Cold stress induces colitis-like phenotypes in mice by altering gut microbiota and metabolites

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Research Article

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Abstract

Objective

The westernized lifestyle has been paralleled by an epidemic of inflammatory bowel disease (IBD). Excessive consumption of cold beverages is especially common among the modern humans. However, whether cold stress contributes directly to the gut barrier and gut-brain axis is not clear. We investigated whether the cold stress affect gut inflammation or not.

Material

: 16 male mice were used in this study.

Treatment:

The mice were treated with 14 consecutive days of intragastric cold or common water administration.

Methods

We observed changes in gut transit and gut barrier in the colon.We also employed RNA sequencing-based transcriptomic analysis to identify the genes potentially driving gut injury, and simultaneously examined the gut microbiota and metabolites in the feces.

Results

Cold stress inhibited the colonic motility and increased gut permeability. A set of core genes related to immune responses were consistently overexpressed in the cold stress group. Additionally, cold stress induced decreased bacterial diversity, ecological network, and increased pathogens mainly belonging to Proteobacteria. The dopamine signaling pathway-related metabolites were largely reduced in the cold stress group.

Conclusion

This study revealed that cold stress could trigger an IBD-like phenotype in mice, implying that cold stress is a possible risk factor for IBD development.

Introduction

The westernized lifestyle has been paralleled by an epidemic of several gastrointestinal diseases worldwide beginning in the 1980s, including irritable bowel disease (IBD) characterized by gut barrier dysfunction (1, 2). Especially, the incidence of IBD is continuously rising among young people (3). Patients with IBD present symptoms similar to those of patients with irritable bowel syndrome (IBS) to some extent, including anxiety and depression (4). These findings suggest a potential link between westernized lifestyle, gut barrier, and gut–brain interaction.

Excessive consumption of cold beverages is highly prevalent in modern population subsets, especially in the youth. Mounting evidence has shown that sweeteners, food additives, and other food ingredients in beverages play important roles in IBD (5). However, whether some other related factors (for instance, cold stress in the gut) might affect IBD occurrence or not, is still not quietly clear. A study has reported that cold meal intake inhibits contractile activity of the gastric (6). Another study has showed that women who consumed excessive cold drinks exhibits an abnormal intestinal response to stress (7).

Interestingly, industrial and domestic refrigeration development is regarded as a key environmental factor in the etiology of IBD since it increases the chance for exposure of human populations to psychrotrophic bacteria (such as *Yersinia*), which exacerbate intestinal inflammation (8). However, only approximately 10% of patients with IBD were found to harbor *Yersinia* in their gut (9); therefore, the theory of psychrotrophic bacteria cannot explain these conditions completely. Consequently, some other related factors (for instance, cold stress in the gut) might affect IBD occurrence.

To untangle the link between cold stress, gut barrier, and the gut–brain interaction, we mimicked excess cold beverage consumption in a mouse model. Metabolic syndrome is a potentially important confounder factor, which can indirectly affect the gut–brain axis by changing a myriad of physiological and endocrine systems in the gut, liver, pancreas, muscle, adipose tissue, and brain. To uncouple the metabolic effects directly caused by excess calorie intake from the beverage rather than the cold stress, we treated mice with cold water intragastrically for 14 consecutive days. We then evaluated the gastrointestinal physiology and stress-related behavior in mice. Moreover, we investigated whether cold water could affect the microbiota and metabolites of fecal contents since gut microbiota and metabolites are essential for bidirectional interactions within the gut–brain axis. These findings could highlight a link between cold beverage consumption on gut barrier dysfunction, gut microbiota, and metabolite disturbance, providing new preventive strategies to quell the creeping up in the incidence of IBD.

Material And Methods

Animals

The C57BL/6 mice (8 week, male) were used in this study. 16 mice were divided into two groups: The mice in the control group (n = 8) were subjected to gastric instillation with a total of 1.0 mL water at threes (from 8 am to 10 am) with room temperature (20–25 ℃). The experience group (n = 8) was subjected to gastric instillation with a total 1.0 mL water at threes (from 8 am to 10 am) with low

temperature (0–4 ℃). In addition, regular drinking water and food were available to the mice at all times. Gut function tests and behavioral tests were carried out after intragastric administration for 2 weeks. During the procedure, the body weight was measured on the same day every week.

Fecal output, fecal moisture content evaluation, and Bristol score

All mice were transferred into separate clean cages, and the feces were numbered and collected for 4 h between 8 am, and 12 am. All the feces during the procedure were collected and placed in tubes, then tubes were weighed and the wet weight were recorded, dried overnight at 60 ℃, then reweighed and the dry weight were recorded. At last the water content was calculated. The criteria for Bristol score was evaluated as described in previous study(10).

Bead discharge test and Gut peristalsis

Assessment of gut transit was performed using a bead discharge test. A 2 mm steel bead was lubricated with petroleum jelly and put into the anus (3 cm distance to the anus) using a smooth rod. The mice were placed in the cage, then the evacuation time of the steel bead was monitored and recorded. Gut peristalsis was assessed as described previously (11). In brief, 0.6 g of carmine was dissolved in 0.5% hydroxymethyl cellulose solution to obtain a 6% carmine solution. A 200 μL aliquot of the 6% carmine solution was administered to mice by gavage. The mice were then placed in separate cages. Gut peristalsis activity was defined as the time between intragastric administration and the first red bolus excreted.

Histological analysis and DAI (disease activity index) score

Hematoxylin-eosin staining of the ileum and colon was performed. Intestinal inflammation was assessed by observing ulcer formation, epithelial cell changes (goblet cells), lymphocyte infiltration, and lymph node formation, according to a previous report (12). DAI score is evaluated according the stool consistency, blood and weight loss as previously described (13).

Western blot and Immunofluorescence

The total protein was extracted, separated, transferred and detected as previously described(14). The antibodies against E-cadherin (1:1000; BD Biosciences,); Occludin (1:1000; ThermoFisher); Claudin 3 (1:1000; ThermoFisher); ZO-2 (1:1000; Cell Signaling Technology) and ZO-1 (1:1000; ThermoFisher) were used. The paraffin section of colon tissue were used for immunofluorescence, the antibody ZO-2 (1:100; Cell Signaling Technology) were used overnight, then secondary antibodies (1:300; Servicebio) for 1 h.

Depression- and anxiety-like behaviors test

The tail suspension test (TST) were used to evaluate depression- like behaviors. As described previously (15, 16), the mice were admitted to suspend for 6 min. All sessions were video-recorded. The time spent struggling in TST within a 6-min session were recorded and evaluated as behaviors for survival. The behaviors with reduced time for struggling were regarded as depression-like behaviors. Additionally,

anxiety-like behaviors were examined using Open Field Test (OFT) as described previously (17). Their locomotor activity were monitored for 15 min. The time spent in the central area were recorded as indicators of exploratory behavior. The behaviors with reduced time in the center were regarded as anxiety-like behaviors.

Feces collection, 16S rDNA Sequencing and Untargeted metabolomic analysis

Collection of fecal samples was performed during 8 am and 11 am in the day before euthanasia. The pellets collected were stored at − 80°C. The feces DNA extraction, polymerase chain reaction, library construction and sequencing were performed as previously described(18). Diversity indices were calculated using QIIME2. Principal coordinate analysis was performed using Calypso online tools. The relative abundances, Spearman correlation coefficients, and heatmap were calculated and compared using T packages. Spearman's rank correlations at the genus level were calculated as our previous study(19).

The liquid chromatography-mass spectrometry (LC-MS) was employed to analyze the fecal metabolome. We used the ACQUITY UPLC system (Waters Corporation, Milford, MA, USA) coupled with an AB SCIEX Triple TOF 6600 System (AB SCIEX, Framingham, MA, USA) in positive- and negative-ion modes as described previously (20, 21).

RNA sequencing and bioinformatics analysis

We used TRIzol reagent (Invitrogen, Carlsbad, CA, USA) to extract the RNA, then assess the RNA integrity by Bioanalyzer 2100 (Agilent, CA, USA). Poly(A) RNA was purified and fragmented into small pieces, and reverse-transcribed to synthesize cDNA, treated with U-labeled double-stranded DNAs with the heat-labile enzyme UDG, then amplified. Lastly, the products were sequenced on an Illumina Novaseq 6000 platform (LC-Biotechnology Co., Ltd., Hangzhou, China).

The Fastp software was used for quality control. HISAT2 was used to map the reads and the StringTie was used to assemble them. The different mRNAs were selected as the following criteria: mRNAs with fold change of > 2 or < 0.5 and $P < 0.05$ in a parametric F-test comparing nested linear models.

Statistical analysis

All data in gut function and behavioral tests were showed as the mean ± SEM. Statistical analysis was performed by using Student's t-test for comparisons between cold water group and control group. Statistical analysis for bioinformatics data of RNA-seq, gut microbiota, and metabolites was performed using the R package vegan. The *P* value less than 0.05 was regarded as statistical significance.

Results

Cold stress inhibits colonic motility and increases gut permeability in mice

A total of 15 mice (8 in control group and 7 in cold water group) were able to complete the experiment, as one mouse died from choking during intragastric administration. In the cold stress group, lower fecal output ($t = 2.493$, $P = 0.027$) (Fig. 1A), lower fecal moisture percentage ($t = 4.815$, $P < 0.001$) (Fig. 1B) and lower Bristol scores ($t = 3.015$, $P = 0.010$) (Fig. 1C) were observed. These results suggest that cold stress induce a decreased gut motility, which are consistent with previous studies(22). Altered fecal properties, bloody stools and weight loss are the most important characteristics in IBD, then we used the DAI scores to evaluate the severity of the colitis. We found that the DAI scores in the cold water group were significantly higher than in the control group ($t = -4.861$, $P < 0.001$) (Fig. 1D). Correspondingly, hematoxylin-eosin staining showed sparse intestinal villus, and edema in the intestinal villi of the jejunum (Fig. 1E), a significant reduction in goblet cells, an enhanced inflammation response, and an elevated histological score in the colon ($t = -2.193$, $P = 0.047$) (Fig. 1F). As to the gut barrier, we found the expression of ZO-1 and ZO-2 were lower by western blot (Fig. 1G), compared to that in the control group. The immunofluorescence results confirmed the lower expression of ZO-2 in the cold water group (Fig. 1H).

Cold stress exacerbates the inflammatory response of the intestinal tissue in mice

To further identify the changes in gene expression in the intestinal tissue after cold stress, we selected out 6 mice from the two groups randomly and investigated the gene expression profiles of the intestinal tissue using RNA sequencing (Fig. 2A). A total of 432 genes with differential expression between groups were identified, with 330 genes upregulated and 102 genes downregulated in the cold water group when compared with the control group (Fig. 2B). Gene Ontology analysis showed that all the genes most strongly enriched in immune function processes, including innate immune response and adaptive immune response, such as the B cell activation and B cell receptor complex, immunoglobulin production, immunoglobulin complex, complement activation, antigen binding and defense response to a bacterium (Fig. 2C). In accordance with these findings, KEGG pathway analysis also showed that the genes were enriched in immune activation in the colon, including the primary immunodeficiency, B cell receptor signaling, intestinal immune network for IgA production, NK cell-mediated cytotoxicity pathways, leukocyte transendothelial migration, Th1, and Th2 cell differentiation, Th17 cell differentiation, cytokine–cytokine receptor interaction, chemokine and Fc gamma R-mediated phagocytosis, all of which are closely related to the immune response in the colon (Fig. 2D).

To further reveal the effects of cold water on the colon, we used gene set enrichment analysis to analyze the genes which revealed substantial upregulation of genes involved in the B and T cell receptor signaling pathway, leukocyte transendothelial migration, FC epsilon RI signaling pathway, chemokine signaling

pathway, and cytokine–cytokine receptor interaction (Fig. 2E). These findings suggested that cold water triggers an IBD-like phenotype in mice.

Cold stress leads to low bacterial diversity and a fragile ecological network in the gut microbiota

In general, IBD is considered to occur when the immune system overreacts to the resident gut microbiota, inducing a chain of inflammatory events that can destroy the gut barrier (23). These findings prompted us to further investigate whether changes in microbiota or bacterial metabolites from the feces under cold water stimulation may regulate the gut barrier and gut–brain interactions.

Eight individual fecal samples from the control group and seven individual fecal samples from the cold stress group were collected and sequenced. The principal component plots with unweighted UniFrac distances showed a clear separation between the cold stress and control groups (Fig. 3A), which suggested that cold stress led to a significant alteration in the gut microbiota composition. The Shannon index also showed a low bacterial diversity in the cold stress group (Fig. 3B). At the operational taxonomic unit (OTU) level, the microbiota were significantly differed (Fig. 3C). The abundances of *OTU198* (*Lachnospiraceae_unclassified*), *OTU337* (*Clostridiales_Incertae_unclassified*), *OTU156* (*Muribaculaceae_unclassified*), *OTU254* (*Erysipelotrichaceae_unclassified)*, *OTU88* (*Duncaniella*), *OTU334* (*Muribaculaceae_unclassified*), *OTU13* (*Paramuribaculum*), *OTU433* (*Proteobacteria_unclassified*), *OTU215* (*Lachnospiraceae_unclassified*), *OTU258* (*Firmicutes_unclassified*), *OTU115* (*Paramuribaculum*), *OTU272* (*Mailhella*), and *OTU34* (*Akkermansia*) were significantly increased in the cold stress group. Additionally, the abundances of *OTU246* (*Anaerotruncus*), *OTU15* (*Alistipes*), and *OTU209* (*Clostridiales_unclassified*) decreased under cold stress (Fig. 3D).

Linear discriminant analysis of effect size further showed that the bacteria with increased abundance in the cold stress group mainly belong to Proteobacteria (Supplementary Fig. 1A). To determine the pattern of bacteria, we constructed their networks in the two group respectively, we found that the network of the cold water-treated mice had a simpler property (nodes/edges = 81/176) than the control group (nodes/edges = 80/234), indicating that cold water may induce vulnerability to environmental stress in the gut microbiota (Fig. 3E).

(A) Principal component analysis (PCoA) plots of unweighted UniFrac distances between the two groups. (B) Shannon diversity scores. (C) Heatmap of key operational taxonomic units (OTUs). (D) Gut bacteria with different abundances at the OTU level. (E) Network analysis at the genus level. V: number of nodes. E: number of edges. (n = 8 in control group and n = 7 in cold water group)

Cold water downregulates metabolites of the dopamine-related pathway in the intestinal flora

As a microbe–host bridge, some metabolites of the intestinal flora can affect host physiology by entering the bloodstream. Therefore, we analyzed fecal metabolites using LC-MS. We found that the metabolic data clusters of the control and cold stress groups were separated from each other in both positive- and negative-ion modes by partial least-squares discriminant analysis (Fig. 4A–B). The heatmap also showed that cold stress led to significant alterations in fecal metabolite levels (Fig. 4C); 1179 metabolites were upregulated, and 1896 metabolites were downregulated with significant changes (Fig. 4D). The most strongly impacted metabolic pathways included cocaine addiction, dopaminergic synapse, amphetamine addiction, and alcoholism addiction (Fig. 4E), all of which were accompanied by a significant reduction in levels of dopamine, l-dopamine, l-tyrosine, and homovanillic acid (Fig. 4F). As one of the most important neurotransmitters, decreased dopamine levels may contribute to anxiety-like and depression-like behaviors in the cold stress mice; Additionally, patients with IBD present some similar symptoms to patients with IBS, including anxiety and depression. Therefore, these behaviors were further evaluated.

Cold water increases depression-like behaviors in mice

In the tail suspended test, the struggling time was significantly decreased in the cold stress group (t = 2.618, P = 0.021) (Fig. 5A), suggesting an increase in depression-like behaviors in mice exposed to cold stress. Furthermore, in the open field test, the center time (t = 2.195, P = 0.047) were reduced in the cold stress group (Fig. 5B), implying a tendency of decreased exploratory behavior and increased anxiety-like behavior in the cold stress group.

Correlations of gut microbiota and metabolic changes

Finally, to explore the functional significance of the metabolite perturbations in the gut microbiota of the cold water-treated group, the 97 annotated metabolites with significant differences were selected, and their Spearman correlation coefficients with different bacteria were calculated. Significant correlations were observed between the gut microbiota and metabolites (Supplementary Fig. 2A), and also observed between metabolites and gut function (Supplementary Fig. 2B).

Discussion

Increased beverage consumption has long been recognized to play an important role in the pathogenesis of metabolic diseases and colorectal cancer (24–26). Although food additives such as emulsifiers, food colorants, titanium dioxide, and aluminum have largely contributed to the development of these conditions, recent evidence have also identified that food additives are pathogenic factors for colitis (26, 27). In this study, we focused on the potential contribution of cold stress associated with beverage consumption to IBD development. Our results revealed that cold stress was a novel risk factor for colitis in mice, including induction of gut barrier injury, increased anxiety-like behaviors, gut microbiota disorder, and metabolite disturbance. These findings suggest that exposure to cold beverages is also a novel risk factor for IBD in humans and a potential trigger for establishing experimental IBD in mice.

Dysregulation of the gut barrier in IBD is caused by environmental factors and genetic predisposition (28), however, identifying specific environmental factors has been difficult. Several food additives are identified as environmental risk factors for IBD (27, 29). Here, we aimed to identify whether the beverage temperature directly affect the development of colitis. Similar to observations in humans, the administration of cold water to mice inhibited gut transit, additionally, the mice exhibited a colitis-like phenotype of gut barrier injury. The fact that increased cold beverage consumption is related to some gastrointestinal symptoms seemingly suggests a link between cold stress and intestinal disorders. Indeed, our results indicate that exposure to cold water not only induces gut transit disturbances but also produces low-grade inflammation. In line with these considerations, cold water exposure expectedly led to activation of ubiquitous inflammatory signaling pathways, suggesting local mucosal immune cell activation and immune cell trafficking, which are characteristics of IBD (30).

Low-grade inflammation in colitis is associated with and may be promoted by gut microbiota dysbiosis (31). The low bacterial diversity and altered gut microbiota observed in the cold stress group in this study are extremely similar to those observed in IBD. For example, microbial diversity studies have demonstrated the overgrowth of Proteobacteria in IBD patients (32). Under normal homeostasis, epithelial cells, tight junctions, and the local immune system prevent the translocation of pathogens in the gut. However, in genetically susceptible individuals, Proteobacteria expand to colonize the lumen and invade the lamina propria, further aggravating disruption of the gut barrier (33). The host recognizes Proteobacteria via nucleotide oligomerization domain-like receptors, Toll-like receptors and retinoic acid-inducible gene I-like receptors. Moreover, microbiota-derived products such as lipopolysaccharide, peptidoglycan, and flagellin from Proteobacteria trigger the activation of immune responses in the mucosa. Our study suggests that the increased abundance of pathogens accompanied by cold stress may contribute to gut barrier disruption in mice by accelerating inflammation in the gut.

Apart from the microbiota-derived products, the metabolites of the gut microbiota are also key actors in the development and exacerbation of IBD. Accumulating evidence suggests that signals from microbial metabolites affect mucosal integrity and immune homeostasis. Moreover, in IBD patients, the metabolites composition and function are disturbed seriously, including bile acids, short-chain fatty acids and tryptophan (34, 35). Significant alteration in the gut microbiota metabolites was also identified under cold stress exposure in this study. Interestingly, we found a remarkable reduction in the levels of several metabolites in the dopamine-related pathway.

Dopamine is an critical catecholaminergic neurotransmitter that present in the peripheral tissues and central nervous system simultaneously, which regulates blood pressure, sodium balance, glucose homeostasis, cognition, memory, the sympathetic nervous system, and mood (36). Dopamine is mainly synthesized in the brain, T cells, dendritic cells, and as well as by gut commensals such as members of the genus Clostridium (36). Dopamine has recently recognized as an important regulator of the immune system. Disturbance of the dopamine pathway affects both innate and adaptive immunity largely, causing the development of inflammatory pathologies (37). A significant proportion of patients with gut barrier injury suffer from anxiety and depression (38), implying a rational connection between the gut and

the brain. Consistently, we examined their behaviors and found that cold water lead to a tendency to depression. Our findings suggest that cold water stress result in reduced neuro-activities in gut microbiota and enhanced pro-inflammatory activity that promotes anxiety and depression.

Nevertheless, our study has several limitations. We handled mice with cold water to explore the effect of cold stress to the gut barrier, microbiota, and metabolites. These findings acknowledge the important role of cold stress, however, it cannot mimic the condition of cold beverage consumption completely in humans because of the variety of beverages used and the differences between human and mouse physiology. In addition, although we find a significant overgrowth of pathogens and decreased dopamine-related metabolites in cold water-treated mice, we cannot completely identify that the alteration in gut microbiota causes the gut barrier injury. Further studies to investigate the role of microbiota and their metabolites with the effect on gut barrier and behavior are also needed to deeply elucidate their causal or accompanied relationships.

Conclusion

In summary, our study shows that exposure to cold stress promotes the development of colitis in mice. Our results may also have implications for human beings, as habit-forming consumption of cold beverages is implicated in IBD development.

Abbreviations

IBD, irritable bowel disease; IBS, inflammatory bowel syndrome; OTU, operational taxonomic unit; Tail Suspension Test (TST); Open Field Test (OFT); Liquid Chromatography-Mass Spectrometry (LC-MS)

Declarations

Acknowledgments

No applicable.

Author contributions

Jianbin Zhang designed the study; Lijuan Sun and Xueying Wang performed the research and wrote the paper; Yue Chen analyzed the data; Jianxia Song and Changting Liang contributed the methods and models.

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Data availability

The dataset of 16S rRNA gene sequencing was deposited in the Short Read Archive of NCBI under project no. PRJNA742186.

Ethical Approval and Consent to Participate

This study and handling of mice in general was conducted in strict accordance with the principles outlined in the EU Directive 2010/63/EU, and all experiments were performed in compliance with the ARRIVE guidelines. The protocol was approved by the Animal Research Ethics Board of The Northwest University.

Consent for publication

No applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

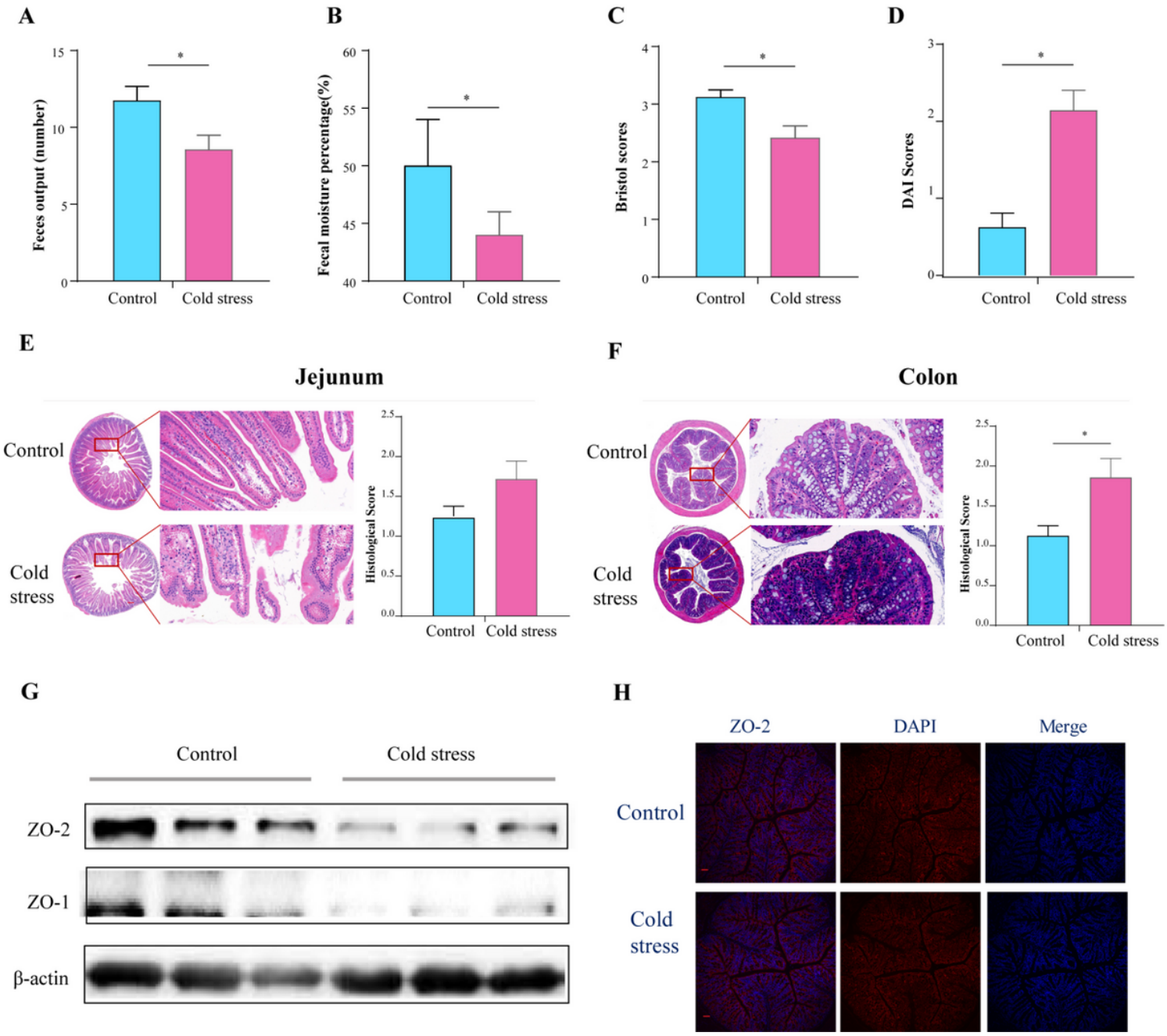


Figure 1

***Cold stress inhibits colonic motility and increases gut permeability in* mice**. (A) Fecal output in 4 h. (B) Fecal moisture percentage. (C) Bristol scores. (D) DAI scores calculating using body weight and fecal characters. (E-F) Hematoxylin and eosin-stained jejunum and colon sections from control and cold water-treated mice and the corresponding histological scores. (G) Immunoblotting of protein markers of tight junctions in the colon. (H) Representative images of ZO-2 immunofluorescence staining in the colon tissue. Scale bars = 50 μm. All the data are expressed as mean±SEM (n = 8 in the control group; n = 7 in the cold water group). *$P < 0.05$.

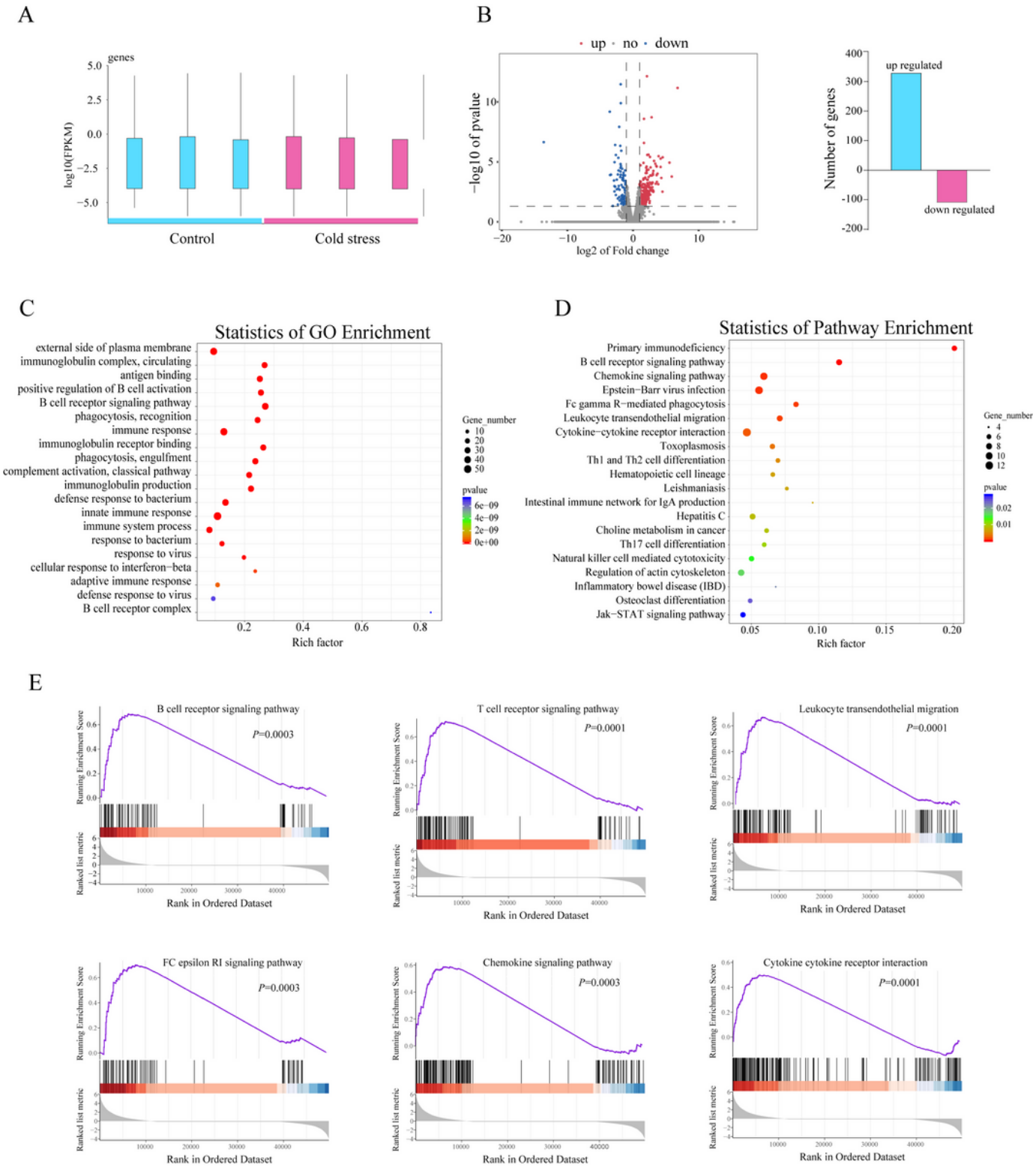


Figure 2

Cold stress exacerbates the inflammatory response of the intestinal tissue **in** ***mice.***

(A) Number of genes upregulated and downregulated in the cold water-treated group.

(B) Volcano plot of genes differentially expressed between the two groups. (C-D) GO and KEGG pathway enrichment analysis between the two groups. (E) Gene set enrichment analysis of the cold water group compared to the control group. Data are representative of three biological repeats (n = 3)

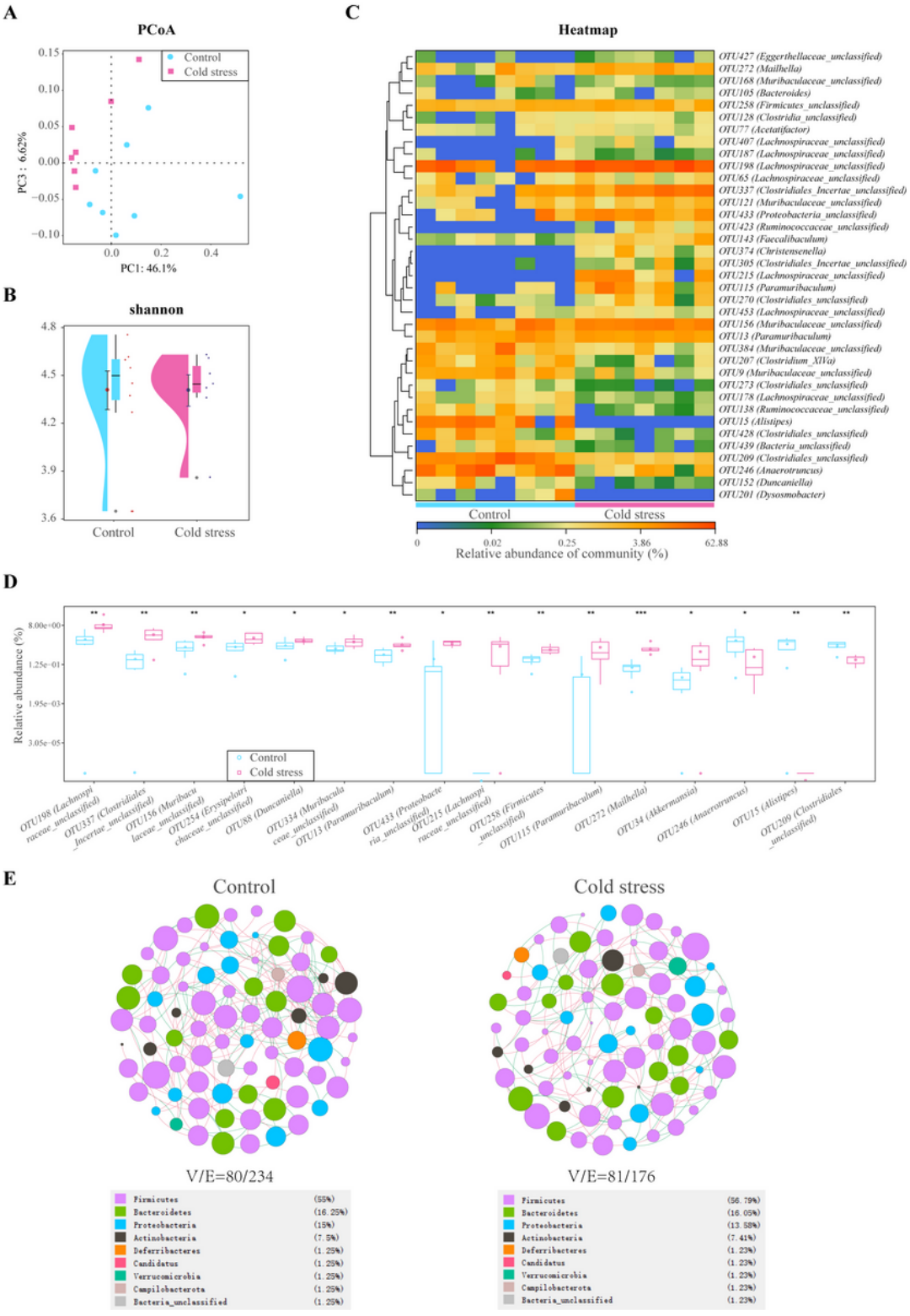


Figure 3

Cold stress leads to low bacterial diversity and a fragile ecological network in the* gut *microbiota.

(A) Principal component analysis (PCoA) plots of unweighted UniFrac distances between the two groups. (B) Shannon diversity scores. (C) Heatmap of key operational taxonomic units (OTUs). (D) Gut bacteria with different abundances at the OTU level. (E) Network analysis at the genus level. V: number of nodes. E: number of edges. (n = 8 in control group and n = 7 in cold water group)

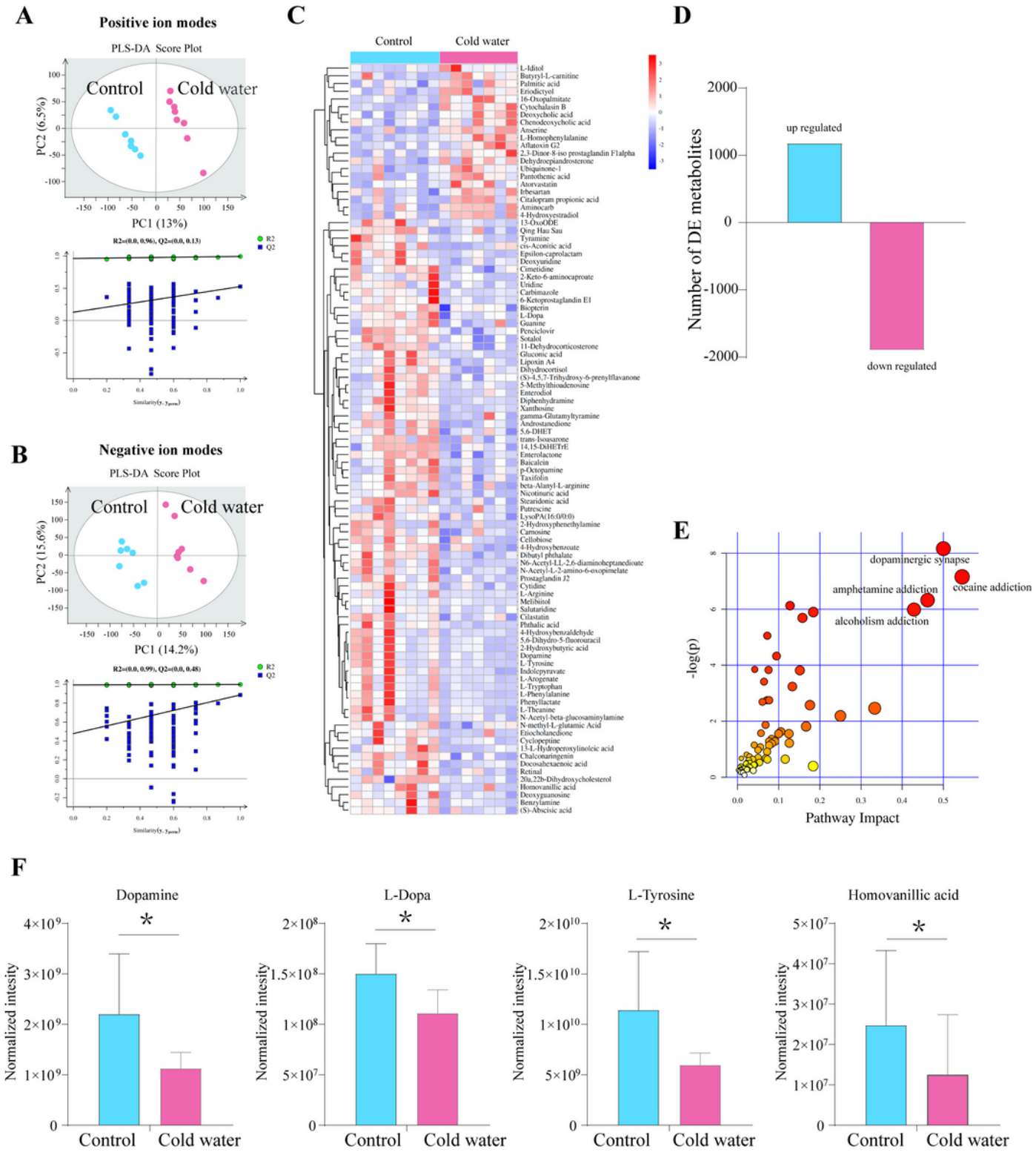
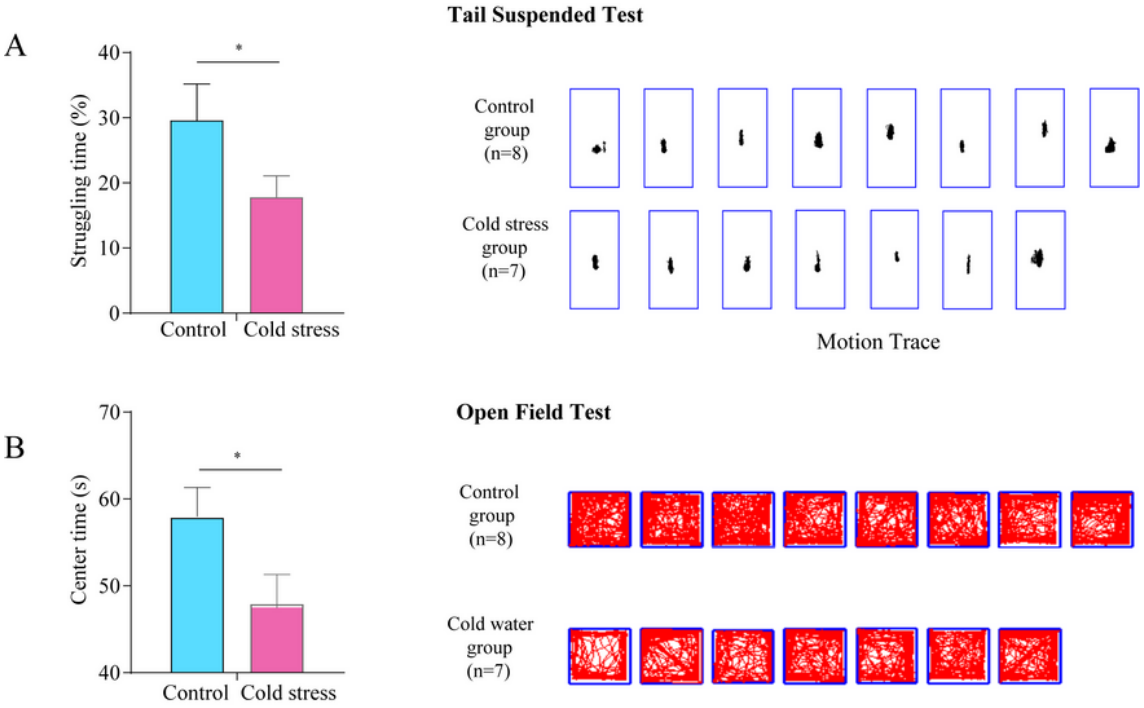


Figure 4

Metabolites in the dopamine-related pathway are downregulated by* cold *stress treatment.

(A) Partial least-squares discriminant analysis (PLS-DA) scores of all peak features in positive-ion mode. (B) PLS-DA scores of all peak features in negative-ion mode. (C) Heatmap of the metabolites that altered in the cold water group. (D) Number of differentially expressed (DE) metabolites between the two groups. (E) Pathways impacted. (F) Metabolites in dopamine-related pathways (dopamine, l-dopamine, l-tyrosine, and homovanillic acid). *P < 0.05 (n = 8 in control group and n = 7 in cold water group)



Motion Trace

Figure 5

Cold stress increases anxiety- and depression-like behaviors.

(A) Struggling time (left) and motion tracking (right) in the tail suspended test. (B) Center time and motion tracking in the open field test. *P < 0.05. (n = 8 in control group and n = 7 in cold water group)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterials.docx