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Sex-specific effects of Semen Cuscutae aqueous extract on behavior, proteomics, and gut microbiota in rats

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Abstract

Background: Sex-based differences often influence the therapeutic efficacy and safety of medications. Semen Cuscutae is a traditional tonic botanical drug with sex-specific characteristics, traditionally indicated for conditions such as impotence (exclusive to males) and restless fetus (exclusive to pregnant females). However, most existing studies have focused on a single sex.

Objective: To evaluate the sex-specific biological effects of Semen Cuscutae in rats and explore its molecular mechanisms, with the aim of uncovering its pharmacological characteristics through a multiomics approach.

Methods: A traditional aqueous extract of Semen Cuscutae (SCA) was used as the experimental material. Forty adult Sprague-Dawley rats (equal numbers of males and females) were randomly divided into 4 groups: male control, male SCA treatment (240 mg/kg), female control, and female SCA treatment (240 mg/kg), with 10 rats in each group. The biological effects were comprehensively evaluated using a combination of open field test, biochemical analyses, proteomics, and gut microbiota profiling.

Results: As a tonic botanical drug, SCA appeared to directly affect the mental and behavioral state of rats. It significantly altered the time spent by rats in the center area during the open field test, showing a sex-dependent reversal of behaviors. Proteomic analysis of brain tissue identified 624 differentially expressed proteins across the groups, with 10 key differentially expressed proteins related to sex differences, including fibroblast growth factor receptor 3, transcription elongation factor A protein-like 1, 40S ribosomal protein S25, neural cell adhesion molecule, and anion exchange protein 2 (SLC4A2). Enrichment analysis revealed that in male rats, SCA upregulated proteins involved in biological processes such as ribosome function and energy derivation, supporting protein synthesis and enhancing energy supply, showing an overall gain effect. In contrast, in female rats, SCA downregulated proteins associated with processes such as positive regulation of target of rapamycin (TOR) signaling and vesicle transport, suggesting suppression of neuronal signaling and material transport, indicative of a shift toward a more restrained physiological state. Furthermore, SCA reduced gut microbiota diversity in female rats but increased it in males, including the abundance of *Akkermansia*, which may serve as a crucial mediator.

Conclusion: Overall, the biological effects of SCA differ significantly between male and female rats, with evidence suggesting greater health benefits in males. These findings help elucidate the scientific basis of its traditional applications and provide guidance for the precise application of SCA as a functional health food.

Keywords: Semen Cuscutae; Proteome; Sex-based differences; Gut microbiota

1. Introduction

Precision medicine represents an irreversible trend for the future, characterized by the targeted application of the most appropriate therapies to diverse populations, varying disease states, and distinct stages of disease progression.[1] Sexual dimorphism in animals is a complex and widespread phenomenon.[2] The manifestation of the same disease can differ between sexes.[3,4] Furthermore, the efficacy and safety of medications may also vary between females and males, necessitating sex-specific therapeutic strategies.[5] However, considering the influence of the female reproductive cycle, most pharmacodynamic studies have been conducted primarily on male animals. This practice often leads to biased conclusions, as the potential impact on females is not adequately evaluated.[6]

Traditional Chinese medicine (TCM) has long embraced the principles of precision and individualized care, with the functions and contraindications of many Chinese medicines varying according to sex. Semen Cuscutae (also known as Tusizi) is a representative example. It refers to the dried, mature seeds of *Cuscuta australis* R. Br. or *C. chinensis* Lam., both belonging to the Convolvulaceae family.[7] Semen Cuscutae is commonly used to treat conditions such as impotence, nocturnal emissions, frequent urination, restlessness during pregnancy, and spleen-kidney deficiency with diarrhea. Traditionally, its indications include impotence (exclusive to males) and restless fetus (exclusive to pregnant females). Studies have demonstrated that Semen Cuscutae has a clear promotive

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effect on male reproductive function[8,9] and offers protective benefits for fertility and bone health in females[10,11]. Although previous studies suggest sex-specific efficacy, direct comparative pharmacological assessments are lacking. Such comparisons are essential for clarifying its pharmacological properties and safety.

Therefore, this study selected adult rats of both sexes and administered a traditional aqueous extract of Semen Cuscutae (SCA) orally for 28 days. Since the TCM concept of "tonifying the liver and kidney" is related to the brain function and reproduction, we conducted the open field test (OFT) to evaluate the effects of SCA on the mental and behavioral states of male and female rats. Biochemical and hematological indicators were then detected to evaluate their basic physiological status. Based on the observed sex-specific effects, the study further analyzed changes in brain proteomics and gut microbiota, identified differentially expressed proteins (DEPs), and performed enrichment analysis to reveal the distinct biological effect profiles of SCA in males and females. These findings aim to elucidate the scientific basis of its traditional applications and to guide the precise application of SCA in health products.

2. Materials and methods

2.1. Preparation of SCA and reagents

The Semen Cuscutae used in this study was collected from Pingluo County, Ningxia Hui Autonomous Region, China. It was identified by Lanping Guo, Research Fellow, as the dried mature seeds of *C. australis*, a plant from the Convolvulaceae family (Fig. 1A). The test substance was a traditional aqueous decoction of Semen Cuscutae. Specifically, 1 kg of raw Semen Cuscutae was boiled twice in 8 L of pure water at 100 °C for 40 minutes each time. The decoction was filtered to collect the extract, which was then concentrated and freeze-dried. The resulting powder was thoroughly ground and mixed to obtain the SCA powder. The yield of SCA was approximately 10%, meaning that 1 g of extract is equivalent to 10 g of the original medicinal material. Liquid chromatography-mass spectrometry (LC-MS) chemical composition analysis of SCA is presented in the Supplementary materials, https://links.lww.com/STCM/A66. The extracts used in this study were confirmed to be stable and reproducible.

The following reagents and materials were used in this study: Environmentally friendly dewaxing liquid (Servicebio, CAT: G1128), tissue fixation fluid (Servicebio, CAT: G1101), hematoxylin-eosin high-definition constant staining kit (Servicebio, CAT: G1076), xylene (Sinopharm Group, CAT: 10023418), FastPure Stool DNA isolation kit (MJYH, CAT: T10-100), FastPfu polymerase (TransGen, CAT: AS221-02), NEXTFLEX rapid DNA-Seq kit (Bioo Scientific, CAT: NOVA-5144), NextSeq 1000/2000 P2 XLEAP-SBS reagent kit (Illumina, CAT: 20100984), ethylene diamine tetraacetic acid (Sinopharm Group, CAT: 10009617), xylene brilliant cyanin G (Sinopharm Group, CAT: C274306-10g), tetraethylammonium bromide (Sigma, CAT: T7408-500ML), trypsin (Promega, CAT:

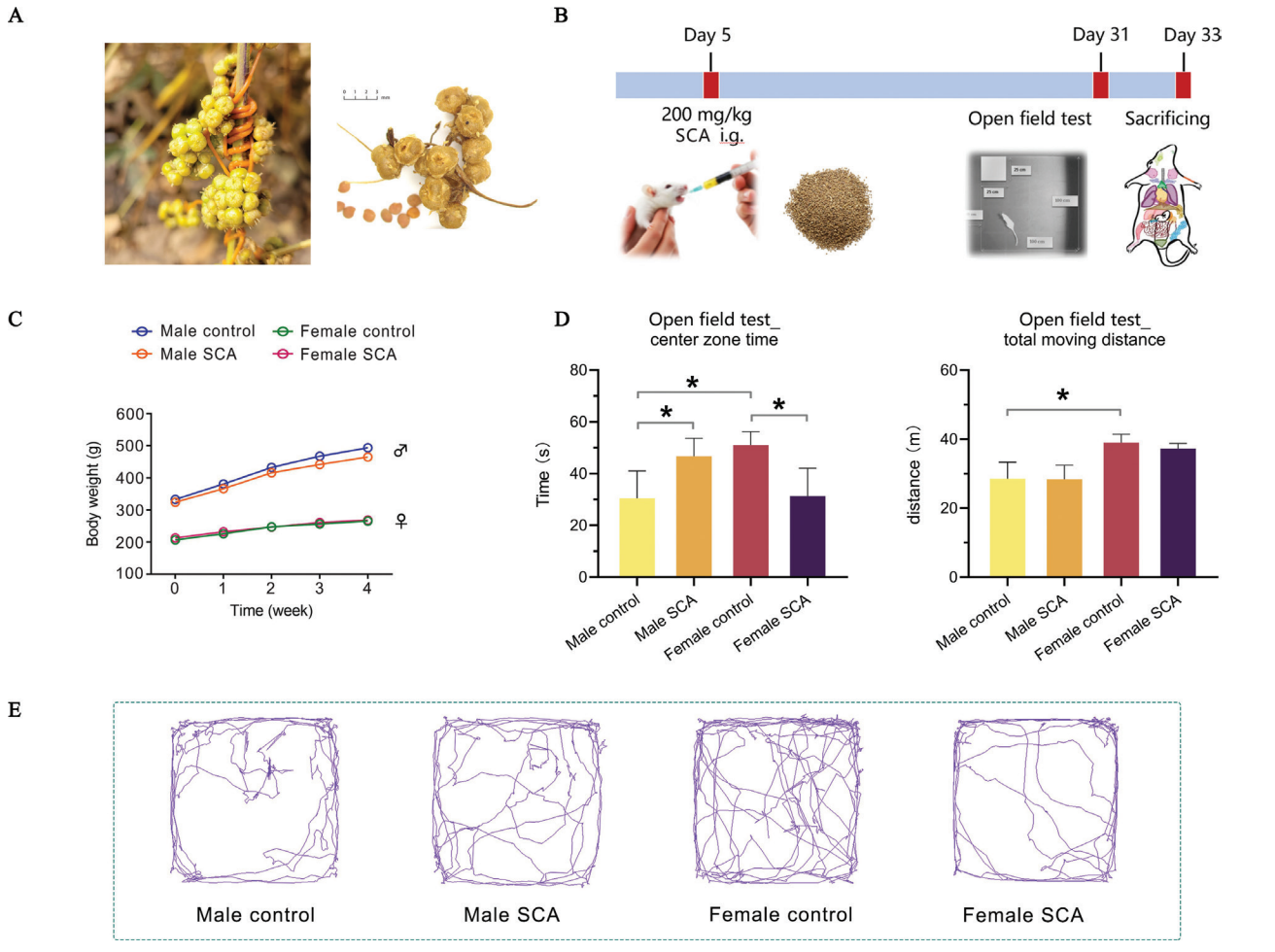

Figure 1. Experimental design, general condition of rats, and open field test. (A) Semen Cuscutae and its origin plant. (B) SCA administration and animal sampling schedule. (C) Body weight of rats. (D) Results of open field test. (E) Track map of rats in the open field test. *P < 0.05, mean ± SD, n = 8. SD, standard deviation.

V5280), marker proteins (Fermentas, CAT: 20352ES76), and BCA protein quantification kit (Beyotime, CAT: P0009).

2.2. Animal experiment design

Forty adult Sprague-Dawley rats, with equal numbers of males and females, were randomly divided into 4 groups: male control (MC), male SCA treatment (MS), female control (FC), and female SCA treatment (FS), with 10 rats in each group. The animals were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., with license No. SYXK(Jing)2023-0011. All animal procedures were approved by the Animal Care and Protection Committee of Beijing University of Chinese Medicine (Ethical Approval No. BUCM-2024062601-2210). After acclimation for 4 days, control groups received gavage with pure water, while experimental groups were administered SCA dissolved in water at 240 mg/kg (Fig. 1B). The administration volume was standardized to 10 mL/kg and was given daily at 10:00 AM. This dose corresponds to twice the clinical dosage of Semen Cuscutae. Treatment lasted 28 days, with weekly body weight measurements. Behavioral testing using the OFT was conducted 2 days before the end of the study. One day before termination, 500 mg of feces was collected, immediately frozen, and stored for gut microbiota analysis. On the final day, rats were fasted for 16 hours (water withheld), anesthetized with pentobarbital sodium, and blood was collected via the abdominal aorta. After cardiac perfusion, brains were harvested and stored at –80 °C for further analyses.

2.3. OFT

The assessment aimed to evaluate locomotor activity and anxiety-related behaviors.[12] Conducted in a softly illuminated room (75 lx) over 6 minutes, the observation arena measured 100 cm × 100 cm × 50 cm (length × width × height). An overhead camera recorded the rats' activity, which was analyzed using EthoVision XT 11.5 software (Noldus Information Technology BV, Wageningen, the Netherlands). The arena was divided into 2 zones: an outer zone, 20 cm wide, and a central zone measuring 60 cm × 60 cm. Key behaviors analyzed included total distance traveled, distance covered in each zone (outer and central), frequency of entries into each zone, and latency to enter the central zone.[13]

2.4. Hematology, clinical chemistry, and histopathology analysis

Blood samples were collected from the abdominal aorta under pentobarbital sodium anesthesia. Hematological assessments were conducted following standard procedures.[14] For biochemical analyses, 0.5 mL of blood was collected in nonheparinized Eppendorf tubes, and serum was separated by centrifugation at 3000 r/min for 10 minutes. Various biochemical parameters were then measured as described in the procedural guidelines.[14] Hematoxylin-eosin staining and histopathological analysis were performed on the liver, kidney, and brain tissues from all 4 groups of rats.

2.5. Gut microbiota analysis

Genomic DNA from microbial communities was extracted from 5 randomly chosen fecal samples per group using the E.Z.N.A. DNA Kit (Omega Bio-Tek, Norcross, Georgia, USA). The extracted DNA was assessed by agarose gel electrophoresis, and its concentration was measured using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA). The hypervariable V3–V4 region of the bacterial 16S rRNA gene was amplified for sequencing. Subsequent sequencing and data analysis were carried out according to the protocol described by Zhao et al.[15]

2.6. Astral-data-independent acquisition (DIA) proteomic analysis

2.6.1. Sample processing

Samples were first retrieved from the –80 °C freezer and ground into powder using liquid nitrogen. An appropriate amount of the powdered tissue was transferred to 1.5 mL centrifuge tubes, followed by the addition of lysis buffer containing 8 mol/L urea, 1 mmol/L PMSF, and 2 mmol/L ethylenediaminetetraacetic acid. Ultrasonic lysis was performed for 5 minutes on ice, after which the samples were centrifuged at 15,000 × *g* at 4 °C for 10 minutes to collect the supernatant. Protein concentration was then determined using a BCA assay kit. Proteolytic digestion and desalting procedures were carried out subsequently.

2.6.2. LC-MS/MS analysis, database retrieval, and quantification

LC was conducted using a nanoElute UHPLC system (Bruker Daltonics, Germany). Approximately 200 ng of peptides were separated over 20 minutes at a flow rate of 0.5 µL/min on a commercial reverse-phase C_{18} column equipped with a CaptiveSpray Emitter (15 cm × 75 µm, 1.6 µm, Aurora Series with CSI, IonOpticks, Australia). The column oven, integrated with a Toaster module, maintained a stable temperature of 50 °C. Mobile phases A and B were composed of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. The LC system was coupled online to a hybrid timsTOF Pro2 mass spectrometer (Bruker Daltonics, Germany) via a CaptiveSpray nano-electrospray ion source. To determine the optimal acquisition windows for dia-parallel accumulation–serial fragmentation (PASEF) mode, the instrument was operated in data-dependent PASEF mode, incorporating 4 PASEF MS/MS frames per cycle. A capillary voltage of 1500 V was applied, and mass spectra for both MS and MS/MS were acquired over the *m/z* range of 100 to 1700. An ion mobility range ($1/K_0$) of 0.85 to 1.3 Vs/cm^2 was utilized. A target value of 10,000 was maintained, and the intensity threshold was set at 1500. Charge states from 0 to 5 were accepted. Collision energy was ramped based on ion mobility, ranging from 45 eV at $1/K_0$ = 1.3 Vs/cm^2 to 27 eV at $1/K_0$ = 0.85 Vs/cm^2. Quadrupole isolation width was set to 2 *m/z* for *m/z* values < 700 and 3 *m/z* for values > 800.

Raw MS data were processed using the library-free approach of DIA-NN (v1.8.1) (Kathryn Lilley Lab, Cambridge, United Kingdom). A comprehensive protein database, uniprotkb_proteome_UP000002494_rat_47930_20240528.fasta, containing 47,930 sequences, was utilized to generate a spectral library employing deep learning algorithms based on neural networks. To improve analysis accuracy, the match between runs feature was enabled, allowing the spectral library to be built from data-independent acquisition (DIA) data and subsequently refined through iterative analysis. The false discovery rate was strictly controlled to remain below 1% at both the protein and precursor ion levels. The resulting identifications were retained for further quantitative analysis.[16,17]

2.6.3. Bioinformatics analysis

Protein quantification data were preprocessed by removing missing values and normalizing the dataset. Statistical analysis was performed using a *t* test. DEPs were identified based on the criteria of a false discovery rate (adjusted *P* value) < 0.05 and a fold change > 1.2 or < 0.83.[18] A heatmap was generated to illustrate the distribution of DEPs across different groups. Protein names and identification numbers were verified and converted using the UniProt database. Subsequently, Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were carried out

on the DEPs to investigate the signaling pathways modulated by SCA. The results were visualized using the Bioinformatics online data analysis platform and the Metware Cloud (Metware Biotechnology Co., Ltd., Wuhan, China) visualization service.[19]

2.7. Statistical analysis

Experimental results are expressed as mean ± standard deviation. Unless otherwise specified, one-way analysis of variance followed by Tukey's post hoc test was applied for group comparisons, using GraphPad Prism 8.02 software (GraphPad Software, Inc., San Diego, California, USA). For comparisons between 2 groups, Student's *t* test was applied. A *P* value of less than 0.05 was considered statistically significant. In the figures, significance is denoted as $P < 0.05$ and $P < 0.01$, respectively[15]. Spearman correlation analysis and visualization were performed using the Metware Cloud platform.

3. Results

3.1. General condition and behavioral assessment

During the experiment, no abnormalities were observed in the mental state, activity, defecation, or sleep patterns of rats across the 4 groups. Body weight increased steadily in all groups (Fig. 1C). The OFT revealed that rats in the FC group exhibited a significantly greater total movement distance and spent more time in the center area compared with the MC group ($P < 0.05$) (Fig. 1D, 1E), consistent with previous reports.[13] Administration of SCA did not affect the total movement distance in either male or female rats, indicating that overall activity levels remained unchanged. Notably, rats in the MS group spent significantly more time in the center area compared with the MC group ($P < 0.05$), whereas female rats in the FS group spent significantly less time in the center area than their controls (Fig. 1D, 1E). These results suggest that SCA affected the rats' mental state in a sex-specific manner, with a clear trend of behavioral reversal between males and females.

3.2. Hematology, clinical chemistry, and histopathology analysis

The results of the hematological analysis are presented in Supplemental Table S1, https://links.lww.com/STCM/A66. In the MS group, the percentage of neutrophils (NEU%) was significantly decreased compared with the MC group ($P < 0.05$), while the percentage of lymphocytes (LYM%) was significantly increased ($P < 0.05$). No other significant abnormalities were observed across the groups.

In female rats, administration of SCA led to significant increases in serum levels of glucose (GLU), urea (UREA), alkaline phosphatase (ALP), phosphorus (P), and potassium (K) compared with the control group ($P < 0.05$) (Supplemental Table S2, https://links.lww.com/STCM/A66). In contrast, male rats exhibited only a significant decrease in UREA levels ($P < 0.05$) after SCA administration. These findings suggest that the biological effects of SCA differ notably between sexes. Histopathological examination revealed that the tissue architecture of the liver, kidney, and brain remained intact and normal across all groups, with no evident pathological lesions observed (Fig. 2).

3.3. Proteomics analysis and DEPs

Protein identification and quantification were performed on the LC-MS data using DIA-NN (v1.8.1) software, resulting in the identification of 68,764 peptides and 7802 proteins across 20 samples. Most peptides ranged from 7 to 20 amino acids in length, consistent with typical patterns expected from enzymatic digestion and MS fragmentation. The peptide length distribution met quality control standards (Fig. 3A). Box plots and violin plots illustrated the dispersion of expression levels (abundance values) and demonstrated good intragroup consistency among biological replicates (Fig. 3B). Principal component analysis showed that sample points clustered closely within each group, while the 4 groups were distinctly separated, confirming the validity of the grouping (Fig. 3C). Notably, the MC, FC, and FS groups clustered relatively near each other, whereas

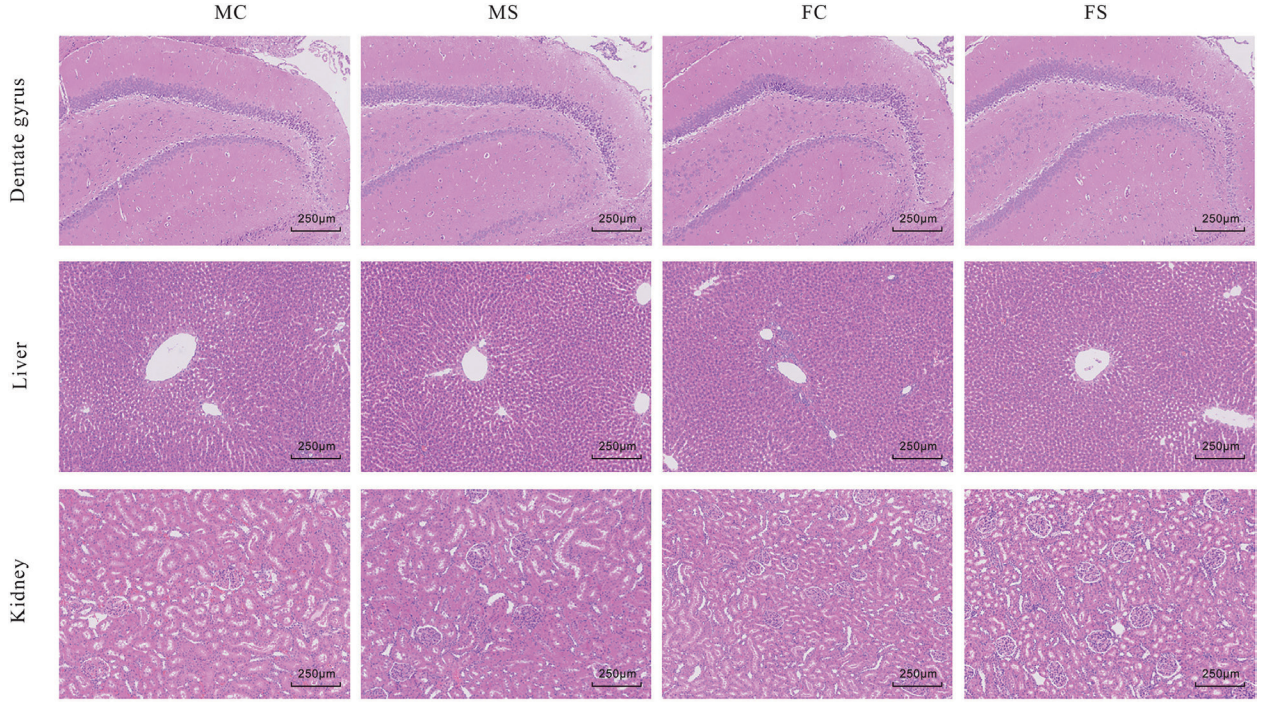

Figure 2. Effect of SCA on the histopathology of the liver, kidney, and brain (hippocampal dentate gyrus) in rats.

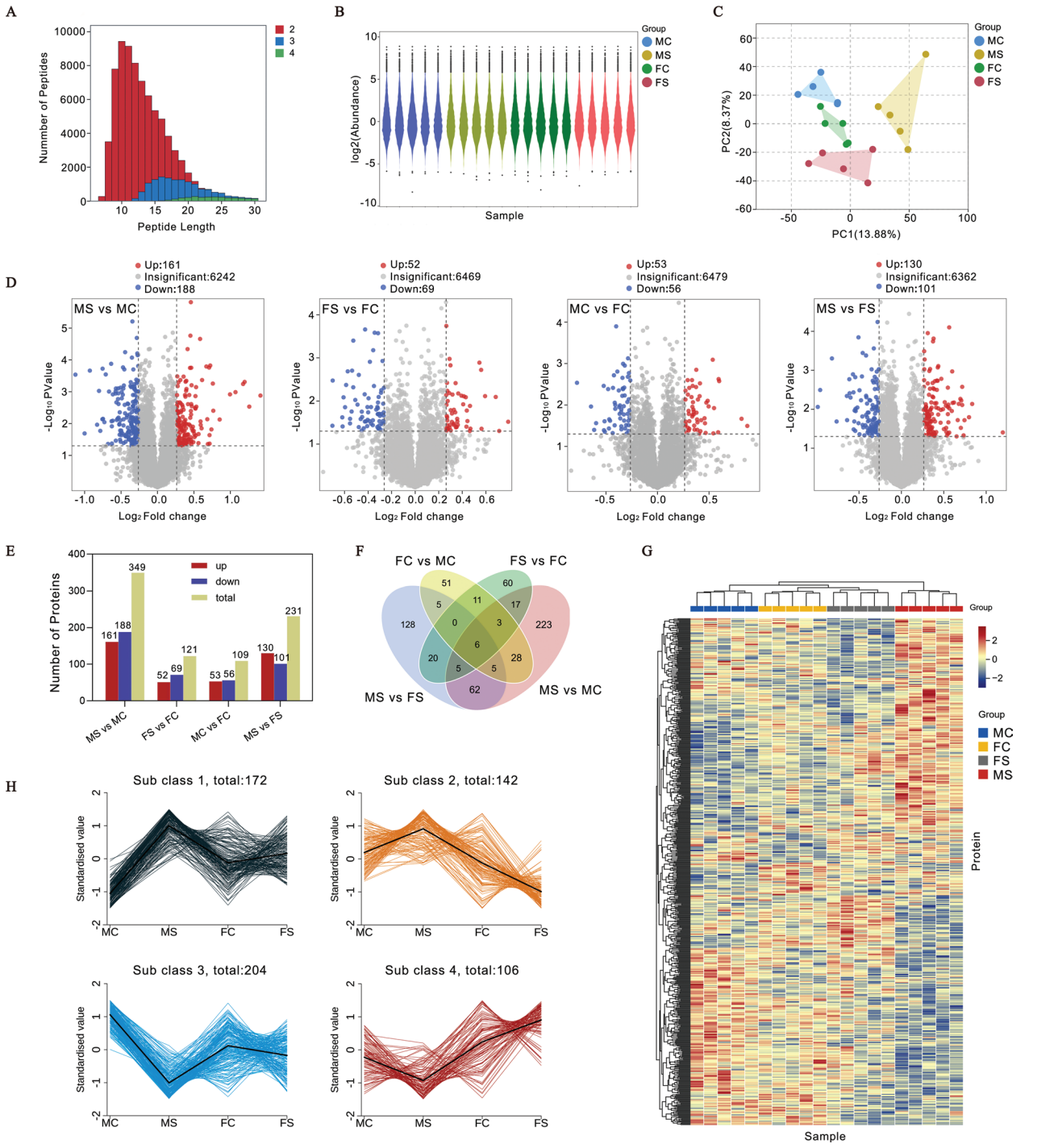

Figure 3. Effects of SCA on brain proteome in male and female rats. (A) Peptides distribution plot. (B) Distribution of protein abundance values across samples. (C) PCA score plot of protein quantification. (D) Volcano plot of DEPs. (E) Summary bar plot of DEPs. (F) Venn diagram of DEPs. (G) K-means clustering analysis of DEPs. (H) Heatmap of DEPs. *n* = 5. PCA, principal component analysis.

the MS group was positioned farther apart, indicating a distinct protein expression profile in male rats treated with SCA.

The volcano plot combines statistical significance (*P* value) and fold change to visually represent protein expression differences and their significance. In this study, DEPs were identified using criteria of $P < 0.05$ and fold change >1.2 or fold change <0.83. Compared with the FC group, 53 proteins were significantly upregulated and 56 were downregulated in the MC group (Fig. 3D). After SCA administration, compared with the MC group, the MS group exhibited 161 upregulated and 188 downregulated proteins. In the FS versus FC comparison, 52 proteins were upregulated and 69 were downregulated (Fig. 3E, 3F). These results suggest that SCA administration differentially affects brain protein expression in male and female rats. A total of 624 DEPs are presented in a clustered heatmap, where red represents significant upregulation and blue represents significant downregulation (Fig. 3G). K-means clustering further categorized these DEPs into 4 major groups, each

reflecting distinct expression patterns (Fig. 3H). When more stringent criteria were applied ($P < 0.01$ and fold change >1.5 or fold change < 0.83), the number of DEPs decreased: MC versus FC showed 3 DEPs (2 upregulated, 1 downregulated), MS versus MC showed 28 DEPs (15 upregulated, 13 downregulated), and FS versus FC showed 6 DEPs (2 upregulated, 4 downregulated). Notably, the male group exhibited a substantially higher number of DEPs after SCA treatment compared with the female group (28 vs. 6).

Interestingly, some DEPs appeared across multiple group comparisons. For example, neural cell adhesion molecule (NRCAM) showed significant differential expression in MC versus FC, MS versus MC, FS versus FC, and MS versus FS comparisons (Fig. 4). Based on these observations, 4 common expression patterns were identified (Supplemental Table S3, https://links.lww.com/STCM/A66). A total of 5 proteins (HSPB8, MRPL23, ARL6IP5, NEU1, and TMEM41B) were significantly upregulated after SCA administration in both the MS and FS groups. In contrast, 16 proteins (TMEM25, SLC7A10, SCAP, PPP1R8, NEDD8, MTPN, etc.) were significantly downregulated in both sexes. Additionally, several proteins exhibited opposite expression trends between males and females after SCA treatment. For instance, ribosomal protein S25 (RPS25), fibroblast growth factor receptor 3 (FGFR3), and transcription elongation factor A protein-like 1 (TCEAL1) were upregulated in males but downregulated in females (Supplemental Table S3, https://links.lww.com/STCM/A66). On the contrary, NRCAM, serine/threonine-protein kinase (MAK), anion exchange protein 2 (SLC4A2), glutamate pyruvate transaminase 2 (GPT2), stomatin (STOM), mitochondrial ribonuclease P catalytic subunit (PRORP), and protein associated with ABC transporters (PAAT) were downregulated in males but upregulated in females (Fig. 4). These findings suggest that the distinct biological effects of SCA in male and female rats may be mediated by these sex-specific DEPs.

3.4. Enrichment analysis based on DEPs

To elucidate the biological significance of the DEPs, functional enrichment analyses were performed using the KEGG and GO online databases. KEGG enrichment analysis identified signaling pathways, biological processes (BPs), diseases, and metabolic pathways associated with the DEPs (Fig. 5A, 5C). Compared with the MC group, the MS group showed enrichment in pathways related to Alzheimer disease, neurodegeneration (multiple diseases), thermogenesis, ribosome function, and the PI3K-Akt signaling pathway. Within the ribosome pathway, 8 proteins were upregulated and 2 were downregulated. Conversely, in the Alzheimer disease pathway, 5 proteins were upregulated, while 10 were downregulated, suggesting that SCA may inhibit these BPs and disease pathways (Fig. 5B). In contrast, the female groups showed a distinct enrichment pattern, including pathways such as peroxisome, folate biosynthesis, PPAR signaling, and fatty acid metabolism. Notably, in the folate biosynthesis pathway, 4 proteins were downregulated with no upregulated proteins detected (Fig. 5D). These findings suggest that SCA

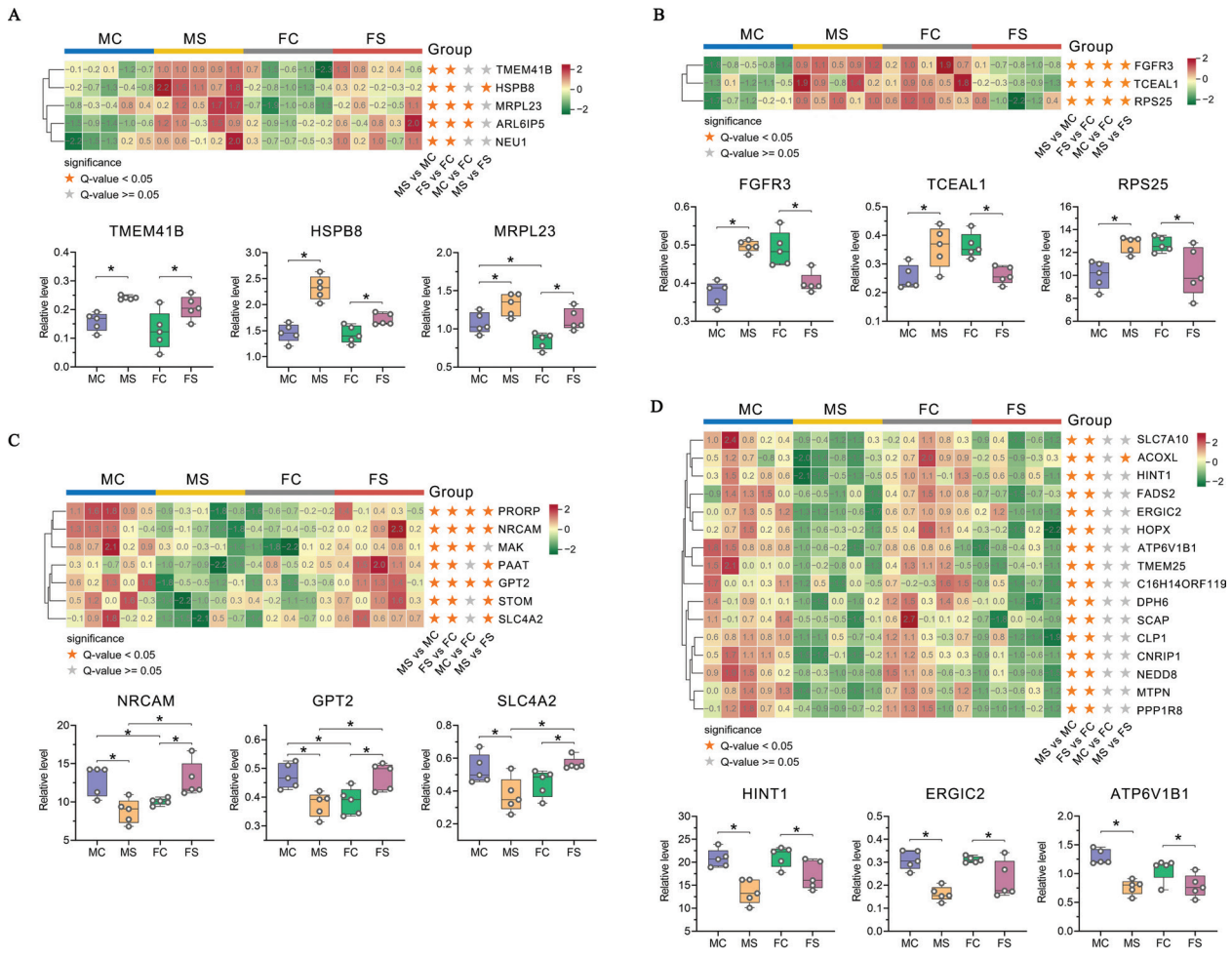

Figure 4. Sex-associated DEPs. (A) DEPs upregulated in both male and female rats. (B) DEPs upregulated in males but downregulated in females. (C) DEPs downregulated in males but upregulated in females. (D) DEPs downregulated in both male and female rats. *$P < 0.05$, mean ± SD, $n = 5$. SD, standard deviation.

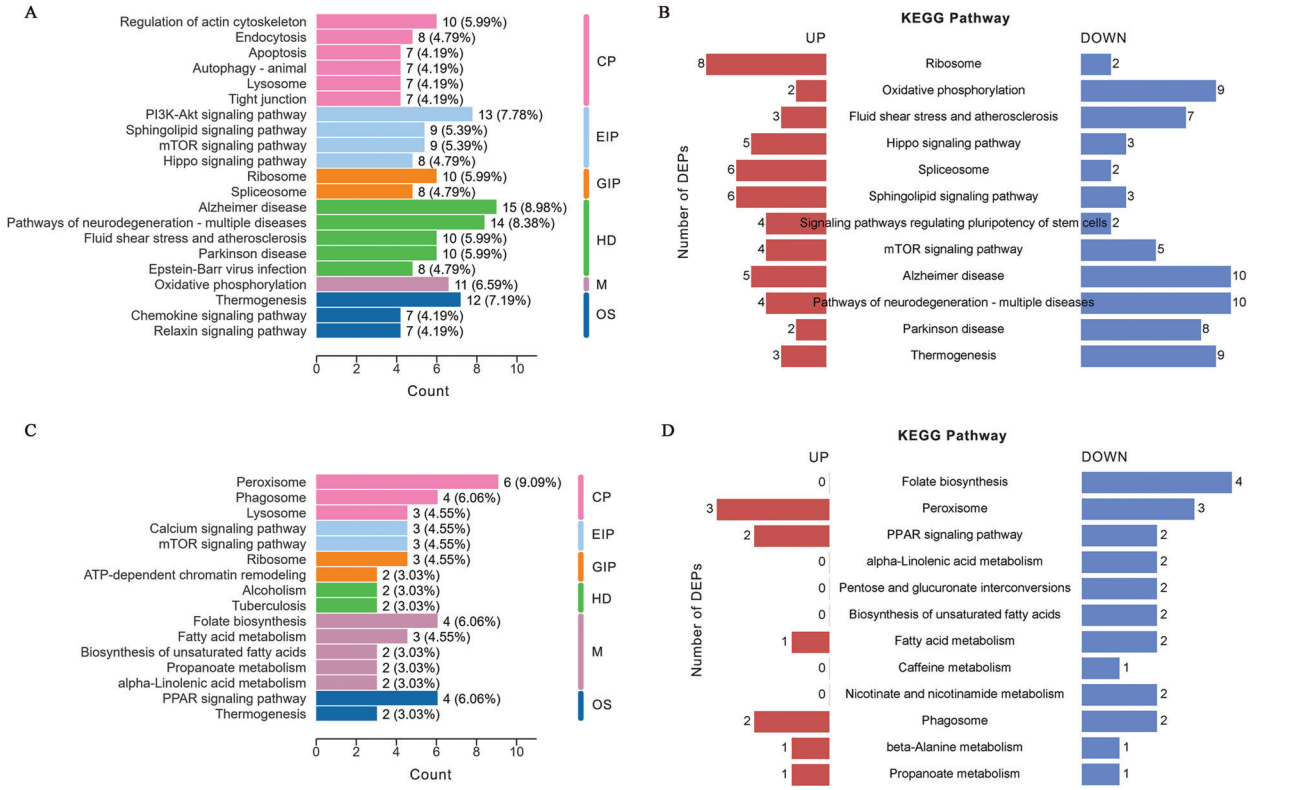

Figure 5. KEGG enrichment analysis based on DEPs. (A) Enrichment pathway in MS versus MC. (B) Distribution of upregulated and downregulated DEPs in MS versus MC. (C) Enrichment pathway in FS versus FC. (D) Distribution of upregulated and downregulated DEPs in FS versus FC. CP, cellular processes; EIP, environmental information processing; GIP, genetic information processing; HD, human diseases; M, metabolism; OS, organismal systems.

administration primarily exerts inhibitory effects on enriched pathways in female rats.

The GO database characterizes the biological significance of proteins across three categories: BP, cellular component (CC), and molecular function. We performed four GO enrichment analyses on upregulated and downregulated proteins from the MS versus MC and FS versus FC comparisons, selecting terms based on enrichment significance and the number of DEPs. The enriched GO terms and their associated DEPs are visualized using bubble plots and circular correlation diagrams (Fig. 6). Compared with the MC group, upregulated proteins in the MS group were enriched in BPs such as energy derivation by oxidation of organic compounds and stem cell population maintenance. Regarding CCs, these proteins were enriched in the ribosome, mitochondrial protein complex, and neuron projection terminus. For molecular function, significant enrichment was observed in structural constituents of the ribosome and mRNA binding, with the ribosome term exhibiting the highest number of DEPs (Fig. 6A). Conversely, downregulated proteins in the MS group were associated with processes including autophagic cell death and pH regulation, as well as components like the vacuolar membrane and Golgi-associated vesicles, along with functions such as phosphatase inhibitor activity, indicating a distinct biological impact pattern (Fig. 6B).

In female rats, compared with the FC group, upregulated proteins in the FS group were enriched in GO terms, including negative regulation of interleukin-17 production, anion transmembrane transport, organellar large ribosomal subunit, and postsynaptic density. Conversely, downregulated proteins in the FS group were enriched in BPs and CCs, such as positive regulation of target of rapamycin (TOR) signaling, alcohol metabolic process, transport vesicle, lysosomal membrane, and FAD binding (Fig. 6C, 6D).

3.5. Gut microbiota analysis

A total of 3,329,767 sequences were obtained through 16S rRNA sequencing, with an average sequence length of 419 base pairs. By applying a 97% similarity threshold to nonredundant sequences, 928 operational taxonomic units were identified, among which 259 operational taxonomic units (27.9% of the total) were shared across all 4 groups (Fig. 7C). Additionally, 334 distinct intestinal bacterial species were classified into 10 phyla, 80 families, and 180 genera. Non-metric multidimensional scaling analysis illustrates that samples from the MC, MS, FC, and FS groups clustered into four distinct regions (Fig. 7D). Notably, the MC and FC groups were widely separated, indicating inherent differences in gut microbiota composition between male and female rats. After SCA administration, samples from the FS group shifted closer to the MC and MS groups, moving away from the normal female group (Fig. 7D). This pattern suggests a sex-dependent reversal in gut microbiota composition, consistent with behavioral findings from the OFT. Regarding diversity indices, the ACE index significantly increased in the MS group but decreased in the FS group. The Shannon index revealed that SCA treatment increased gut microbiota diversity in male rats but had no significant effect on female rats (Fig. 7A, 7B).

Species composition and differential analysis revealed that SCA treatment significantly altered the gut microbiota, exhibiting distinct changes between the sexes (Fig. 7E). After SCA administration, the abundance of *Bacteroides* increased in both males and females, while *Blautia* decreased in both sexes. Notably, *Phascolarctobacterium* abundance increased in males but decreased in females. Additional sex-specific changes included increases only in females of *Alistipes* and *unclassified_f_Lachnospiraceae*, and decreases only in females

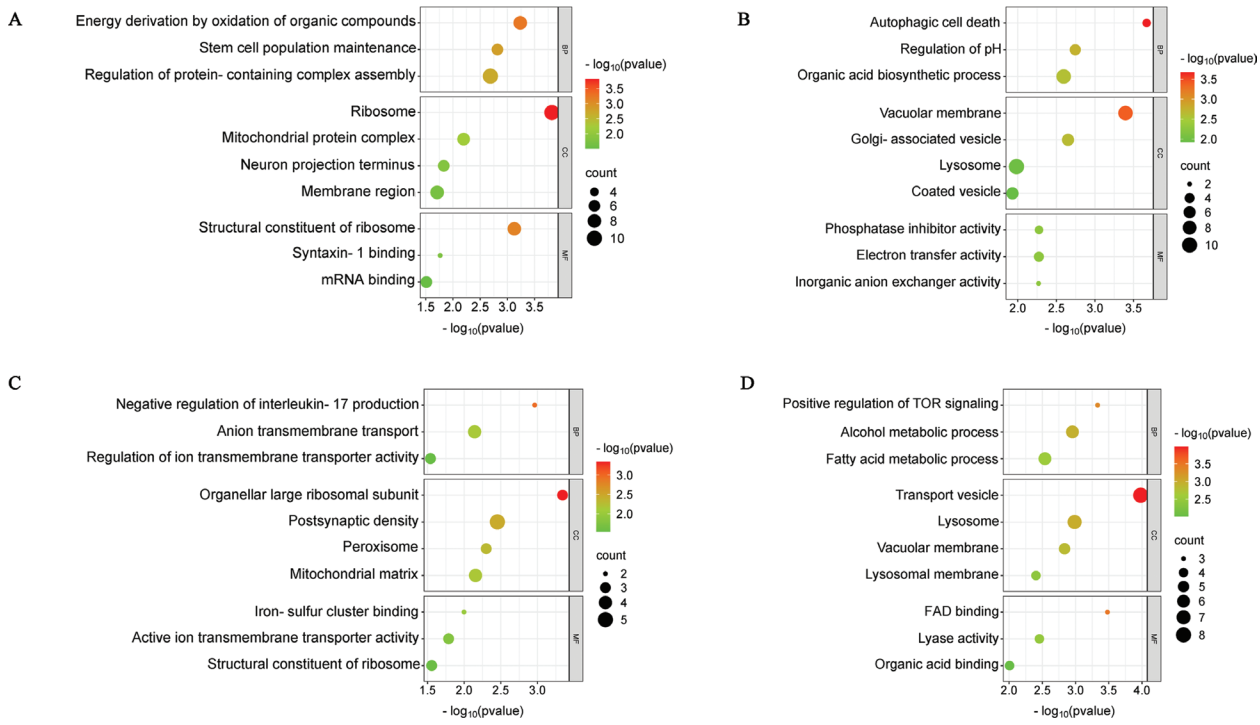

Figure 6. GO enrichment analysis based on DEPs. (A) Bubble map of upregulated DEPs in MS versus MC and corresponding relevance circle graph. (B) Bubble map of downregulated DEPs in MS versus MC and corresponding relevance circle graph. (C) Bubble map of upregulated DEPs in FS versus FC and corresponding relevance circle graph. (D) Bubble map of downregulated DEPs in FS versus FC and corresponding relevance circle graph; BP. biological process; CC. cellular component; MF. molecular function.

of *Phascolarctobacterium*. In males, *Akkermansia*, *norank_f_Muribaculaceae*, *Phascolarctobacterium*, *Prevotellaceae_UCG-001*, and *Ruminococcus* increased exclusively, whereas *Lactobacillus*, *Romboutsia*, *Streptococcus*, and *Turicibacter* decreased only in males (Fig. 7F, 7G).

3.6. Correlation analysis

Spearman correlation analysis was conducted on the expression levels of key DEPs, the relative abundances of various bacterial genera, and OFT data (Fig. 8). Notably, the OFT results showed a significant positive correlation with the relative abundances of *Akkermansia* ($\rho = 0.55$, $P < 0.05$) and *unclassified_f_Lachnospiraceae* ($\rho = 0.46$, $P < 0.05$), suggesting that SCA may influence the open field behaviors of male and female rats by modulating these two bacterial genera. Additionally, the relative abundance of *Akkermansia* was significantly negatively correlated with NRCAM protein expression ($\rho = -0.49$, $P < 0.05$), while *unclassified_f_Lachnospiraceae* was significantly negatively correlated with ERGIC2 protein levels ($\rho = -0.52$, $P < 0.05$). These findings imply that NRCAM and ERGIC2 may be targets through which the gut microbiota affects rat behavior. Furthermore, the relative abundances of *Alistipes* and *Ruminococcus* also showed significant correlations with multiple DEPs such as HSPB8, TMEM41B, and HINT1, highlighting their potentially crucial roles (Fig. 8A, 8B).

4. Discussion

As a plant seed, Semen Cuscutae contains not only primary metabolites that serve as nutritional components but also flavonoid compounds such as hyperoside, calycosin, and quercetin, as well as terpenoid compounds like australiside A.[20,21] Among these, flavonoids are widely regarded as the primary active metabolites of Semen Cuscutae and have attracted significant attention.[22] Notably, Semen Cuscutae also contains resin glycosides, commonly found in plants of the Convolvulaceae family, which exhibit broad biological activities and contribute to the high viscosity of its aqueous solutions. Consequently, the SCA forms a gel-like structure, offering good stability, biocompatibility, and adsorptive properties.[23] Existing pharmacological studies on Semen Cuscutae often focus on the reproductive system, such as its protective effects on male sperm and estrogen-like effects in females.[10,24] However, conventional disease-focused research approaches do not fully capture its comparative effects across sexes. Therefore, this study was designed to investigate the sex-specific effects of Semen Cuscutae. Omics technologies, with their high-throughput and large-scale experimental capabilities, offer advantages in sensitivity and comprehensiveness.[25] Compared with traditional DIA techniques, 4D-DIA Astral quantitative proteomics significantly enhances proteomic analysis in terms of depth, sensitivity, and throughput.[26]

During the 28-day continuous gavage of SCA, both female and male rats exhibited normal growth. However, the observed abnormalities in NEU% and LYM% suggest that SCA might affect the immune response to viral challenges in male rats. Biochemical analyses revealed that female rats displayed more pronounced abnormalities in several parameters, including GLU, ALP, UREA, K, and P, after SCA administration, indicating a potential impact on glucose metabolism and renal excretory function, which may pose safety concerns. Based on these sex-specific effects, behavioral tests were employed to visually assess changes in the mental state of the rats.

The OFT assesses spontaneous activity levels, anxiety-related behavior, exploratory tendencies, and motor abilities. In the control groups, female rats typically exhibited longer movement tracks and more frequent entries into the central area compared with males, reflecting stronger exploratory behavior and lower anxiety levels.[13] Interestingly, SCA administration reversed this pattern. Without affecting the total distance traveled, male rats displayed increased boldness, while females became more

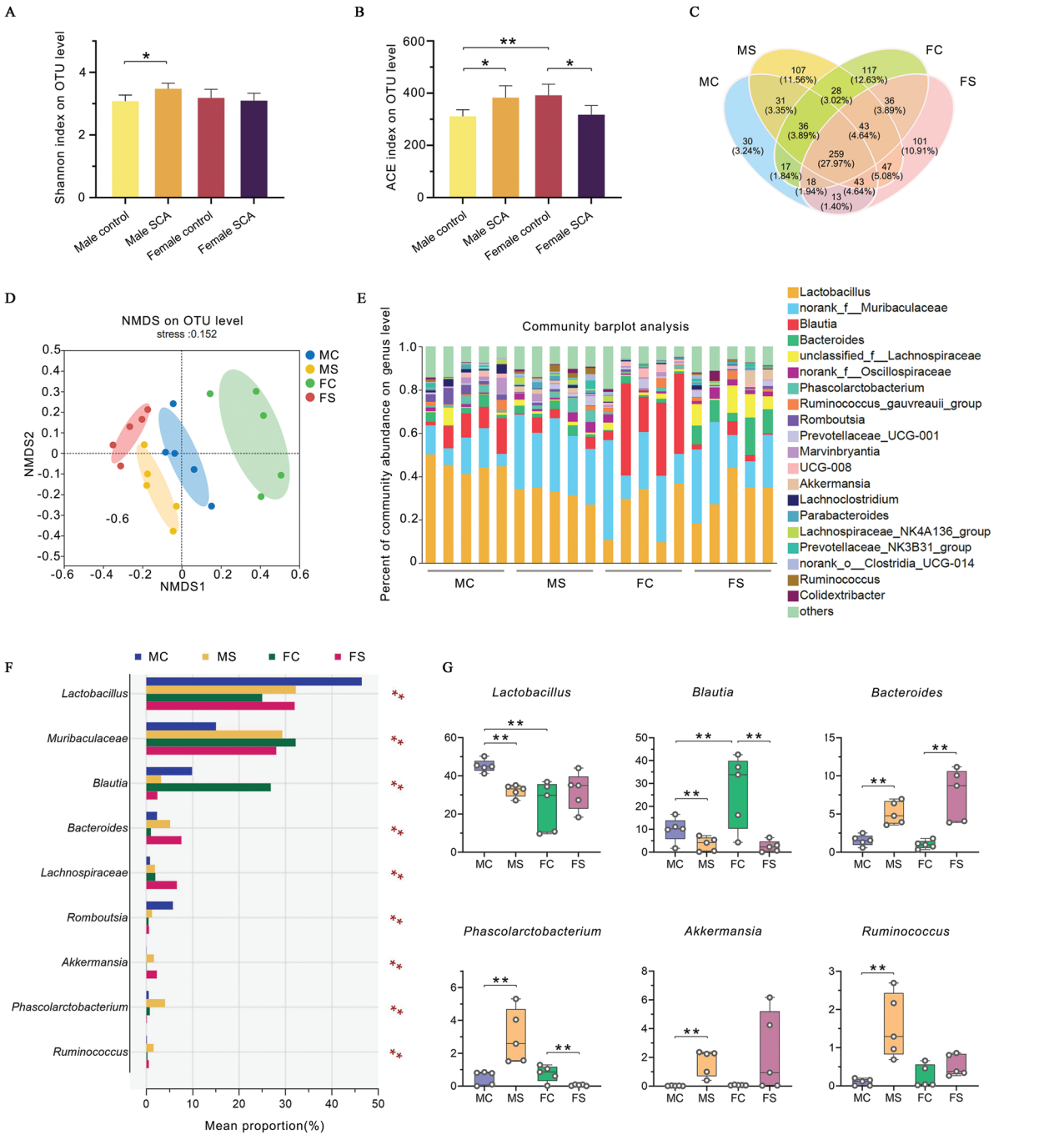

Figure 7. Effect of SCA oral administration on gut microbiota in rats. (A) Shannon index at the OUT level. (B) ACE index at the OUT level. (C) Venn diagram at the OUT level. (D) NMDS diversity analysis plot at the OUT level. (E) Gut microbiota diversity at the genus level. (F),(G) Comparison of bacterial genera between groups. *$P < 0.05$, **$P < 0.01$, mean ± SD, $n = 5$. SD = standard deviation; NMDS, Non-metric multidimensional scaling.

cautious. The normal histological structure of brain tissue suggests that these behavioral changes are not due to organic brain damage. This marked contrast further underscores the significant sex-dependent functional differences induced by SCA. Previous literature has reported that Semen Cuscutae modulates open field behavior in male depression model mice, potentially via mechanisms involving gut microbiota and neuroinflammation.[27]

The chemical constituents of botanical drugs undergo extensive gastrointestinal metabolism, with only a small fraction entering systemic circulation. The role of gut microbiota in mediating the biological effects of TCMs has gained increasing recognition.[28] Beyond regulating the intestinal microenvironment, gut microbiota can indirectly influence mental and emotional states via the gut-brain axis.[29] In this study, SCA increased gut microbiota diversity in male rats, enhancing both evenness and genus richness, while reducing the number of bacterial genera in female rats. Importantly, no increase in harmful bacteria was detected in any group. After SCA gavage, male rats exhibited a significant rise in gut microbiota diversity, including increased abundance of potentially beneficial bacteria such as *Akkermansia*.[30] Correlation analysis further suggests *Akkermansia* may be a key mediator for the effects of SCA on sex-specific mental behaviors. *Akkermansia* plays a positive role

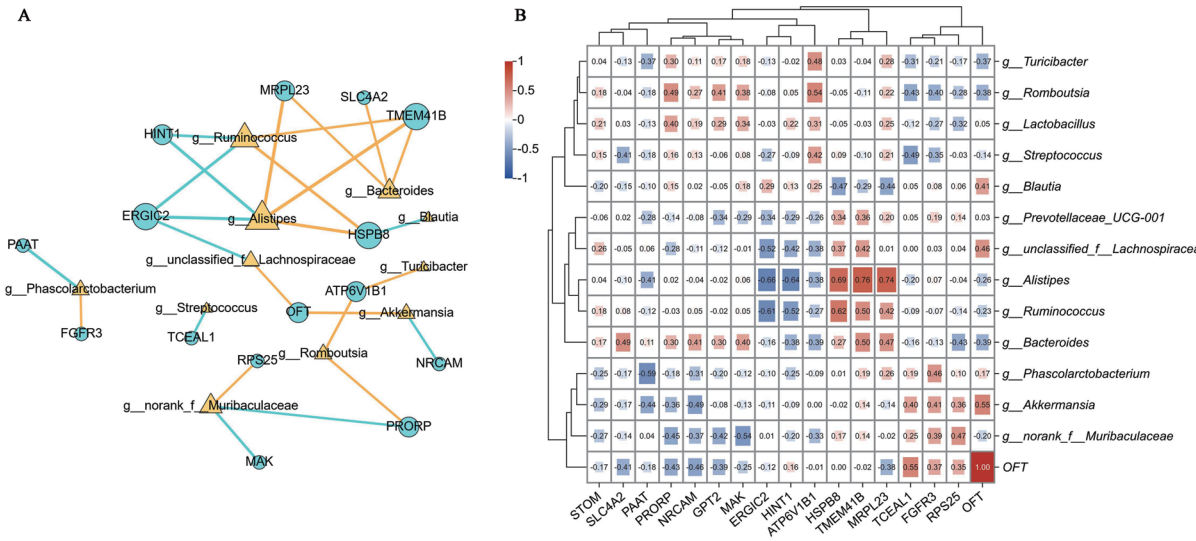

Figure 8. Correlation analysis between the microbiome and proteome. (A) Correlation network. (B) Correlation clustering heatmap.

in maintaining intestinal barrier function and regulating immunity and metabolism. Previous studies have demonstrated its neuroprotective functions through modulation of brain-derived neurotrophic factor and serotonin pathways.[31] These probiotics may affect the mental behavior through their metabolic products, short-chain fatty acids, which are recognized messengers on the gut-brain axis. Further studies will be conducted to explore these mechanisms. In contrast, the gut microbiota of female rats did not show a shift toward a healthier composition. While these results do not directly prove a causal link between behavioral changes and gut microbiota, they clearly demonstrate sex differences in the biological activity of SCA.

Behavioral differences are closely related to brain function, and in TCM, the brain is considered a key component of the concept of "kidney." Therefore, we selected brain tissue for proteomic analysis to elucidate the underlying mechanisms behind SCA's sex-specific effects. Notably, protein expression changes were more pronounced in male rats than in females, especially for proteins showing highly significant differences (28 in males vs. 6 in females). Of particular interest, some proteins exhibited completely opposite expression changes between the sexes after SCA administration. These opposing trends may reveal the scientific basis of the sex-specific effects. For example, SCA upregulated FGFR3, TCEAL1, and RPS25 in male rat brains but downregulated these proteins in females (Fig. 4B). All 3 proteins are involved in regulating gene expression at the cellular level. Specifically, FGFR3 affects transcriptional activity via signal transduction, TCEAL1 directly modulates transcription factor activity, and RPS25 indirectly regulates gene expression by controlling protein synthesis. By enhancing protein synthesis, RPS25 may contribute to neuroplasticity, synaptic remodeling, and neuronal signaling, thus impacting brain regions related to emotion. Notably, deficiency of TCEAL1 is associated with a rare neurological disorder characterized by significant neuronal damage.[32] The upregulation of these proteins in male rat brains may promote normal neurodevelopment and renewal, thereby exerting positive biological effects.

In contrast to the previously described trend, 7 proteins showed decreased expression in male rats but were increased in female rats. These proteins are primarily involved in cellular signal transduction, membrane stability, and ion transport functions. For example, NRCAM plays a key role in cell-cell adhesion, neurodevelopment, and neural plasticity. Deletion of NRCAM in astrocytes has been shown to reduce the number of inhibitory synapses without affecting excitatory synapses.[33] SLC4A2 is an anion transporter responsible for exchanging Cl^- and HCO_3^- ions. The opening of Cl^- channels can modulate action potentials and thus inhibit nerve conduction.[34] GPT2 is an enzyme involved in amino acid metabolism, catalyzing the conversion of pyruvate and glutamate into aspartate and α-ketoglutarate. Given that glutamate is a potent excitatory neurotransmitter[35], elevated GPT2 expression may lead to neuroinhibition. Collectively, the upregulation of these proteins in female rats likely exerts neuroinhibitory effects, which could underlie the observed reduction in exploratory behavior in the central zone during the OFT.

Enrichment analysis helps reveal the potential roles of specific gene sets in BPs or disease mechanisms by extracting key functional information. KEGG enrichment results showed that SCA activated proteins involved in ribosome-related pathways in male rats, while inhibiting proteins associated with disease processes such as Alzheimer's disease. Consistently, GO enrichment analysis demonstrated that SCA upregulated proteins related to ribosome function, energy derivation, and mRNA binding in males, promoting protein synthesis and energy supply. Conversely, in female rats, SCA downregulated proteins involved in pathways such as positive regulation of TOR signaling and transport vesicles, thereby affecting cell growth and proliferation. Transport vesicles are crucial in the nervous system, participating in neurotransmitter synaptic transmission and axonal transport, which influence the efficiency and accuracy of nerve conduction.[36,37] Overall, the enrichment analysis suggests suppression of nerve conduction-related proteins in female rats, aligning with the reduced exploratory behavior observed in the OFT and providing a potential mechanism for this effect.

Based on our comprehensive analysis, we speculate that SCA exerts an overall enhancing effect on vital activity in male rats, while producing an inhibitory effect in females. In males, SCA helps maintain normal gene expression and translation, boosts energy supply, promotes nerve conduction, and reduces sensitivity to environmental changes, thereby fostering a stronger exploratory drive.[38,39] In contrast, SCA inhibits nerve conduction, cell proliferation, and substance transport and metabolism in female rats, leading to a more conservative physiological state with reduced exploratory behavior. The traditional efficacy of Semen Cuscutae primarily lies in "tonifying the liver and kidney." According to TCM theory, the "liver" and "kidney" correspond to reproductive and brain functions, which differ

between sexes. Females tend to embody "Yin" qualities—calmness—while males lean toward "Yang"—excitability. Overall, the tonifying function of Semen Cuscutae manifests as distinct physiological effects in males and females, aligning well with traditional understandings.

SCA also induced abnormalities in certain biochemical indicators, which may be related to the reduced diversity of gut microbiota. However, no lesions were observed in histopathological examinations, indicating that these abnormal parameters did not cause significant liver or kidney damage. SCA appears to confer health benefits in male rats, consistent with cognitive improvements reported in previous studies.[8,9,38,39] In female rats, SCA inhibited proteins involved in folate biosynthesis, a finding that requires further validation. Furthermore, this study did not control for the estrous cycle in female rats, so hormonal fluctuations may have influenced behavioral outcomes. Future experiments should include dose gradients and larger sample sizes to confirm the reliability of these results.

Compared with mRNA, differences at the protein level more directly reflect changes in biological function. However, the pathways identified still require precise validation through further experimental methods. Moreover, living organisms possess intrinsic regulatory mechanisms, and changes in protein expression do not necessarily indicate an absolute functional outcome. The ultimate goal of harnessing the biological effects of a medicine is to restore dynamic balance and promote overall health.

5. Conclusions

This study demonstrated that SCA significantly altered the time spent in the central area during the OFT, showing a sex-dependent reversal of behavior. Brain proteomic analysis identified 624 DEPs between groups, with 10 proteins, including FGFR3, TCEAL1, RPS25, NRCAM, and SLC4A2, showing opposite expression changes in male and female rats. Enrichment analysis revealed that SCA upregulated proteins involved in BPs such as ribosome function and energy derivation in males, indicating an overall enhancing effect. Conversely, in females, SCA downregulated proteins related to positive regulation of TOR signaling and transport vesicle functions, thereby inhibiting neurocomputation and substance transport, reflecting an overall suppressive effect. Additionally, SCA decreased gut microbiota diversity in female rats, which corresponded with abnormalities in certain serum biochemical parameters. Further validation with larger sample sizes is required to confirm these findings.

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Statement of ethics

All animal experiments were conducted in accordance with the guidelines of the Beijing Municipal Ethics Committee for the Care and Use of Laboratory Animals and were approved by the Animal Care and Protection Committee of Beijing University of Chinese Medicine (Ethical Approval No. BUCM-2024062601-2210). The study complied with all relevant local legislation and institutional regulations.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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Data availability statement

All data supporting the findings of this study are available from the corresponding author upon reasonable request.

Author contributions

Zihan Zhao: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing—Original Draft. Yaling Yang: Formal analysis, Investigation. Junhui Zhou: Formal analysis, Writing—Review and Editing. Jie Ren: Writing—Review and Editing. Zhiqiang Luo: Investigation, Validation. Ruibin Bai: Investigation. Jian Yang: Conceptualization, Writing—Review and Editing, Project administration, Funding acquisition.

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