCFA)-producing bacterium [59]. Thus, decreasing *Odoribacter* may cause gut microbiota problems and metabolic illnesses.

In homoeothermic mammals, *S24-7* is a critical intestine bacteria [43]. In the present work, SIc enhanced the S24-7 family. Most studies relate S24-7 to metabolic illnesses and few to CNS disorders. S24-7 family members' abundance also changes with environmental circumstances. In diabetic rats given a high-fat diet, *S24-7* was greater in quantity [37]. Although these findings are confined to mouse studies, they suggest that *S24-7* engages in host-microbial interactions and impacts intestine function and health.

In our study, we used male mice based on scientific justifications. Males often exhibit more pronounced behavioral and physiological changes in response to stress and social isolation. Focusing on male mice minimizes confounding factors and allows for easier comparison with existing literature. However, we recognize the significance of studying both male and female animals to fully understand the impact of sex differences on the relationship between social isolation, the gut microbiome, and behavioral outcomes. Recent research has highlighted the importance of considering sex as a biological variable in preclinical studies. However, future research should also consider studying females to explore sex-related differences in responses.

5. Conclusion

Social isolation led to anxiety and behavior disorders, suggesting abnormalities in mouse brain tissue. Eight weeks of social isolation destroyed the intestinal mucosa tight junction. They increased the permeability of intestinal mucosa, while 15 weeks of social isolation further damaged the integrity of the intestinal mucosal barrier, resulting in intestinal inflammation and an imbalance of gut microbiota. The increase in intestinal mucosal permeability preceded the intestinal inflammatory response and imbalance of gut microbiota in mice. This suggests that social isolation caused damage to the intestinal barrier, further leading to the intestinal inflammatory response and imbalance of gut microbiota.

6. Ethics approval and consent to participate

All methods were performed following the relevant guidelines and regulations of Dalian Medical University and approved by the Dalian Medical University Animal Care and Research Ethics Committee.

Funding

National Nature Science Foundation of China (Grant No.31600614). The role of the funder is to provide funding for the experiment.

CRediT authorship contribution statement

Yue Wang: Methodology, Conceptualization. **Hidayat Ullah:** Writing – original draft, Data curation. **Ting Deng:** Methodology. **Xinxiu Ren:** Methodology. **Zinan Zhao:** Methodology, Formal analysis. **Yi Xin:** Writing – review & editing, Supervision, Project administration, Investigation, Conceptualization. **Juanjuan Qiu:** Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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