



Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: <https://www.sciopen.com/journal/2097-0765>

Anxiolytic activity of nutritional interventions with soybean protein and its derived peptides in mice: from the gut-brain axis to the HPA axis

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ABSTRACT: Anxiety disorders have emerged as global neurological disorders with serious detrimental effects, while dietary supplementation offers new insights for alleviating them. Although bioactive peptides have demonstrated anxiolytic properties, a systematic comparison between low-molecular-weight soybean peptides (SPep) and intact soybean proteins (SPro) through nutritional interventions remains unexplored. This study evaluated the effects of SPro and SPep nutritional interventions on anxiety-like behaviors induced by chronic unpredictable mild stress in mice, exploring their underlying mechanisms. The results revealed that both SPro and SPep nutritional interventions increased glutamate and glutamine levels in mouse brain tissue, but only the SPep diet was effective in alleviating anxiety-like behaviors and reducing c-Fos expression. Distinct alterations in gut microbiota composition were observed for each diet, with the SPep diet notably increasing the relative abundance of *Bacteroidales* and *Lachnospiraceae*, and elevating isobutyric acid, valeric acid, and isovaleric acid levels. Additionally, the SPep diet significantly reduced corticosterone levels and specifically modulated the StAR gene, contributing to the normalization of the hypothalamic-pituitary-adrenal (HPA) axis. Importantly, correlation analysis underscored the critical roles of the gut-brain and HPA axes. Collectively, these findings suggest that the SPep diet exerts its anxiolytic effects primarily through the gut-brain axis, with the HPA axis serving as the central regulatory mechanism.

Keywords: Soybean protein, soybean peptides, anxiety, gut-brain axis, HPA axis

1. Introduction

Anxiety disorders represent a major global health burden, affecting approximately 25% of the population worldwide ^[1]. Its widespread prevalence, chronicity, and co-morbidities have posed a significant threat to human health, and it has been listed by the World Health Organization as the ninth most common cause of disability related to health ^[2]. Anxiety disorders contribute to both mental health deterioration and physical conditions including cardiovascular and gastrointestinal diseases ^[3, 4]. Current treatment strategies, including psychological interventions and medications, can relieve symptoms to a certain extent. However, medications

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Received 24 January 2025

Received in revised form 6 March 2025

Accepted 3 April 2025

may induce side effects such as fatigue, drowsiness, and cognitive impairment. Additionally, the issue of some patients developing resistance to medications poses a considerable challenge to effective treatment [5]. Therefore, it is crucial to find alternative treatments that have fewer side effects and are easier to manage in the long term, and nutritional interventions are becoming a hot topic of research due to their safety and efficacy [6, 7].

Protein, as a core macronutrient, is critical in nutritional interventions for its efficacy. Its hydrolyzed product, peptide, shows greater digestibility and physiological effects than intact proteins due to its smaller molecular weight and higher biological activity [8]. While the anxiolytic effects of hydrolyzed animal proteins, such as those derived from milk, fish, and eggs, have been extensively studied [9]. The potential of plant proteins, particularly soybean protein, as sustainable protein sources for nutritional interventions to ameliorate mood disorders is equally noteworthy. Soybean protein (SPro) is a high-quality plant protein with rich nutrients and balanced amino acids. Its sustainability and global accessibility render it an ideal choice for dietary interventions aimed at managing anxiety disorders [10]. Soymorphine-5 (YPFVV), -6 (YPFVVN), -7 (YPFVNA), and β CG α 323-333 (FLSSTEAAQSY), which are hydrolyzed products of soybean protein, have exhibited anxiolytic activity when orally administered to ddY mice [11, 12]. However, studies on the anxiolytic mechanisms of low molecular weight soybean peptides (SPep) through nutritional interventions are still insufficient, especially regarding the comparison of efficacy with intact SPro.

The gut microbiota, a complex community, is essential for sustaining dynamic metabolic homeostasis [13]. In recent years, numerous studies have revealed the pivotal role of the gut microbiota in the gut-brain axis, an interaction that profoundly influences emotional regulation, stress management, pain sensitivity, and the equilibrium of the brain's neurotransmitter systems [14, 15]. Nutritional interventions enhance beneficial gut microbiota and suppress harmful ones by optimizing dietary structure. This approach has emerged as a novel strategy to alleviate symptoms of mental disorders, such as anxiety and depression [16]. Our previous study demonstrated the differential effects of SPro and SPep diets on gut microbiota composition and diversity, highlighting the benefits of SPep [17]. Notably, the hypothalamic-pituitary-adrenal (HPA) axis serves as a critical neuroendocrine link between gut microbiota and central nervous system stress responses [18]. Long-term exposure to a variety of chronic stressors can lead to an elevation of corticosterone (CORT) and cortisol (COR), hyperactivation of the HPA axis, and trigger various stress-related disorders like anxiety and depression [19]. Trp oligopeptide [20], Peptidyss [21], YL dipeptides [22], and Ganoderma lucidum spore polysaccharides peptides [23] have all been found to reduce the CORT levels in stressed mice, modulating the HPA axis to alleviate anxiety and depression. Therefore, the gut microbiota and HPA axis may play a critical role in the anxiolytic effects exerted by SPep, and a correlation may exist with the gut-brain axis.

This study investigated the effect of SPro and SPep nutritional interventions on chronic unpredictable mild stress (CUMS)-induced anxiety-like behaviors in mice. Anxiety-like behaviors (elevated plus maze and open field tests) and neuronal status (c-Fos positive cell expression and neurotransmitter levels) were assessed in mice. Subsequently, the composition of the gut microbiota and levels of short-chain fatty acids (SCFAs) in

the mouse cecal contents and metabolites in mouse serum were analyzed to identify key differential metabolites and metabolic pathways. Hormone expression within the HPA axis and stress-related genes were further examined. Ultimately, correlation analyses were performed to determine the crucial role of the gut-brain axis. This study compares the effects of SPro and its hydrolyzed peptides on anxiety for the first time and explores their key roles in the gut microbiota and HPA axis. These findings will provide theoretical support for the development of plant protein-based functional foods and open new avenues for dietary supplement intervention in anxiety disorders.

2. Materials and methods

2.1 Preparation and compositional determination of SPep

The SPep was prepared as described in a previous study [24]. In brief, an SPro solution containing 10% protein was enzymatically digested using four commercial proteases. This was followed by separation and purification using a combination of ultrafiltration membranes and metal membranes, and the SPep powder was obtained after sterilization and spray drying. The composition of the SPep was assessed according to the previous methods [25, 26]. Protein content was determined using the Kjeldahl method, amino acid content was measured by an amino acid analyzer, and molecular weight distribution was detected by high performance liquid chromatography (HPLC).

2.2 Animals and diets

Male specific-pathogen-free C57BL/6J mice, aged 6–7 weeks and weighing 22.1 ± 0.7 g, were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All the mice were housed in groups of four per cage under controlled conditions: temperature maintained at $25 \pm 2^\circ\text{C}$, relative humidity at 60%, and a 12 h dark/light cycle. They had *ad libitum* access to food and water. All experimental procedures were conducted following the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines and the Animal Management Rules of the Ministry of Health of the People's Republic of China (documentation no. 55, 2001, the Ministry of Health of the P. R. China). The protocol received approval from the Ethics Committees for Animal Research of Beijing Huayuan Time Technology Co., Ltd. (HYS2021-12, Beijing, China).

The four experimental diets were formulated based on the AIN-93M standard diet, with casein (140 g) replaced by SPro (148 g) and SPep (139 g) in the SPro and SPep diets, respectively, while maintaining consistent protein levels across all diets. The composition and specific group assignments are detailed in Table S1. After one week of acclimatization, mice were randomly divided into four groups ($n = 16$ per group): (1) a control group (Control), (2) a CUMS group (CUMS), (3) a CUMS + SPep diet group (SPep), and (4) a CUMS + SPro diet group (SPro). Subsequently, 52 days of CUMS modeling and 56 days of feeding different diets were performed. Behavioral tests were conducted on days 53 and 54. During this period, the body weight and food intake of mice were recorded weekly. On day 56, all mice were sacrificed to collect serum, brain tissues, and cecal contents for further analysis. The specific schedule is shown in Figure 1A.

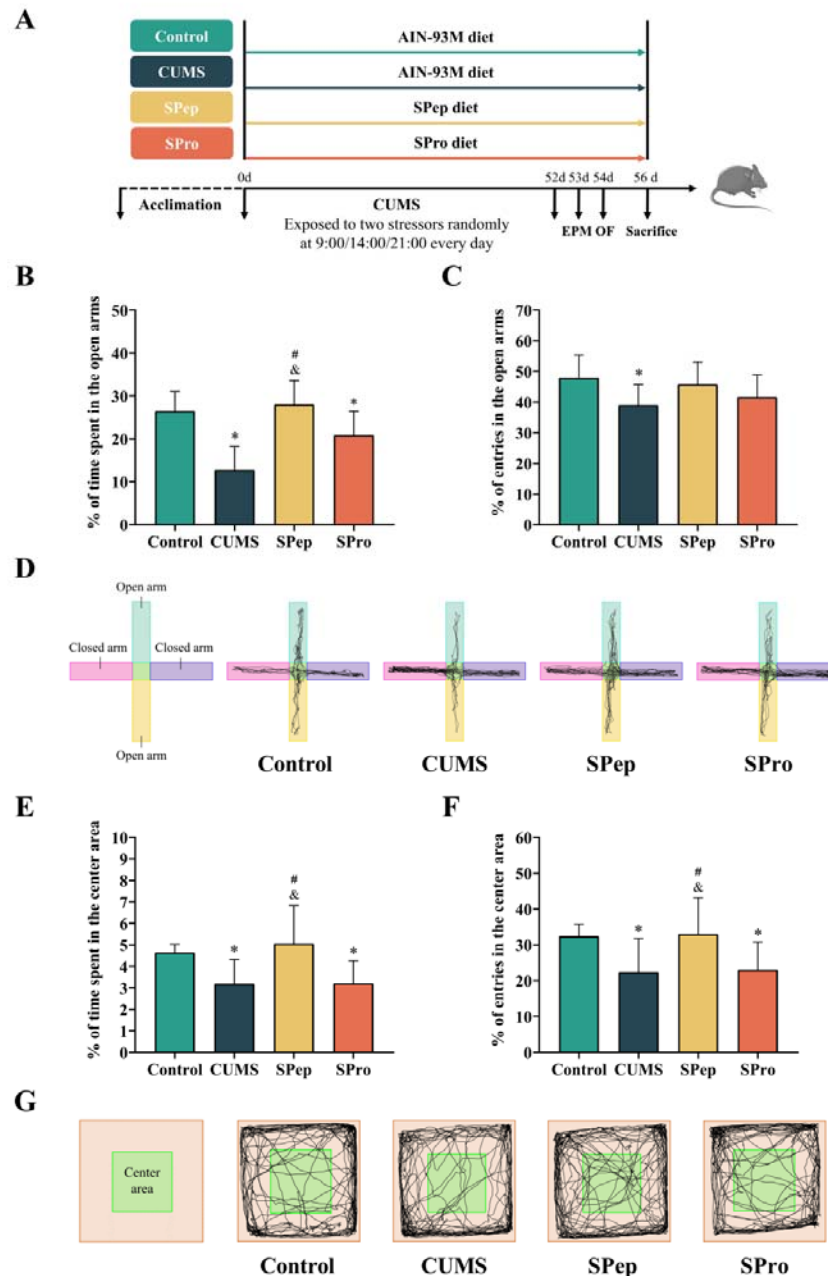


Fig. 1 Effects of SPro and SPep nutritional interventions on anxiety-like behavior in CUMS-induced mice. (A) The animal experiment schedule. (B) Percentage of time spent in the open arms, (C) Percentage of entries in the open arms, and (D) Representative tracking map of the elevated plus maze. (E) Percentage of time spent in the center area, (F) Percentage of entries in the center area, and (G) Representative tracking map of the open field. Data are represented as mean $\pm$ SD ($n=8$). * $P < 0.05$ vs control group; # $P < 0.05$ vs CUMS group; & $P < 0.05$ vs SPro group. No markers indicate no significant difference ($P > 0.05$).

2.3 CUMS procedure

The CUMS procedure was adapted from a previously established method^[27]. Mice were exposed to two stressors randomly at 9:00, 14:00, and 21:00 every day for 52 days. As shown in Table S2, the protocol includes eight different stressors: restraint stress for 2 h, water bath at 30°C for 30 min, damp sawdust for 4 h, without sawdust for 4 h, cage tilted at 45° for 4 h, swimming in 4°C cold water for 1 min, food and water deprivation for 12 h, and light/dark cycle reversal for 12 h. Mice in the control group were free of any stressors.

2.4 Neurobehavioral tests

2.4.1 Elevated plus maze (EPM) test

The EPM test was performed based on previous studies with minor modifications [28, 29]. The EPM consisted of two open arms (30 cm × 8 cm) and two closed arms (30 cm × 7 cm) connected with a central square platform (7 cm × 7 cm). The closed arms were surrounded by 24 cm high walls, while the open arms had no walls. The height of the device was 50 cm above the ground. Mice were individually placed on the square platform facing a closed arm and allowed to explore for 5 min.

2.4.2 Open field (OF) test

The OF test was performed according to the procedures reported previously with minor modifications [30]. The OF device was a square animal cage (50 cm × 50 cm × 40 cm) and was divided into a peripheral area and a center area (25 cm × 25 cm). Mice were individually placed in the center of the device and allowed to explore for 15 min.

2.5 Immunohistochemical staining

Mice were anesthetized with pentobarbital sodium and perfused transcardially with saline, followed by fixed liquid (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). The brains were then extracted and post-fixed in the same fixed liquid. Tissues were embedded in paraffin wax, sectioned to a thickness of 4 μm, and stored at 4°C. During the staining process, sections were incubated in 3% H₂O₂ for 15 min and blocked in 5% bovine serum albumin for 30 min. Following blocking, sections were incubated with a rabbit monoclonal anti-c-Fos primary antibody (1:1000, Abcam, ab222699) at 4°C overnight. The next day, sections were incubated with a goat anti-rabbit IgG (H+L) HRP-conjugated secondary antibody (1:200, Affinity biosciences, S0001) at room temperature for 1 h. Subsequently, all sections were stained with 3'-diaminobenzidine and re-stained with hematoxylin for 3 min. Finally, sections were examined under an inverted microscope (AxioVert.A1, ZEISS, Germany) and the number of c-Fos positive cells was quantitatively assessed.

2.6 Neurotransmitters levels analysis

Neurotransmitter levels in mouse brain tissue were quantified using LC-MS/MS. Metabolite were extracted from tissues using pre-cooled acetonitrile/water solution (4:1, v/v). Then, 50 μL ultrapure water and 200 μL methanol-acetonitrile solution (1:1, v/v) were added, and the mixture was homogenized for 30 mg of tissue. Proteins were precipitated at -20°C for 1 h after the metabolites were sonicated twice for 30 min at 4°C. Following 20 min of centrifugation at 14,000 g and 4°C, the supernatant was collected and freeze-dried, and the freeze-dried samples were resolubilized in a mixture of aqueous 25 mmol/L ammonium formate with 0.1% formic acid and acetonitrile with 0.1% formic acid (9:1, v/v). An Agilent 1290 Infinity UPLC (Agilent, Santa Clara, CA, USA) equipped with a 5500 QTRAP mass-spectrometer (AB SCIEX, Foster City, CA) was used for the LC-MS analysis. Chromatographic separation was achieved on an ACQUITY UPLC BEH Amide column (100 mm × 2.1 mm, 1.7 μm) at 45°C. The mobile phase was composed of A (aqueous with 0.1%

formic acid and 25 mmol/L ammonium formate) and B (acetonitrile with 0.1% formic acid). A 2 μ L injection volume and a flow rate of 0.3 mL/min were used to detect the analytes. The following was the programming for the gradient elution: (0-18) min, 10%-60% A; (18-18.1) min, 60%-10% A; and (18.1-23) min, 10% A. The primary MS parameters for the positive mode of electrospray ionization were as follows: source temperature of 450°C, ion source gas pressure of 160 psi, ion source gas pressure of 260 psi, curtain gas pressure of 30 psi, ion spray voltage of 5000 V.

2.7 16S rRNA sequencing

The cecum contents were collected for 16S rRNA gene sequencing. DNA was extracted from the mouse cecal content using the Omega Mag-bind soil DNA kit (Omega, M5635-02). The hypervariable V3-V4 regions of the bacterial 16S rDNA gene were amplified by PCR using the primers 338 F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Subsequently, sequencing libraries were constructed using the TruSeq® Nano DNA LT Library Prep Kit (Illumina, USA) and sequenced on the Illumina NovaSeq 6000 platform. Bioinformatic processing were performed with QIIME2 (2019.4) [31]. Raw sequence data were quality filtered, denoised, merged, and chimera removed using the DADA2 plugin to generate amplicon sequence variants (ASVs). Species composition analysis was visualized using the R ggplot2 package, while species difference analysis was performed using the LefSe method, employing the R ggtree and ggplot2 packages. The linear discriminant analysis (LDA) effect size (LefSe) was set to $P < 0.05$ and LDA score > 4 as the screening criteria.

2.8 SCFAs analysis

Extraction was performed from the cecal contents using 50 μ L of 15% phosphoric acid, 100 μ L of 125 μ g/mL 4-methylpentanoic acid solution, and 400 μ L of ether. After 1 min of vortexing, the mixture was centrifuged at 12,000 g for 10 min at 4°C. The supernatant was transferred to a vial for GC-MS analysis. SCFAs in mouse cecum were determined using a Trace 1300 gas chromatograph (Thermo Fisher Scientific, USA) equipped with an ISQ 7000 mass spectrometer (Thermo Fisher Scientific, USA). Chromatographic separation was achieved on the Agilent HP-INNOWAX capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m). The system program was as follows: the initial temperature was 90°C, raised to 120°C at 10 °C/min, then to 150°C at 5 °C/min, and finally to 250°C at 25 °C/min. The temperatures of the ion source and interface were 300°C and 250°C, respectively. The injected sample volume was 1 μ L, with each analysis run lasting 15 min. Helium served as the carrier gas at a flow rate of 1 mL/min and a split ratio of 10:1. The mass spectrometer operated in electron impact ionization mode (70 eV) with selected ion monitoring (SIM) at m/z 60 (acetic acid, butyric acid, isovaleric acid, valeric acid, caproic acid), m/z 74 (propionic acid), and m/z 73 (isobutyric acid).

2.9 HPA axis hormone analysis

The levels of corticotropin-releasing hormone (CRH), adrenocorticotrophic hormone (ACTH), and CORT in mouse serum were analyzed using commercial enzyme-linked immunosorbent assay (ELISA) kits (Elabscience, Wuhan, China) according to the manufacturer's instructions.

2.10 Untargeted metabolomics analysis

Untargeted metabolomics was used to detect metabolites in mouse serum. An aliquot of the sample was mixed with 400 μ L of methanol, vortexed for 1 min, and then centrifuged at 12,000 g for 10 min at 4°C. The supernatant was concentrated, dried, and resolubilized in 150 μ L of 2-chloro-L-phenylalanine solution (dissolved in 80% methanol-water). LC-MS/MS was carried out using a Vanquish UHPLC system (Thermo Fisher Scientific, USA) connected to a Q Exactive mass spectrometer (Thermo Fisher Scientific, USA) equipped with an ESI ion source and an ACQUITY UPLC® HSS T3 column (150 $\times$ 2.1 mm, 1.8 μ m). The flow rate was set to 0.25 mL/min, the injection volume was 2 μ L and the column was maintained at 40°C. For LC-ESI (+) - MS analysis, the mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), with a gradient elution profile: (0-1) min, 2% B; (1-9) min, 2%-50% B; (9-12) min, 50%-98% B; (12-13.5) min, 98% B; (13.5-14) min, 98%-2% B; (14-20) min, 2% B. The LC-ESI (-) - MS used 5 mmol/L ammonium formate water (A) and acetonitrile (B) with a similar gradient, except the final hold at 2% B was extended to 17 min. The MS parameters included a sheath gas pressure of 30 arb, an auxiliary gas flow of 10 arb, spray voltages of 3.50 kV for ESI (+) and -2.50 kV for ESI (-), a capillary temperature of 325°C, and an MS range of m/z 100-1000. Full MS resolution was set at 70,000, with MS/MS resolution at 17,500 and normalized collision energy at 30 eV.

The R XCMS package was used for peak detection, filtering, and alignment during data analysis. Statistical analyses, including principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis (OPLS-DA), were performed using the Ropls package (v1.22.0). Metabolite identification was performed by matching MS/MS secondary spectra with the HMDB (<http://www.hmdb.ca>), MassBank (<http://www.massbank.jp/>), LipidMaps (<http://www.lipidmaps.org>), and mzCloud (<https://www.mzcloud.org>) databases. Differential metabolites were screened based on $P < 0.05$ and a variable importance in projection (VIP) score > 1 . Significantly differential metabolites were further analyzed for pathway enrichment using the KEGG database.

2.11 Real-time quantitative polymerase chain reaction (RT-qPCR) assay

Total RNA was extracted and purified from the hypothalamus, pituitary, and adrenal gland using the TransZol Up Plus RNA kit (Cat. No. ER501-01-V2, TransGen Biotech, Beijing, China). Agarose gel electrophoresis was performed to evaluate the purity of the RNA, and the concentration and A260/A280 ratio values of the RNA were determined using a Nanodrop 2000c spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA). Then, cDNA was synthesized using the TransScript® Uni All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (Cat. No. AU341-02-V2, TransGen Biotech, Beijing, China). Real-time quantitative PCR (RT-qPCR) was conducted using the PerfectStart® Green qPCR SuperMix (Cat. No. AQ601-01-V2) on a Real-time PCR System (Bio-Rad, CA, USA). The primer sequences used are listed in Table S3. Gene expression was calculated using $2^{-\Delta\Delta C_t}$, with Actb serving as the housekeeping gene.

2.12 Statistical analysis

The data are expressed as mean $\pm$ standard deviation (SD). For data that conformed to normal distribution, differences between groups were analyzed using one-way ANOVA followed by Duncan's *post hoc* test. Otherwise, non-parametric tests (Kruskal-Wallis H Test) were used. All data were analyzed using SPSS 23.0 software. $P < 0.05$ indicated significant differences between the groups. The graphs were created using the GraphPad Prism 8.0.1 software.

3. Results

3.1 Compositional analysis of SPep

The protein content of SPep was high ($90.7\% \pm 0.7\%$), with molecular weights mainly below 3000 Da, and 71.1% of the peptides had molecular weights below 1000 Da, as shown in Table S4. As for amino acids, SPep had a rich and balanced amino acid profile, with high contents of glutamic acid (24.83 ± 0.61) g/100 g and aspartic acid (11.63 ± 0.18) g/100 g, and other amino acid contents were shown in Table S5. The results indicated that SPep is a high quality nutritional supplement characterized by high protein content, low molecular weight and balanced amino acid composition.

3.2 Effects of the SPro and SPep nutritional interventions on anxiety-like behavior in CUMS-induced mice

During the 52 days CUMS modeling and 56 days dietary intervention, all groups showed similar progressive increases in body weight and food intake ($P > 0.05$, Figure S1). Behavioral tests confirmed that CUMS successfully induced anxiety-like behaviors. Compared to the control group, CUMS-exposed mice exhibited significant reductions in both time spent and entries in the open arms in the EPM test ($P < 0.05$, Figure 1B, C), as well as decreased percentage of time spent and entries in the center area ($P < 0.05$, Figure 1E, F). Notably, SPep treatment ameliorated these anxiety-like behaviors. In the EPM test, SPep-treated mice spent a significantly higher percentage of time in the open arms ($28.0\% \pm 5.6\%$) compared to CUMS group ($12.7\% \pm 5.5\%$, $P < 0.05$, Figure 1B). Similarly, in the OF test, the percentage of time spent in the center area ($5.0 \pm 1.8\%$) and entries ($33.0 \pm 10.2\%$) were both higher in the SPep group than in the CUMS group ($3.2\% \pm 1.1\%$, $22.4\% \pm 9.4\%$, $P < 0.05$, Figure 1E, F). However, the SPro diet did not produce statistically significant effects ($P > 0.05$, Figure 1B, C, E, F). In addition, the above results are visually supported by the representative tracking maps shown in Figure 1D and G.

3.3 Effects of the SPro and SPep nutritional interventions on the c-Fos expression in the hippocampus of CUMS-induced mice

Increasing evidence suggests that anxiety is associated with heightened neuronal excitability in the hippocampus [32]. Neuronal activity can be detected by the expression of the immediate early gene c-Fos [33]. To evaluate the effects of SPro and SPep diets on neuronal excitability in mouse brains, the expression of c-Fos was measured. Immunohistochemical staining revealed c-Fos positive cells, which appeared red-brown as indicated by the red arrows in Figure 2A. Quantification of these cells was performed across all sections (Figure 2B). After CUMS exposure, a significant elevation in the number of c-Fos positive cells was observed in the hippocampus compared to the control group ($P < 0.05$), suggesting increased abnormal neuronal

activity. Notably, compared to the CUMS group, the SPep diet dramatically decreased the number of c-Fos positive cells in the hippocampus of mice ($P < 0.05$), whereas the SPro diet had no significant effect ($P > 0.05$). The results suggest that the SPep diet can modulate neuronal excitability in the mouse brain, thereby alleviating anxiety-like behavior.

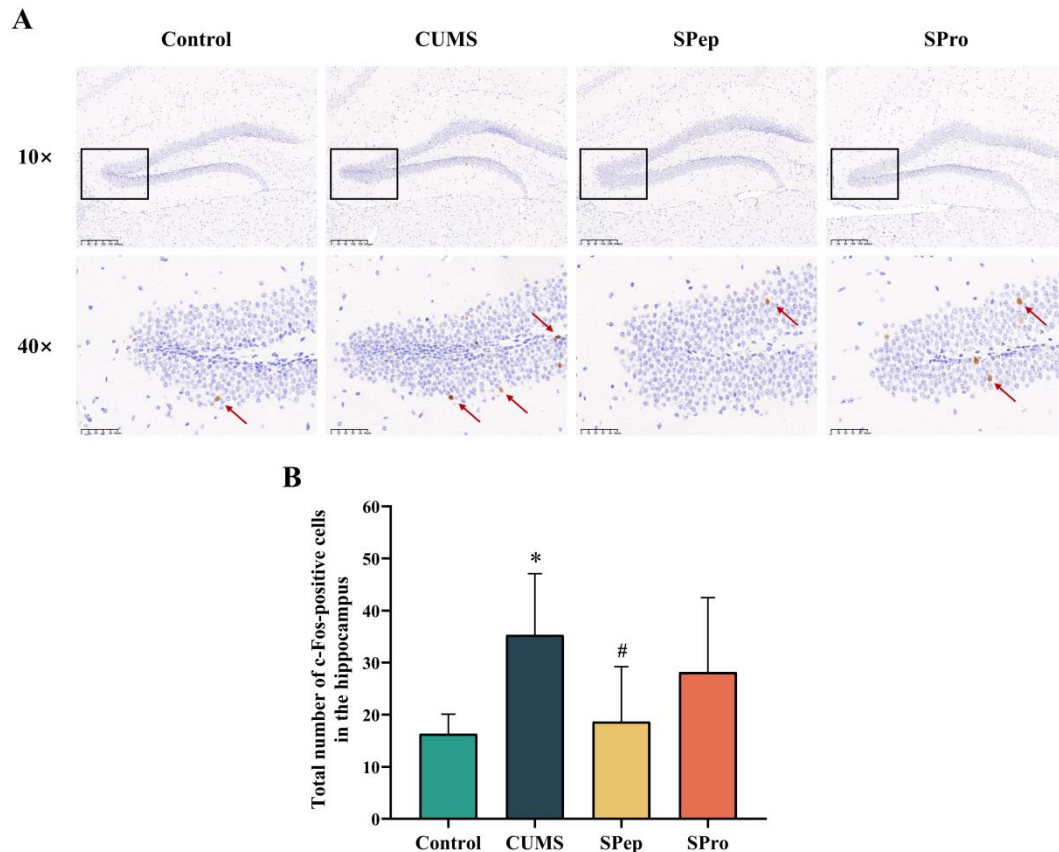


Fig. 2 Effects of SPro and SPep nutritional interventions on c-Fos expression in the hippocampus of CUMS-induced mice. (A) Representative images of immunohistochemistry staining. (B) Total number of c-Fos-positive cells in the hippocampus. Data are represented as mean $\pm$ SD ($n=6$). * $P < 0.05$ vs control group; # $P < 0.05$ vs CUMS group. No markers indicate no significant difference ($P > 0.05$).

3.4 Effects of the SPro and SPep nutritional interventions on neurotransmitter levels in the brain of CUMS-induced mice

Neurotransmitters play a crucial role in anxiety disorders. To investigate the effects of SPro and SPep diets on neurotransmitters in anxiety-like mice, the levels of 23 neurotransmitters were measured in the brain tissue of mice. Compared with the control group, 13 neurotransmitters were significantly downregulated in the brain tissue of CUMS group mice, including glutamate, glutamine, tyrosine, threonine, phenylalanine, arginine, ornithine, rac 4-hydroxy-3-methoxyphenylethylene glycol, normetanephrine, 3,4-dihydroxymandelic acid, tryptophan, 3,4-dihydroxyphenylethylene glycol, and 5-hydroxy-L-tryptophan (Table 1, $P < 0.05$). Following the intervention, both the SPro and SPep diets significantly increased the levels of glutamate and glutamine compared to the CUMS group ($P < 0.05$). Furthermore, the SPro diet also markedly elevated the levels of tyrosine, normetanephrine, and 3,4-dihydroxymandelic acid ($P < 0.05$). These results revealed that both SPro and SPep diets can modulate neurotransmitter levels associated with anxiety in

the brain. However, only the SPep diet exerted protective effects against CUMS-induced anxiety-like behavior and abnormal neuronal excitability. Thus, the underlying mechanisms of the positive effects of the SPep diet need further study.

Table 1 Effect of SPro and SPep diets on neurotransmitter levels in the brain of CUMS-induced mice.

Neurotransmitter	Unit	Control	CUMS	SPep	SPro
Glutamate	μmol/g	12.05 ± 0.64	7.97 ± 1.05*	11.13 ± 0.70 [#]	10.00 ± 1.46 ^{*#}
Glutamine	μmol/g	6.82 ± 0.30	5.92 ± 0.58*	6.96 ± 0.42 [#]	7.02 ± 0.50 [#]
Tyrosine	μmol/g	0.24 ± 0.05	0.12 ± 0.02*	0.16 ± 0.01*	0.17 ± 0.04 ^{*#}
Threonine	μmol/g	0.79 ± 0.09	0.52 ± 0.05*	0.61 ± 0.04*	0.59 ± 0.05*
Phenylalanine	μmol/g	0.12 ± 0.03	0.08 ± 0.01*	0.09 ± 0.01*	0.09 ± 0.01*
4-Aminobutyric acid	μmol/g	2.17 ± 0.12	1.95 ± 0.16	2.06 ± 0.08	2.07 ± 0.21
Serine	μmol/g	1.19 ± 0.18	1.10 ± 0.15	1.04 ± 0.13	1.17 ± 0.17
Arginine	nmol/g	22.51 ± 4.88	16.85 ± 3.44*	18.47 ± 3.49	18.94 ± 2.25
Ornithine	nmol/g	0.05 ± 0.01	0.04 ± 0.01*	0.04 ± 0.01	0.05 ± 0.01
rac					
4-Hydroxy-3-methoxyp	nmol/g	1.02 ± 0.22	0.61 ± 0.14*	0.66 ± 0.15	0.52 ± 0.06*
henylethylene Glycol					
Normetanephrine	nmol/g	31.98 ± 7.00	14.29 ± 2.26*	19.17 ± 1.70*	20.76 ± 4.09 ^{*#}
3,4-Dihydroxymandelic	nmol/g	6.14 ± 1.18	3.36 ± 0.65*	4.40 ± 0.26*	4.58 ± 0.85 ^{*#}
acid					
Tryptophan	nmol/g	32.88 ± 4.93	21.99 ± 2.43*	25.03 ± 2.71*	24.20 ± 4.20*
3,4-Dihydroxyphenyleth	nmol/g	1.21 ± 0.13	0.83 ± 0.16*	0.92 ± 0.11*	0.97 ± 0.09*
yleneglyco					
5-hydroxy-L-tryptophan	nmol/g	0.80 ± 0.15	0.60 ± 0.12*	0.32 ± 0.03 ^{*#}	0.56 ± 0.16 ^{*&}
Ethanolamine	nmol/g	87.71 ± 5.02	90.29 ± 7.46	80.44 ± 7.82	82.50 ± 6.79
DL-Norepinephrine	nmol/g	3.83 ± 1.25	2.92 ± 1.13	2.63 ± 0.51	2.78 ± 0.36
5-Hydroxyindole-3-acet	nmol/g	3.06 ± 1.93	1.23 ± 0.22	1.28 ± 0.62	1.17 ± 0.21
ic acid					
3,4-Dihydroxyphenylac	nmol/g	2.10 ± 0.59	3.44 ± 4.64	1.65 ± 0.59	1.30 ± 0.17
etate					
Serotonin	nmol/g	1.70 ± 0.72	1.34 ± 0.57	0.70 ± 0.38	0.68 ± 0.07
Dopamine	nmol/g	0.84 ± 0.07	1.93 ± 2.40	0.80 ± 0.09	0.79 ± 0.02
Homovanillic acid	nmol/g	0.71 ± 0.39	1.08 ± 1.73	1.26 ± 1.72	0.39 ± 0.09
3-Methoxytyramine	nmol/g	0.12 ± 0.08	0.45 ± 0.72	0.10 ± 0.07	0.07 ± 0.05
hydrochloride					

Note: Data are represented as mean ± SD (n=5). Different letters represent significant differences in four groups for each neurotransmitter ($P < 0.05$). * $P < 0.05$ vs control group; [#] $P < 0.05$ vs CUMS group; & $P < 0.05$ vs SPro group. No markers indicate no significant difference ($P > 0.05$).

3.5 Effects of the SPro and SPep nutritional interventions on the gut microbiota in CUMS-induced mice

To further explore the mechanism behind the anxiolytic effects of SPep, the gut microbiota in the cecum contents of mice was analyzed. The gut microbiota of mice was mainly composed of *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* (Figure 3A, B). CUMS altered the microbiota balance, leading to a decrease in the relative abundance of *Firmicutes* ($59.6\% \pm 12.1\%$) and an increase in *Proteobacteria* ($27.6\% \pm 15.6\%$) compared to the control group ($71.9\% \pm 14.3\%$, $14.3\% \pm 16.9\%$). Conversely, both SPro and SPep diets restored *Firmicutes* ($65.2\% \pm 16.0\%$, $66.6\% \pm 13.7\%$) and *Proteobacteria* ($17.8\% \pm 13.8\%$, $17.0\% \pm 6.4\%$) to the same levels as the control group. At the family level, the CUMS group showed a decrease in the relative abundances of *Erysipelotrichaceae* and *Lactobacillaceae*, but an increase in the relative abundances of *Desulfovibrionaceae*, *Ruminococcaceae*, and *Coriobacteriaceae*. These alterations were reversed by SPro and SPep diets. Additionally, a LEfSe analysis (Figure 3C, D) was

performed to determine the specific microbiota affected by SPro and SPep diets. The analysis highlighted significant differences in the relative abundance of *Actinobacteria* and *Bifidobacterium* in the control group, *Lactobacillales* and *Akkermansia* in the SPro group, and *Bacteroidales* and *Lachnospiraceae* in the SPep group ($P < 0.05$, LDA > 4). The results showed that SPro and SPep diets improved the gut microbiota of mice with significant differences, but whether the SPep diet could exert anxiolytic effects through the gut microbiota remains unclear.

3.6 Effects of the SPro and SPep nutritional interventions on the SCFAs in CUMS-induced mice

Considering the changes in the gut microbiota, SCFA levels were further measured. As shown in Figure 3E, CUMS treatment resulted in a decrease in the levels of acetic acid, isobutyric acid, valeric acid, and isovaleric acid, with isovaleric acid showing significant differences ($P < 0.05$, Figure 3F). Acetic acid levels were correlated with SPro diet and were significantly increased compared to the CUMS group ($P < 0.05$). Isobutyric acid, valeric acid, and isovaleric acid were closely related to the SPep diet, as their levels were significantly increased in the SPep group compared to the CUMS and SPro groups, with (52.0 ± 14.4) , (67.4 ± 15.7) , and (43.0 ± 12.2) $\mu\text{g/g}$, respectively ($P < 0.05$, Figure 3F-H). The results showed that SPro and SPep diets had significantly different effects on SCFA enhancement, with SPep showing more prominent effects.

3.7 Effects of the SPro and SPep nutritional interventions on metabolites in serum of CUMS-induced mice

In addition to the gut microbiota, untargeted metabolomics was performed to detect the metabolite changes in mouse serum. Using LC-MS scans in both positive ion mode (POS) and negative ion mode (NEG), a total of 3,405 and 9,916 metabolites were identified, respectively (Figure S2A, B). OPLS-DA score plots in POS and NEG were obtained (Figure 4A and S2C), which provided reliable results with clustered samples within the four groups and dispersed samples between them. Further metabolite identification based on MS/MS fragmentation patterns discovered 253 metabolites, including: 51 carboxylic acids and derivatives, 22 fatty acyls, 19 organooxygen compounds, 14 benzene and substituted derivatives, 9 indoles and derivatives, 9 steroids and steroid derivatives, 8 phenols, 8 pyridines and derivatives, 6 organonitrogen compounds, and other substances. Figure 4B displays a heatmap that visualizes the upregulation and downregulation of differential metabolites between the four groups. Comparing the SPep group with the remaining three groups separately, 10, 17, and 16 metabolites were upregulated, while 26, 32, and 16 metabolites were downregulated in the comparisons of SPep vs. CUMS, SPep vs. control, and SPep vs. SPro, respectively (Figure 4C and S3A, B).

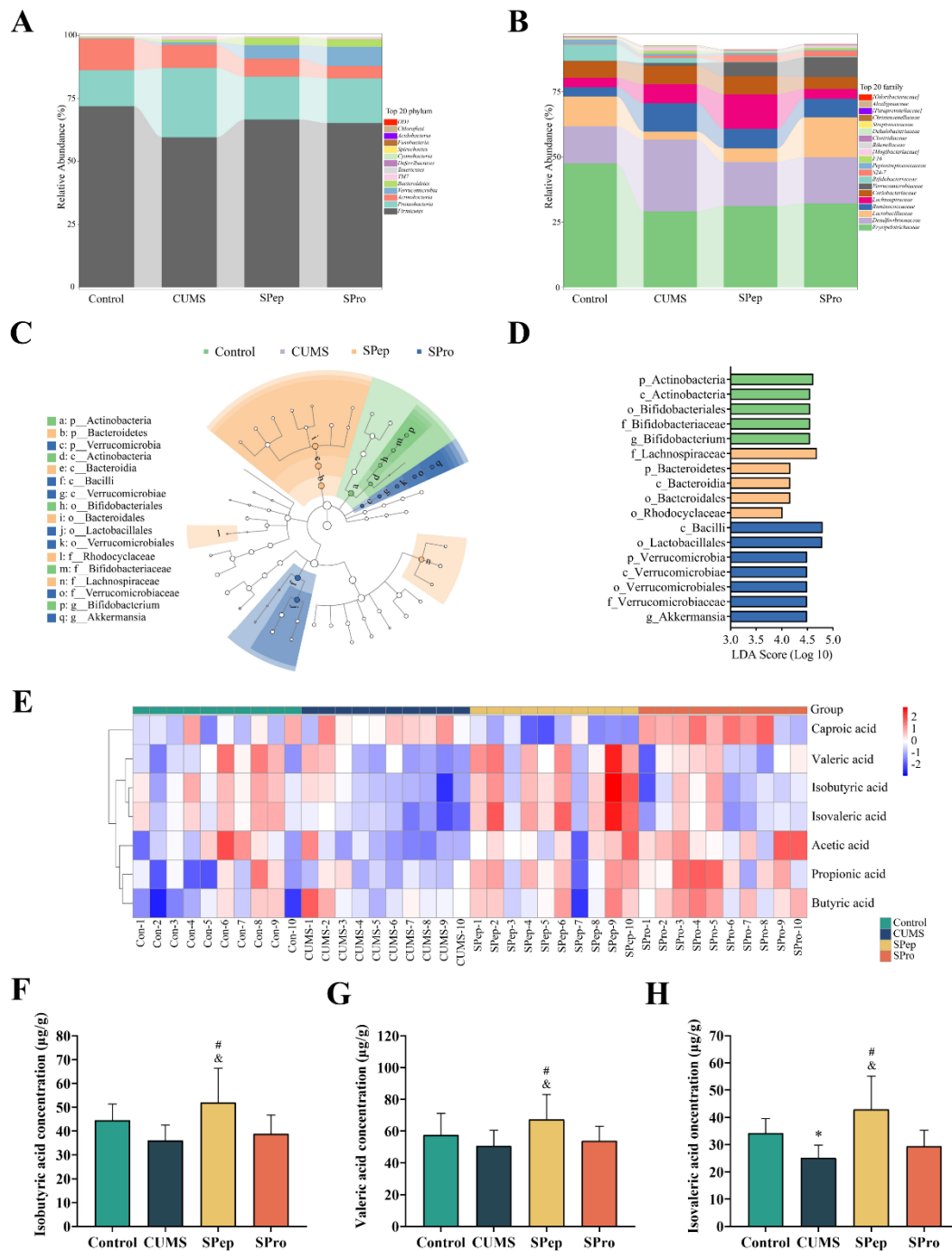


Fig. 3 Effects of SPro and SPep nutritional interventions on the gut microbiota and SCFAs in CUMS-induced mice. Gut microbiota composition at the (A) phylum level and (B) family level. LEfSe differential analysis (C) Cladogram (D) LDA score plot. (E) Clustering heat map analysis of SCFAs (F) Isobutyric acid (G) Valeric acid (H) Isovaleric acid. Data are represented as mean $\pm$ SD, $n=8$ for A-D, $n=10$ for E-H. * $P < 0.05$ vs control group; # $P < 0.05$ vs CUMS group; & $P < 0.05$ vs SPro group. No markers indicate no significant difference ($P > 0.05$).

The differential metabolite pathways were further analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG). The impact and contribution of differential metabolites on metabolic pathways were analyzed, and the metabolic pathway impact factor bubble plots for comparisons of SPep vs. CUMS, SPep vs. control, and SPep vs. SPro are shown in Figure 4D and S3C, D. Specifically, in the SPep vs. CUMS comparison, the SPep diet affected 41 metabolic pathways, in which the Prostate cancer, Pathways in cancer, Tyrosine metabolism, Phenylalanine, tyrosine and tryptophan biosynthesis, Prion disease, Steroid hormone

biosynthesis, Protein digestion and absorption, Neuroactive ligand-receptor interaction, Cortisol synthesis and secretion, Synaptic vesicle cycle, and other pathways played major roles. Figure 4E, S3E, F visually illustrates the metabolic pathways and the differential metabolite changes in SPep vs. CUMS, SPep vs. control, and SPep vs. SPro. It is noteworthy that the downregulation of COR, CORT, and dehydroepiandrosterone (DHEA) was involved in most pathways ($P < 0.05$, $VIP > 1$). COR and CORT are major products of the HPA axis, which plays a central role in regulating the stress response [34]. The results suggested that the SPep diet significantly downregulated the levels of COR, CORT, and DHEA, thereby affecting the HPA axis and alleviating stress-induced anxiety symptoms.

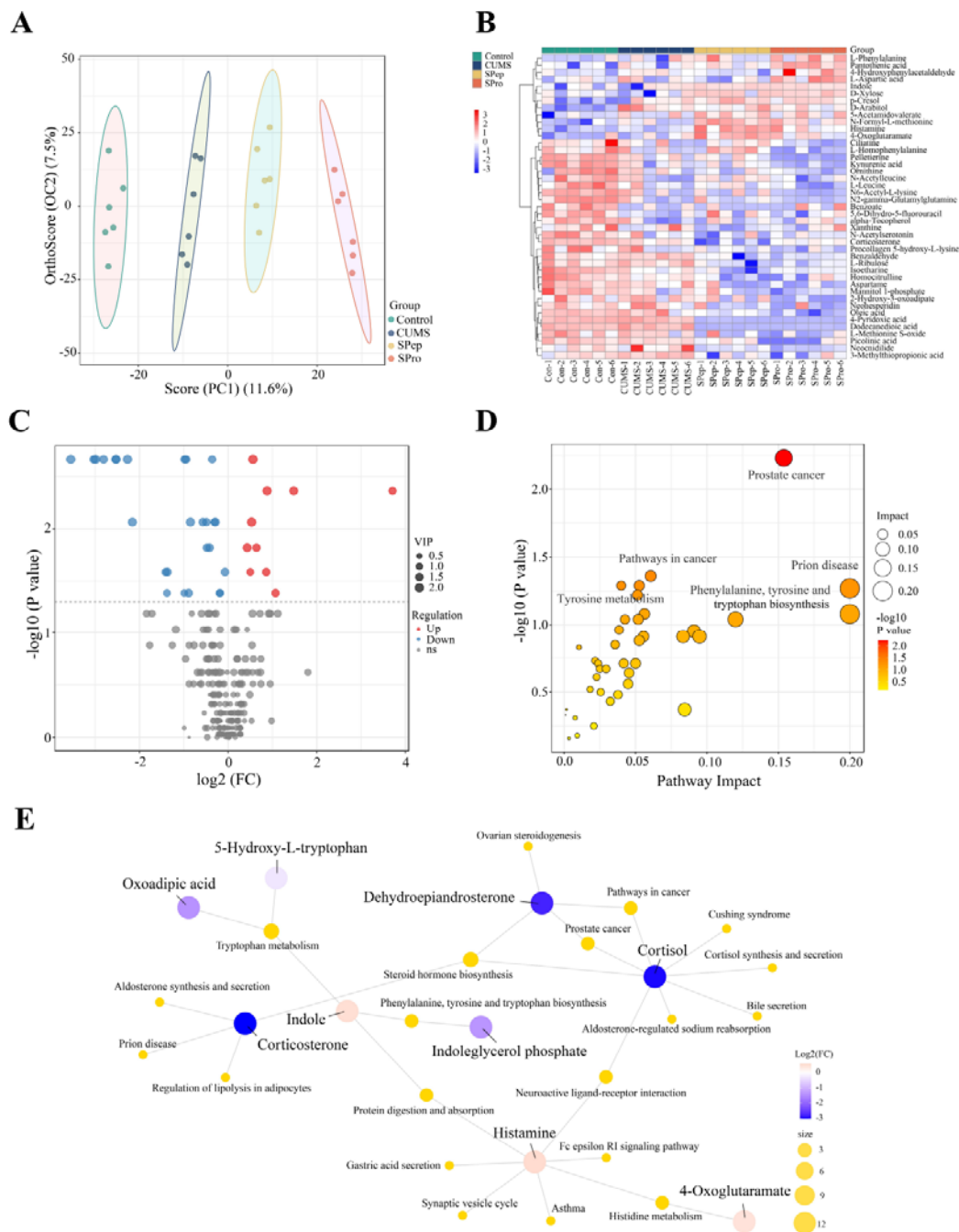


Fig. 4 Effects of SPro and SPep nutritional interventions on serum metabolites in CUMS-induced mice. (A) OPLS-DA score plot for the positive ions mode. (B) Difference metabolite heatmap. (C) Differential metabolite volcano plot of SPep vs. CUMS. (D) Metabolic pathway impact factor bubble plot of SPep vs. CUMS. (E) Network analysis of different metabolites and pathways of SPep vs. CUMS (n=6).

3.8 Effects of the SPro and SPep nutritional interventions on HPA axis hormones and gene expression in CUMS-induced mice

To further investigate the effects of the SPep diet on the HPA axis in mice, levels of CORT, ACTH, and CRH were quantified, along with the expression of relevant genes. As shown in Figure 5A-C, compared with the control group, CUMS significantly elevated levels of CORT, ACTH, and CRH to (222.2 ± 27.5) ng/mL, (50.0 ± 7.4) pg/mL, and (2.2 ± 0.4) pg/mL, respectively ($P < 0.05$). In contrast, the SPep diet significantly reduced the levels of CORT (141.39 ± 31.81) ng/mL and ACTH (36.34 ± 2.79) pg/mL compared to that of the CUMS group ($P < 0.05$) and reached the same level as the control group, while the SPro diet did not show a significant effect ($P > 0.05$).

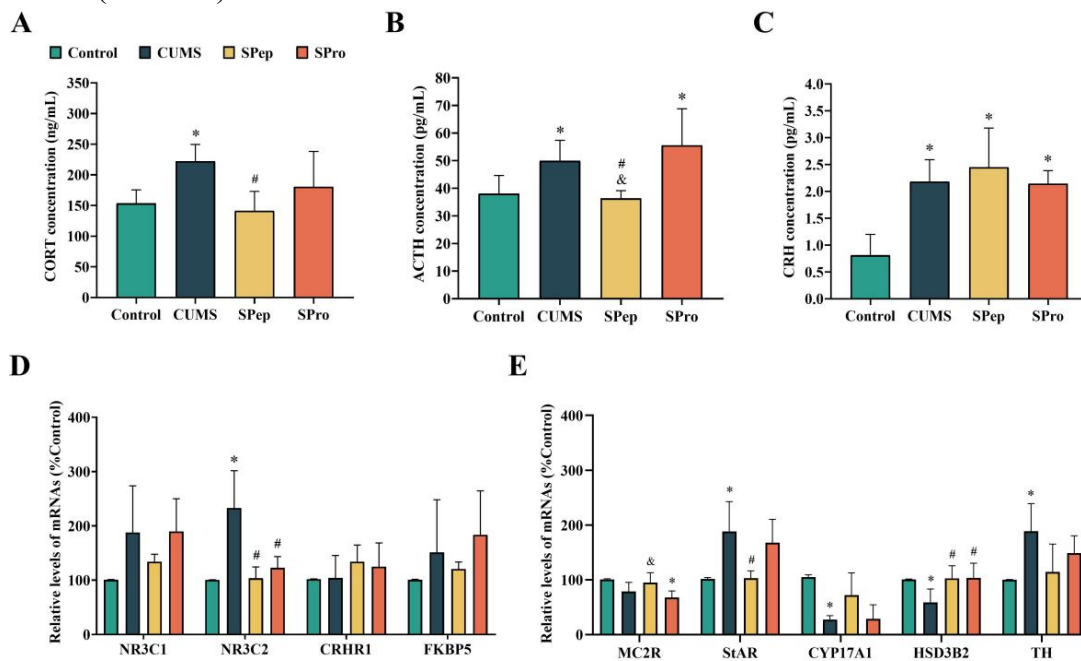


Fig. 5 Effect of SPro and SPep nutritional interventions on the HPA axis. (A) CORT (B) ACTH (C) CRH. mRNA expression level of genes in the (D) hypothalamus and pituitary gland, and (E) adrenal gland. Data are represented as mean $\pm$ SD, $n=6$ for A-C, $n=3$ for D-E. * $P < 0.05$ vs control group; # $P < 0.05$ vs CUMS group; & $P < 0.05$ vs SPro group. No markers indicate no significant difference ($P > 0.05$).

Moreover, RT-qPCR was utilized to quantify the expression of key genes in the hypothalamus, pituitary, and adrenal glands. As depicted in Figure 5D, in the CUMS group, expression of the NR3C2 gene was significantly upregulated in the hypothalamus, which was 2.3-fold of that in the control group ($P < 0.05$). Both the SPro and SPep diets significantly downregulated their expression to 0.5 and 0.4-fold of that in the CUMS group ($P < 0.05$). In the adrenal glands, CUMS treatment resulted in significant upregulation of the StAR and TH genes and substantial downregulation of the CYP17A1 and HSD3B2 genes ($P < 0.05$, Figure 5E), with expression levels being 1.9-, 1.9-, 0.3-, and 0.6-fold of those in the control group, respectively. SPep diet restored the expressions of StAR and HSD3B2 genes to the same level as the mice in the control group. However, the SPro diet only significantly increased the expression of the HSD3B2 gene compared to the CUMS group ($P < 0.05$). Collectively, these results demonstrate that the SPep diet more effectively reduces CORT and ACTH levels and regulates the expression of NR3C2, StAR, and HSD3B2 than the SPro diet.

3.9 Correlation analysis of behavioral test indicators, neuronal activity, neurotransmitters, SCFAs, and HPA axis hormones

To further investigate whether the SPep diet exerts anxiolytic effects via the gut-brain axis, we performed Pearson correlation analyses among various indicators, including behavioral tests, neuronal activity, HPA axis hormones, neurotransmitters, and SCFAs. As shown in Figure 6, the gut microbiota metabolites SCFAs play an important role in alleviating anxiety behaviors, suggesting that the SPep diet may act through the gut-brain axis. Specifically, levels of isobutyric, valeric, and isovaleric acids showed a significant positive correlation with the percentage of time spent in the open arms ($r=0.85, 0.84, 0.76, P < 0.05$), the entries into the open arms ($r=0.65, 0.65, 0.61, P < 0.05$), and the time spent in the center area ($r=0.61, 0.72, 0.74, P < 0.05$). In addition, isobutyric, valeric, and isovaleric acids correlated positively with levels of glutamate ($r=0.65, 0.67, 0.67, P < 0.05$) and glutamine ($r=0.66, 0.60, 0.60, P < 0.05$). Isobutyric and valeric acids showed a significant negative correlation with c-Fos ($r=-0.56, -0.53, P < 0.05$). Crucially, the HPA axis may be the primary mechanism by which SPep exerts anxiolytic effects via the gut-brain axis. CORT and ACTH levels showed significant negative correlations with glutamate and glutamine, significant positive correlations with c-Fos, and significant negative correlations with levels of isobutyric acid, valeric acid, and isovaleric acid.

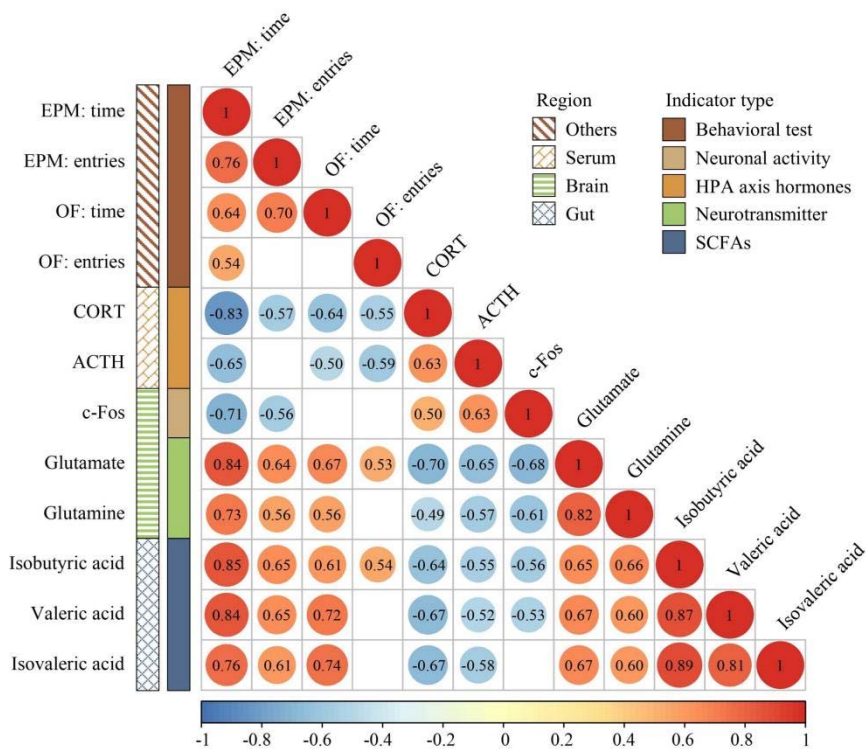


Fig. 6 Correlation analysis of behavioral test indicators ($n=8$), neuronal activity ($n=6$), neurotransmitters ($n=5$), SCFAs ($n=10$), and HPA axis hormones ($n=6$). Pearson correlation analyses were performed by Control, CUMS, and SPep groups. The size and color of the circle indicate the level of the correlation coefficient (r). Larger circles have a larger r . Red is positive and blue is negative.

4. Discussion

In this study, we systematically evaluated the effects of SPro and SPep diets on anxiety-like behaviors in CUMS-induced mice. The results revealed that the SPep diet effectively alleviated anxiety behaviors and

neural excitability in mice, significantly reduced the levels of CORT and COR, and regulated StAR gene expression, thereby normalizing the HPA axis. Meanwhile, the SPro and SPep diets had similar effects on neurotransmitter regulation in mice, and both remodeled gut microbiota composition and SCFA levels. Correlation analyses demonstrated that the SPep diet may act via the gut-brain axis, with the HPA axis playing a potential bridging role.

CUMS is a widely-used model to simulate anxiety and depression behaviors in rodents [35, 36]. Consistent with previous studies, CUMS induced anxiety-like behaviors in the EPM and OF tests [37, 38], increased c-Fos-positive cells in the hippocampus, and decreased neurotransmitter levels. Conversely, the SPro and SPep diets attenuated anxiety-like behaviors and suppressed nervous excitability, with the SPep diet reaching a significant difference. Both diets increased levels of glutamate and glutamine, which may be attributed to their high glutamate content as detailed in Table 1. Glutamate, a major excitatory neurotransmitter, supports various neural functions, while glutamine serves as its precursor, participating in the glutamate-glutamine cycle that regulates excitatory signaling [39]. Correlation analyses showed significant positive correlations between glutamate, glutamine, and behavioral indicators (Figure 6), suggesting that the SPro and SPep diets may alleviate anxiety by modulating neurotransmitters, but the prominence of the SPep diet in behavioral and neuronal activity needs to be explored in depth.

The gut microbiota is integral to gut-brain communication and is tied to neuropsychiatric and neurological disease development [15, 40]. Microbial metabolites such as SCFAs contribute significantly to this signaling [41]. Increasing evidence supports that SCFAs, particularly those elevated by the SPep diet (e.g., isobutyric acid, valeric acid), may act as critical metabolic messengers that bridge gut microbial activity and extra-intestinal systems, including the brain and endocrine organs [42]. Anxiety alters the composition and stability of the gut microbiota and SCFAs [43, 44]. In the present study, CUMS disrupted the gut microbiota balance with a decrease in the relative abundance of *Firmicutes* and an increase in the relative abundance of *Proteobacteria* and caused an overall downregulation of SCFAs, which was significantly mitigated by SPro and SPep diets. Specifically, the SPro diet significantly elevated the relative abundance of *Lactobacillales* and *Akkermansia*, which have been linked to reduced anxiety [45, 46], and also raised acetic acid levels. The SPep diet, on the other hand, significantly increased the relative abundance of *Lachnospiraceae* and *Bacteroidales* and increased concentrations of isobutyric, valeric, and isovaleric acids. A significant increase in the relative abundance of *Lachnospiraceae* was found in both studies exploring the anxiolytic effects of tryptophan-rich diets and *Schisandra chinensis* [38, 47]. Additionally, a study on sesamin highlighted its ability to increase *Bacteroidales*, which correlates with reduced stress-induced behavioral disorders [48]. In addition to acetic acid, propionic acid, and butyric acid, SCFAs are also produced by fermenting proteins and amino acids to produce branched-chain fatty acids, such as isobutyric and isovaleric acids [49–51]. The SPep diet mainly increased levels of isobutyric acid, valeric acid, and isovaleric acid, which was attributed to the SPep diet increasing the level of proteins and amino acids reaching the colon, providing more fermentation substrates for the gut microbiota. Changes in SCFAs were positively correlated with both behavioral indicators,

glutamate, and glutamine levels, suggesting a potential involvement of the gut-brain axis in exerting anxiolytic activity of the SPep diet.

The HPA axis, comprising the hypothalamus, pituitary, and adrenal glands, is strongly associated with stress-related neurological disorders [52, 53]. In this pathway, the hypothalamus secretes CRH which stimulates the pituitary to release ACTH, in turn promoting the steroid hormone biosynthesis pathway in adrenal cells, converting cholesterol into glucocorticoids-cortisol (COR) in humans and corticosterone (CORT) in rodents [54-56]. Recent studies suggest that SCFAs may regulate the HPA axis through both central and peripheral mechanisms. SCFAs such as butyrate can cross the blood-brain barrier and epigenetically regulate hypothalamic genes (e.g., CRHR2) by enhancing histone acetylation. Additionally, SCFAs may activate vagal afferents and modulate peripheral inflammation, thereby indirectly influencing CRH neuron activity. These pathways indicate that SCFAs predominantly act upstream on the HPA axis, rather than directly targeting adrenal glands [57]. Our untargeted metabolomics study showed that the SPep diet significantly reduced the levels of COR, CORT, and DHEA, implicating multiple metabolic pathways. Subsequent targeted measurements showed that SPep also decreased circulating ACTH and CORT. Additionally, gene expression analysis revealed that both the SPro and SPep diets significantly regulated the expression levels of NR3C2, StAR, and HSD3B2 genes, with the SPep diet specifically targeting the StAR gene. StAR is a membrane-bound protein that is primarily responsible for facilitating the transport of cholesterol to the steroid hormone synthesis pathway [58]. Vagnerová et al. found that acute restraint stress significantly elevated the expression of the StAR gene in the adrenal glands of mice, which is consistent with our findings [59]. However, the SPep diet effectively counteracted this response, resulting in reduced cholesterol transport into mitochondria and consequently leading to decreased levels of COR and CORT.

Correlation analysis showed that CORT and ACTH in the HPA axis correlated with indicators in brain tissue (c-Fos, glutamate, glutamine) and in the gut (isobutyric acid, valeric acid, and isovaleric acid), respectively, suggesting a potential role for the HPA axis in the anxiolytic activity of the SPep diet via the gut-brain axis. While anatomically outside the brain and gut, the adrenal glands serve as critical effectors in the gut-brain axis by releasing glucocorticoids that exert both central and peripheral effects. Glucocorticoids modulate immune responses by suppressing pro-inflammatory cytokines and promoting anti-inflammatory mediators, thereby reducing neuroinflammatory inputs that can exacerbate anxiety-like behaviors. This supports a functional gut-brain-immune axis in which SCFA-induced modulation of the HPA axis contributes to behavioral improvement through both neuroendocrine and immunological pathways [54].

Overall, this study demonstrates the alleviating effects of SPro and SPep diets on anxiety-like behaviors in mice, with SPep showing a more pronounced effect. It is noteworthy that our intervention was initiated simultaneously with CUMS exposure, aiming to attenuate the development of stress-related disorders. This stands in contrast to pharmacological agents, which are typically administered after disease onset for therapeutic purposes. Therefore, we recommend adjusting protein intake in humans by increasing the

proportion of plant proteins, particularly SPep, as a potential strategy for preventing and alleviating symptoms of anxiety.

5. Conclusion

In conclusion, our study provides evidence for the beneficial effects of SPro and SPep diets on CUMS-induced anxiety in mice. The potential anxiolytic activity of SPep via the gut-brain axis is highlighted, with the HPA axis serving as an influential communication mechanism. However, the exact roles of gut microbiota and metabolites and their association with the HPA axis need to be further elucidated, and the investigation of the anxiolytic mechanism needs to be more in-depth. Despite this, the present study opens new perspectives on the potential of the SPep diet for the prevention and treatment of anxiety disorders.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by the National Key Research and Development Program of China [No. 2021YFD2100402], and the National Natural Science Foundation of China [No. 81903275].

Appendix A. supplementary data

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.***.

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