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ARTICLE

Deoiled sunflower seeds ameliorate depression by promoting the production of monoamine neurotransmitters and inhibiting oxidative stress

Xiaomeng Lu,[a,c†] Ce Qi [a,†] ,Long Jin [b], Jie Zheng, [b] Mei Sun, [b] Xue Tang, [c] Duo li and Jin Sun [a*]

We aimed to evaluate the antidepressant activity of deoiled sunflower seeds (SFS), which are rich in tryptophan, in our mouse model and explored a possible mechanism of action. Male C57BL/6J mice were subjected to chronic unpredictable mild stress (CUMS) and were administered a diet containing SFS as the main protein source. SFS alleviated CUMS-induced depression-like behaviors, compared to the effects of a whey protein-based diet. This effect was related to increases in the levels of serotonin, dopamine, norepinephrine, acetylcholine, and brain-derived neurotrophic factor in SFS-fed mice. These changes accompanied the amelioration of inflammatory abnormalities and oxidative stress. SFS increased the aromatic amino acid levels, and the ratio of tryptophan to neutral amino acids. Furthermore, the antidepressant-like effects of SFS were involved in lipid, nucleotide, and amino acid metabolism. In summary, SFS was found to attenuate depression-like symptoms in mice. These antidepressant effects may be related to the increase in the levels of aromatic amino acids and neurotransmitters, amelioration of oxidative stress and inflammation, and the regulation of the levels of abnormal metabolites to the normal levels.

Introduction

Depression, also known as depressive disorder, is a common psychiatric condition. It is characterized by anhedonia, low mood, weight loss, sleep cycle changes, decreased physical activity, hopelessness, or feelings of guilt.[1] According to the World Health Organization, approximately 350 million people worldwide have depression, and approximately two-thirds of these individuals commit suicide.[2]

One of the most widely accepted etiological hypothesis of depression is the monoamine hypothesis, which suggests the dysfunction of monoamine neurotransmitters in depressive disorders.[3] Monoamine neurotransmitters, such as 5-hydroxytryptamine (5-HT), norepinephrine (NE), and dopamine (DA), are involved in physiological responses, including mental activities and emotional responses. Tryptophan (Trp) is a precursor of 5-HT and determines its activity. Dietary Trp is positively related to 5-HT activity.[4] Moreover, Trp intake is inversely associated with self-reported levels of depression, and is positively associated with sleep duration.[5] The enzyme tryptophan hydroxylase converts Trp to 5-HT, and is responsible for the concentration and availability of Trp in blood; Trp, in turn, increases the synthesis and release of 5-HT.[6] Considerably decreased levels of Trp have been observed in the plasma of patients with depression.[7] Additionally, depression is closely associated with oxidative stress and inflammation.[8] Oxidative stress impairs the antioxidant defense system, causing oxidative damage by altering the balance between oxidant and antioxidant factors. Stress-induced oxidative damage has been implicated in the etiology of depression and anxiety.[9] Increased levels of pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α), have also been observed in stressed animals.[10] Moreover, depression is accompanied by decreased brain-derived neurotrophic factor (BDNF) levels.[11] Metabolomics, a systematic assessment for the comprehensive and quantitative analysis of the overall metabolic changes in biological matrices, has been increasingly applied as a multifunctional tool for discovering molecular biomarkers, exploring potential mechanisms of various diseases, and evaluating the therapeutic effects of drugs. Stress-induced depression is also involved in the regulation of several metabolic pathways, including those involving amino acids, fatty acids, and phospholipids.[12] Although great progress has been made in understanding the pathogenesis of depression, most current antidepressants have limited efficacy and multiple side effects.[13]

The chronic unpredictable mild stress (CUMS) protocol is a classical model to evaluate depression-like behaviors in

[a.] *Institute of Nutrition and Health, School of Public Health, Qingdao University, Qingdao 266071, Shandong, China.*
[b.] *National R&D Center for Nuts Processing Technology, Qiaqia Food Co., Ltd, Hefei, 230601, Anhui, China.*
[c.] *Centre for Food Nutrition and Functional Food Engineering, School of Food Science and Technology, Jiangnan University, Wuxi 214122, Jiangsu, China.*
† These authors contributed equally to this work.
* Corresponding author: Jin Sun (sunj@qdu.eud.cn)
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rodents.[14] This model is based on the application of a series of unpredictable and mild stressors to simulate social stresses in daily life. It has good face validity and is similar to depression-like symptoms.[15] The CUMS protocol induces decreased responsiveness to rewards (anhedonia), alteration in locomotor activity and investigative behavior (helplessness and despair), deterioration of coat state, and altered sexual activity.[16]

In recent years, dietary Trp intervention has been used to prevent depression and ameliorate anxiety-like states.[17] Dietary Trp is positively correlated with brain 5-HT activity. Increased dietary Trp consumption enhances 5-HT synthesis in the brain, inducing beneficial effects in terms of anxiety and depression.[7] Natural products containing large amounts of Trp have been studied. For example, deoiled gourd seed combined with glucose was found to improve anxiety, with clinical effects similar to those of pharmaceutical-grade Trp,[18] and Trp-enriched whey protein (WPC)-based diets were found to ameliorate depressive behaviors in mice, compared with a low-Trp containing casein-based diet.[19] Trp-abundant foods with favorable Trp/protein ratios include milk-derived proteins/peptides and certain nuts, in addition to WPC, α-lactalbumin, γ-[Glu] n-Trp (EW), whey protein hydrolysate (WPH), sesame seeds, and sunflower seeds.[20, 21]

Sunflower seeds are a type of food with a favorable tryptophan/protein ratio, up to 1.74 g/100 g protein, second only to milk and sesame seeds. It has been reported that high fat ice cream sprinkled with sunflower or sesame seeds can decrease patient anxiety during daily life activities.[21] Moreover, sunflowers are widely planted worldwide, and many of its by-products have been developed and utilized. Diets rich in WPC were found to promote the release of 5-HT and exert antidepressant effects in mice.[6] However, there are very few studies that have explored the effects of Trp-enriched sunflowers seeds to alleviate depression-related states. We hypothesized that deoiled sunflower seeds (SFS, Trp: 2.35 g/100 g protein) could have a beneficial impact on depression, similar to WPC. Nevertheless, SFS contains large amounts of polyphenolic compounds, such as chlorogenic acid (CGA), accounting for up to 70% of total polyphenols. CGA is readily oxidized to quinone, which then covalently binds to polar groups, causing poor color and digestibility of the protein.[22] In addition, CGA is also associated with antidepressant-like effects, which can exert neuroprotection and promote the release of 5-HT by enhancing synapsin I expression.[23] Therefore, deoiled and dechlorogenic acid-enriched sunflower seeds (RSFS) group was set up to exclude the interference of CGA on the digestion and absorption of SFS in mice on the one hand, and on the other hand, its antidepressant-like activity.

To date, the antidepressant-like effects of SFS have not been reported. Here, we explored the antidepressant-like effects of SFS in mice subjected to CUMS.

Materials and methods

Reagents. Detection kits for catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Enzyme-linked immunosorbent assay (ELISA) kits for detection of IL-1, IL-6, TNF-α, 5-HT, DA, NE, acetylcholine (ACh), and BDNF were purchased from Jiangsu Meimian Industrial Co., Ltd (Yancheng, China).

Diets. Sunflower seeds were obtained from Qiaqia Food Co., Ltd. (Hefei, Anhui, China). Whey protein and soy protein were obtained from Qinuo Food Co., Ltd. (Zhengzhou, Henan, China) and Jiangsu Fu Shing Tak Biological Engineering Co., Ltd. (Nanjing, Jiangsu, China), respectively. SFS were obtained after cold-pressing without heating in the same press (Bestday, Berlin, Germany). The protein, amino acid and phenol compositions of the diet sources are presented in Table S1.

Animals. Fifty male C57BL/6J mice weighing 18–21 g (6 weeks old) were provided by Cavens Laboratory Animals Co., Ltd. (Changzhou, Jiangsu, China). The mice were fed in the Experimental Animal Center of Jiangnan University with a 12-h light/dark cycle (lights on 08:00–20:00 h), constant temperature (23 ± 2°C), constant humidity (60% ± 5%), and *ad libitum* food and water. All animal experiments were conducted in accordance with the National Guidelines for Experimental Animal Welfare (China), and the experimental protocol was approved by Jiangnan University Animal Care and Use Ethics committee (approval number: JN. No. 20181230c0480710 [281]).

After a week of adaptation, the mice were randomly divided into 5 groups (n = 10/group) according to their body weight. The operation was as follows: BASIC programming was performed using statistical methods and computer technology. The mice were numbered and weighed sequentially, the data automatically recorded, and mice were weighed again the next day. By analyzing body weight variance distribution, the qualified animals were sifted and randomized automatically. The procedure was terminated when the mean body weight of each group was similar and not significantly different ($P > 0.05$).[24] An appropriate sample size was computed when the study was being designed. The mice were fed with the following diets for 7 weeks according to dietary intervention time used by other researcher[25]: soy protein (normal control, CON; model control, MOD), SFS, RSFS, and WPC (positive control). The diet formulation referred to the AIN-93M diet formulation, and the composition of each diet is listed in Table S2.[26] After 4 weeks, all mice, except those in the CON group, were housed individually and exposed to CUMS for 3 weeks, according to modeling time used in other study.[27] The experimental design is depicted in Fig. 1.

CUMS. The CUMS procedures were performed with minor modifications.[28] Briefly, mice were exposed to different types of stressors 2 times a day for 3 weeks, including tilting cages to approximately 45° (without bedding, 2 h), 3 min tail pinch (1 cm from the tip of tail), removing bedding (4 h), placing mice in the same cage (5 per cage, 3 h), food and water deprivation (12 h), exposing mice to feces of rats (3 h), removing bedding, adding water to a depth of 0.25 inches (3 h), and altering the normal 12-h/12-h light/dark cycle.

Behavioral tests

Sucrose preference test (SPT). The SPT was conducted to evaluate anhedonia, as previously described.[29] During a 24-h training period, mice were administered a 1% sucrose solution and water, and the bottles were switched in the middle. After 12 h of food and water deprivation, mice were provided free access to sucrose solution and water for 12 h. The bottles were weighed before and after the test. The sucrose preference was calculated as follows: sucrose preference = (sucrose solution consumption/total consumption) × 100.

Forced swimming test (FST). As described previously,[30] mice were placed individually in a cylindrical container with a height of 25 cm and diameter of 15 cm, filled with water up to a height of 10 cm at 25°C. The cumulative immobility time was recorded for the last 4 min of the total 6-min test duration. The mice were considered immobile when they stopped struggling or appeared to float, with only small limb movements to keep their head above water. No other body movements were observed.

Open field test (OFT). The OFT was conducted to assess locomotor activity. The apparatus was divided into 16 squares (10 × 10 cm) on a computer tracking system with a four-sided white box (40 × 40 × 30 cm). The four central squares were defined as the center area. Mice were placed in the center of the apparatus and allowed to explore new situations freely for 30 min. The activity and behaviors were recorded using the ANY-maze video tracking system (Stoelting, Wisconsin, USA).

Sample collection. After behavioral tests, the mice were sacrificed by cervical dislocation, and their blood was collected and centrifuged at 4000 *g* for 10 min at 4°C. The hippocampus was collected, snap-frozen in liquid nitrogen, and stored at −80°C. The cerebral cortex was rapidly dissected out and homogenized. The homogenate was immediately used to determine reactive oxygen species (ROS) levels.

Determination of oxidative stress factor levels. The activities of CAT, SOD and GSH-Px, as well as MDA levels in the cortex, were determined strictly according to the manufacturer's instructions. The ROS levels were measured using a method described by Kobayashi et al.[31]

Determination of inflammatory factor levels. IL-1, IL-6, and TNF-α levels in plasma were measured according to the manufacturer's instructions. The procedure was as follows: standards and samples were added to the Microelisa strip-plate, incubated for 30 min at 37°C and washed five times. HRP-Conjugate reagent was then added and incubated for 30 min at 37°C, washed 5 times. Chromogen Solution A and B were added and kept in the dark for 10 min at 37°C. Finally, the absorbance of the samples was measured at 450 nm after adding Stop Solution. The sample concentration was calculated according to a standard curve, and then multiplied by the dilution multiplier to obtain the actual concentration of the sample. The limits of detection for IL-1, IL-6, and TNF-α were 3.5 ng/L–120 ng/L, 3 pg/mL–120 pg/mL, and 25 ng/L–800 ng/L, respectively. The coefficients of variation within batches for IL-1, IL-6, and TNF-α were 5.6%, 5.8% and 5.7%, respectively. The coefficients of variation between batches for IL-1, IL-6, and TNF-α were 7.2%, 7.1%, and 7.4%, respectively.

Determination of amino acid levels. The procedure for assaying plasma amino acids (Trp; phenylalanine, Phe; tyrosine, Tyr; valine, Val; leucine, Leu; isoleucine, Ile) is detailed below. Amino acid standard dilutions and 50 μL of the test samples were added to the standard and sample wells, respectively. Ninhydrin hydrate (100 μL) and 0.1% ascorbic acid (50 μL) were added to each well. Finally, the Microelisa strip plate was placed in boiling water and heated for 15 min. It was then removed and quickly cooled with cold water. When the color changed from red to blue-purple, the absorbance of the samples was read at 570 nm.

Determination of neurotransmitter and BDNF levels. 5-HT, DA, NE, ACh, and BDNF levels in the hippocampus were measured according to the manufacturer's instructions. The procedure for detecting the neurotransmitter and BDNF levels by ELISA kits was the same as above. The limits of detection for 5-HT, DA, NE, ACh, and BDNF were 8 pg/mL–240 pg/mL, 2 pg/mL–48 pg/mL, 3 pg/mL–120 pg/mL, 15 pmol/L–400 pmol/L, and 3 ng/L–160 ng/L, respectively. The coefficients of variation within batches for 5-HT, DA, NE, ACh, and BDNF were 5.7%, 5.3%, 5.3%, 5.6%, and 5.8%, respectively. The coefficients of variation between batches for 5-HT, DA, NE, ACh, and BDNF were 7.4%, 7.4%, 7.1%, 7.5%, and 7.1 %, respectively.

Quantitative reverse transcription-polymerase chain reaction (RT-qPCR). Total RNA was extracted from the hippocampus using TRIzol reagent (Biomiga, Shanghai, China). The concentration and quality of RNA were determined by measuring the A_{260}/A_{280} ratio. Total RNA was reverse transcribed to complementary DNA (cDNA) according to the manufacturer's instructions (Thermo Fisher, Shanghai, China). The cDNA was diluted 10-fold and mixed with specific primers (Table S3) and SYBR Green I dye (SYBR Green Master Mix, Vazyme,Nanjing, Jiangsu, China). Real-time PCR (Monad Biotech Co., Ltd, Suzhou, China) was performed with the following cycling conditions: initial denaturation, 95°C for 5 min; 40 cycles at 95°C for 20 s, 60°C for 30 s, and 72°C for 20 s; with a final extension at 72°C for 2 min. Relative gene expression data were analyzed using the $2^{-\Delta\Delta CT}$ method.[32]

Ultra-performance liquid chromatography-tandem mass spectrometer (UPLC-MS/MS) analysis of hippocampal tissue

Sample preparation. We retrieved hippocampal samples (50 mg) and homogenized them with 1 mL of ice-cold methanol/water (70%). We then added cold steel balls to the mixture and homogenized at 30 Hz for 3 min. The mixture was then whirled for 1 min and centrifuged at 12,000 × *g* for 10 min at 4°C.

UPLC-MS/MS spectrometry acquisition conditions. Sample extracts were analyzed using an LC-ESI-MS/MS system (UPLC, Shim-pack UFLC SHIMADZU CBM30A, https://www.shimadzu.com/; MS, QTRAP® System, https://sciex.com/). The analytical conditions were as follows: UPLC: column, Waters ACQUITY UPLC HSS T3 C18 (2.1 mm × 100 mm, 1.8 μm); mobile phase, water (0.04% acetic acid): acetonitrile (0.04% acetic acid), flow rate: 0.4 mL/min; elution gradient, 95:5 V/V at 0 min, 5:95 V/V at 11.0 min, 5:95 V/V at 12.0 min, 95:5 V/V at 12.1 min, 95:5 V/V at 14 min; injection volume: 2 μL.

Linear ion trap (LIT) and triple quadrupole (QQQ) scans were acquired on a QQQ-linear ion trap mass spectrometer (Applied Biosystems 4500 QTRAP), QTRAP® LC-MS/MS System, equipped with an ESI Turbo Ion-Spray interface, operating in positive and negative ion mode and controlled by Analyst v.1.6.3 software (Sciex, Connecticut, USA). The ESI source operation parameters were as follows: ion source, turbo spray; source temperature, 550°C; ion spray voltage, 5500 V; ion source gas I (GSI), gas II (GSII), and curtain gas were set at 55, 60 and 25.0 psi, respectively; the collision gas was high. In the QTRAP, each ion pair was scanned based on declustering potential (DP) and collision energy (CE). Instrument tuning and mass calibration were performed with 10 and 100 μmol/L polypropylene glycol solutions in QQQ and LIT modes, respectively. The DP and CE for individual multiple reaction monitoring (MRM) transitions were performed with further DP and CE optimization. A specific set of MRM transitions was monitored for each period according to the metabolites that were eluted within this period. Qualitative and quantitative metabolite analysis was based on a semi-quantitative method, as described in Supplementary Methods.

Normalized data were imported into SIMCA-P v.14.1 software (Umetrics, Goettingen, Germany) for multivariate statistical analysis. First, in unsupervised mode, principal component analysis (PCA) was initially performed to determine the differentiation of all groups. Specific samples were eliminated in subsequent analyses. Thereafter, in supervised mode, partial least squares discriminant analysis (PLS-DA) was performed to determine the classification information. R^2X, R^2Y, and Q^2 values close to 1 suggested that the model had good fitness and predictive ability. Lastly, the data were subjected to orthogonal PLS-DA (OPLS-DA) to obtain an S-plot and values of variable importance (VIPs) plots, which were then used to discover potential biomarkers. When the VIP was > 1.00, these variables were considered to have significant differences; the larger the value, the greater the corresponding contribution of the corresponding metabolite to the differences. Metabolites with VIP > 1.00 were imported into IBM SPSS v.20.0 software (Chicago, USA) for statistical analysis (independent samples, Student's *t*-test). When the metabolites satisfied both conditions: VIP > 1.00 and $P < 0.05$, they were identified as potential metabolic markers.

Statistical analysis. The data were analyzed using SPSS Statistics v.20.0. Results are expressed as means ± standard error of mean (SEM) and were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's test. Statistical significance was set at $P < 0.05$.

Results

Effects of SFS on body weight. Throughout the experiment, non-stressed mice appeared to display normal behaviors with regard to eating, drinking, exercise, and body weight (Fig. S1). In the fourth week (before modeling), the body weights of the SFS and RSFS groups were higher than the other 3 groups ($P < 0.05$). This might be related to better food palatability in the SFS and RSFS groups. Additionally, body weights in the CON group were lower than in the MOD group, but there was no significant difference between the two groups. CUMS induced significant weight loss during the first week of modelling. This might be due to the mice being stressed for the first time. And this has also been observed in a previous study.[33] The lost weight was recovered gradually in subsequent weeks. The MOD mice exhibited slower weight gain, inactivity, and lack of energy compared with CON mice ($P < 0.05$). Over the modeling period, SFS, RSFS, and WPC supplementation caused significantly greater weight gain and elevated appetite compared with the MOD group ($P < 0.05$). No significant differences were observed in weight gain among the SFS, RSFS, and WPC groups.

Effects of SFS on sucrose preference. Sucrose preference in the MOD group was lower than that in the CON group ($P < 0.05$, Fig. 2A), indicating the model was successfully established. Compared with the MOD group, SFS and RSFS supplementation reversed this reduction, with the RSFS group exhibiting the most significant difference ($P < 0.05$).

Effects of SFS on immobility time in FST. FST is a classic experiment used to evaluate depression-like behaviors (Fig. 2B). Here, stressed mice showed longer immobility time than those in the CON group, whereas immobility time among the SFS, RSFS and WPC groups was significantly reduced compared with the MOD group ($P < 0.05$).

Effects of SFS on locomotor activity in OFT. As shown in Fig. 2C, there was a significant difference in total distance, time spent in the center, and entries to the center between the CON and MOD groups ($P < 0.05$). The stressed mice demonstrated a shorter total distance and time spent in the center, and a reduction in entries to the center compared with the SFS, RSFS and WPC groups ($P < 0.05$). Moreover, RSFS supplementation increased the central distance traveled compared to the MOD group ($P < 0.05$). In general, there were no differences between the SFS and RSFS groups.

Effects of SFS on oxidative stress in cortex. The effects of SFS on oxidative stress in the cortex are presented in Fig. 3. In the MOD group, the activities of GSH-Px, SOD and CAT were dramatically reduced ($P < 0.05$), whereas the MDA and ROS levels were increased compared to the CON group ($P < 0.05$). Furthermore, SFS, RSFS and WPC supplementation significantly elevated GSH-Px, SOD and CAT activities, and alleviated the elevation of MDA and ROS levels compared with the MOD group ($P < 0.05$).

Effects of SFS on plasma inflammatory cytokines. Levels of pro-inflammatory cytokines, including IL-1, IL-6, and TNF-α in plasma, were measured (Fig. 4). Chronic stress resulted in elevated levels of these cytokines compared to the CON group ($P < 0.05$). Treatment with SFS and RSFS returned the levels of these cytokines to physiological levels, relative to the MOD group ($P < 0.05$). In addition, SFS and RSFS treatments caused a decrease in IL-1 and IL-6 levels compared to the WPC treatment ($P < 0.05$).

Effects of SFS on plasma amino acid levels. As shown in Fig. 5, large neutral amino acids (LNAAs) include Phe, Tyr, Tyr, Val, Leu, and Ile. The MOD group showed a decrease in Trp, Phe and Tyr levels relative to the CON group ($P < 0.05$). There were

no differences in the Trp/LNAAs ratio between the CON and MOD groups. After SFS, RSFS and WPC supplementation, Trp levels and the Trp/LNAA ratio showed a tendency to return to the normal levels ($P < 0.05$).

Effects of SFS on neurotransmitter levels in hippocampus. The CUMS-treated mice showed a significant reduction in 5-HT, DA, NE, and BDNF levels ($P < 0.05$, Fig. 6), whereas there was no significant difference in the ACh levels, relative to those in normal mice ($P > 0.05$, Fig. 6D). In addition, SFS, RSFS and WPC supplementation elevated 5-HT, DA, NE, ACh, and BDNF levels compared with that of the MOD group ($P < 0.05$). SFS had a greater effect on neurotransmitters than RSFS.

Effects of SFS on mRNA expression in hippocampus. Glucocorticoid receptor (*GR*), indoleamine 2, 3-dioxygenase (*IDO*), *BDNF, IL-1β,* and *IL-6* mRNA expression levels are shown in Fig. 7. The results demonstrated that *IDO* mRNA expression was not different in the hippocampus among these groups ($P > 0.05$). *IL-1β* and *IL-6* mRNA expression levels were significantly higher in the MOD group than in the CON group ($P < 0.05$). There were no significant differences in *BDNF* and *GR* mRNA expression between the two groups. Alternatively, SFS and RSFS treatment increased *BDNF* mRNA expression, and decreased *IL-1β* and *IL-6* mRNA expression relative to the MOD group ($P < 0.05$).

Effects of SFS on hippocampal metabolic profiles. To further investigate the effects of SFS on hippocampal metabolites in mice, PCA, PLS-DA, and OPLS-DA models were established to analyze metabolic characteristics (Fig. 8). PCA plots demonstrated that the normal and CUMS-treated mice were clearly separated, indicating that there were significant differences between the two groups. In addition, the remaining 3 groups were located between the CON and MOD groups, indicating that SFS and WPC could modulate metabolic disturbances induced by CUMS. However, two samples fell outside the oval shape of the PCA plot in the WPC group and were eliminated in subsequent analyses. Furthermore, the PLS-DA plot and the validated model indicated that the differences between these groups were significant. The PLS-DA plot was appropriately validated without overfitting.

In addition, the OPLS-DA plot was established to identify potential differential metabolites, and the corresponding S-plot was used to filter potential biomarkers. Meanwhile, we used one-way ANOVA to evaluate the significance of these differential metabolites in groups. Detailed information regarding the differential metabolites is summarized in Table S4.

Compared with the CON group, the levels of 25 metabolites were significantly changed in CUMS-treated mice, of which 17 and 8 metabolites were upregulated and downregulated, respectively. Compared with the MOD group, the levels of 22, 24, and 18 metabolites were significantly changed in the SFS, RSFS, and WPC groups, respectively; these metabolites are involved in lipid-, nucleotide-, and amino acid metabolism, respectively. The results indicated that the SFS, RSFS, and WPC treatment could significantly alleviate metabolic disorders in CUMS-treated mice. In short, the effects of the SFS and RSFS treatments were not significantly different.

To better visualize changes in metabolic pathways and the effects of SFS and WPC supplementation, MetaboAnalyst software was used to analyze endogenous differential metabolites in the hippocampus (Fig. 9A). Metabolic disorders in stressed mice mainly involve aminoacyl-tRNA biosynthesis; glycerophospholipid metabolism; Val, Leu, and Ile biosynthesis; as well as amino sugar and nucleotide sugar metabolism. This suggested that depression might be a complex mental disease involving multiple metabolic pathway changes. Furthermore, a PLS biplot was established to conduct a rounded analysis of all variables, including metabolite variation (X variables), biochemical indicators (Y variables), and grouping or clustering (observations) (Fig. 9B). Higher levels of neurotransmitters (5-HT, DA, NE, and ACh) and BDNF in hippocampus, Trp and Trp/LNAA in plasma, and oxidative stress factors (SOD, GSH-Px and CAT) in the cortex of normal, SFS and WPC-fed mice were correlated with lysophosphatidylcholine (LPC; C16:0, C18:0, C18:1, C18:3), lysophosphatidylethanolamine (LPE; C16:0, C18:0, C18:1), L-lysine, and L-leu in the hippocampus. In contrast, greater variations in the levels of endogenous metabolites, such as L-dihydroorotic acid, cis-3-hexenylacetate, 2-nonanone, phosphocholine, D-fructose, and 6-phosphogluconic acid trisodium salt were correlated with higher levels of MDA, ROS, and inflammatory factors (IL-1, IL-6 and TNF-α) in stressed mice.

Correlation analysis of behavioral and biochemical indicators. As shown in Fig. S2, based on cluster analysis of correlation coefficients for different indicators, behavioral and biochemical indicators were divided into 3 and 6 categories, respectively. In the classification of biochemical indicators, G1, G2, and G5 are dominated by neurotransmitters, neutral amino acids and the Trp/LNAAs, the precursors of neurotransmitters, and stress mediators, respectively, while G3 and G4 are mainly stress mediators and inflammatory factors and their mRNA expression levels. In behavioral tests, the sucrose preference and immobility time were significantly correlated with neurotransmitters, oxidative stress, inflammatory factors, and aromatic amino acids, suggesting that these parameters are sensitive indicators of depression-like behaviors. In addition, the results of OFT were also closely related to G1–G5. Overall, there was a strong association between these biochemical indicators and depression-like behaviors.

Discussion

Biochemical interpretation of behavioral and biochemical indices. We explored the impact of SFS supplementation on depression-like behaviors and anxiety in the CUMS animal model. We found that such supplementation improved locomotor activity and anxiety-like behaviors. Additionally, it resulted in antidepressant-like activity. Moreover, SFS and RSFS supplementation were not significantly different.

The CUMS rodent model has been widely used to demonstrate chronic stress and depressive symptoms. It simulates the routine unpredictable stressors in human life with good predictive and construct validity.[34] WPC was found to alleviate

CUMS-induced depression-like symptoms in mice.[19] Additionally, WPC improves sociability, exhibits anxiolytic and antidepressant-like activities, and prolongs the efficacy of mental states in mice.[6] In our study, mice exposed to chronic stress displayed retarded weight gain, a remarkable reduction in sucrose preference, and prolonged immobility in the FST, suggesting the induction of depression. Sucrose preference is recognized as an indicator of anhedonia-like behavior, including loss of pleasure and responsiveness to rewards.[35] Chronic treatment with SFS and WPC restored weight gain and sucrose preference, and inhibited increased immobility time. These results were consistent with an earlier report.[36] Furthermore, stressed mice exhibited decreased locomotor activity and exploratory behaviors in the OFT. Nevertheless, this effect was reversed by chronic SFS, RSFS, and WPC treatment. RSFS treatment performed better than WPC to elevate mobility in stressed mice. Similarly, relative to a casein diet, α-lactalbumin caused a remarkable increase in sucrose consumption, and the number of visits to the center of the open field.[7] Taken together, the behavioral tests indicated that SFS, RSFS and WPC alleviated depression-like behaviors in mice subjected to CUMS. Such antidepressant-like activity might be associated with increased levels of 5-HT in the brain.[37]

Reductions in brain 5-HT and NE levels are commonly observed in patients with depression. Drugs typically act by increasing the bioavailability of monoaminergic transmitters.[38] Therefore, we determined monoamine neurotransmitter levels. The results showed that CUMS reduced the 5-HT, DA, NE, and ACh concentrations in the hippocampus, whereas SFS and RSFS intake remarkably reversed this reduction, comparable to that of WPC. This confirmed previous findings that WPC isolate intake elevated brain 5-HT, DA, and NE levels, compared to fluoxetine intake. Interestingly, SFS treatment led to higher levels of DA and NE than RSFS and WPC administration. One possible reason is related to the availability of brain Phe and Tyr, essential amino acid precursors of DA and NE. BDNF is a mediator of synaptic function and neuroplasticity via BDNF–TrkB signaling, associated with anhedonia symptoms and learning-memory impairment.[39] CUMS exposure in our study resulted in a decrease in BDNF levels and *BDNF* mRNA expression. Our findings were consistent with the existing literature indicating that chronic stress significantly reduces BDNF expression in stressed animals.[40] Importantly, this effect was reversed by SFS, RSFS and WPC treatment. Increased levels of *BDNF* mRNA expression regulate neuronal survival and brain plasticity.[41] Consequently, the observed antidepressant-like activity could be attributed to an increase in monoamine neurotransmitters and BDNF expression in the hippocampus.

Changes in brain 5-HT levels are mostly due to the availability of its precursor, Trp, entering the brain. This availability is reflected by the plasma Trp/LNAA ratio, considered to be a sensitive index of brain Trp availability.[42] We found that CUMS not only led to a significant decrease in Trp abundance and the Trp/LNAA ratio, but also reduced 5-HT levels. In addition, SFS, RSFS and WPC intake restored Trp levels, as well as the ratio. Similarly, it has been proposed that WPC supplementation leads to an increase in the Trp/LNAA ratio.[43] The increased availability of Trp promoted the synthesis of 5-HT. Our results showed that SFS and RSFS enhanced Trp levels and the Trp/LNAA ratio. This promoted the entry of Trp into the brain, thereby increasing the synthesis and release of 5-HT.

Emerging evidence suggests that oxidative stress and inflammation are the main contributors to the pathophysiology of major depressive disorder, in which patients show an increase in inflammation and oxidative stress biomarkers.[44] The imbalance between ROS levels and antioxidants leads to oxidative stress.[45] Excessive ROS production can lead to the development and complications of various diseases.[46] In our study, the activities of GSH-Px, SOD and CAT were significantly reduced, whereas MDA and ROS levels were increased in stressed mice. This might be due to the activation of immune-inflammatory processes and increased monoamine catabolism, leading to overproduction of ROS, which impairs the redox balance of the body. These results were consistent with previous studies.[47, 48] Nevertheless, this effect was restored by SFS, RSFS and WPC treatment. One possible reason might be that SFS treatment inhibited the overproduction of ROS, and the results of ROS investigations here confirmed this. It was also found that WPH significantly decreased MDA levels, and elevated SOD and CAT activities in stressed mice.[49] Hence, SFS demonstrated strong protective effects against oxidative stress involved in the pathophysiology of depression.

Inflammation is closely related to the complicated pathogenesis involved in major depressive disorder.[50] In the present study, chronic stress markedly increased pro-inflammatory cytokine levels and *IL-1β* and *IL-6* mRNA expression. ROS have been found to invoke inflammatory reactions by modification of biomolecules.[51] Thus, these phenomena may be a result of excessive ROS generation, and increased levels of ROS were observed in stressed mice. Significant increases in IL-6 and TNF-α levels can be used as a marker of depression, and elevated IL-6 was also proposed as a strong predictor of depression.[52] Furthermore, SFS and WPC intake restored normal IL-6 and TNF-α levels. In addition, SFS decreased *IL-1β* and *IL-6* mRNA expression in hippocampus. RSFS was found to be the most efficient treatment for improving inflammation. This is in agreement with earlier reports, which demonstrated that CUMS significantly upregulated the levels of pro-inflammatory cytokines and promoted depression-like behaviors, and that WPH can return the concentrations of these cytokines to normal levels.[36, 53] In addition, the anti-inflammatory effects of SFS might be also related to inhibition of p38 mitogen-activated protein kinases,[54] and the increased availability of the key co-factor tetrahydrobiopterin in the synthesis of all monoamines.[55] However, the mechanisms underlying these phenomena require further investigation.

Biological explanation of changes in the levels of different metabolites

Lipid metabolism. LPC levels are closely associated with oxidative stress and immune inflammation.[56] Unsaturated LPC is primarily synthesized via the hydrolysis of hepatic

phosphatidylcholine (PC) by phospholipase A2, while saturated LPC is generally synthesized by lecithin cholesterol acyltransferase , which transfers sn-2 fatty acid from PC.[57] The phospholipase *A2* gene is also involved in bipolar disorder.[58] In the present study, saturated LPC (C16:0, C18:0) in CUMS-treated mice was significantly reduced compared to non-stressed mice, indicating that the activity of lecithin cholesterol acyltransferase was reduced, consistent with the literature.[59] In addition, these findings were consistent with a study that reported that lecithin cholesterol acyltransferase activity was remarkably decreased in patients with major depression.[60] Unsaturated LPC (C18:1, C18:3) levels were significantly decreased in stressed mice. This is contrary to a study that revealed that unsaturated LPC (C16:1, C18:1, C20:4) levels are significantly increased in stressed rats, and that phospholipase activity is significantly increased in patients with depression.[61] This might be because oxidative stress decreases PC hydrolysis and phospholipase A2 activity. Unsaturated LPCs are closely connected with the body's immune functions and inflammation. Unsaturated LPC was reduced in stressed mice, indicating that the function of the immune system might be impaired. LPC effectively regulates cell activation, and may enhance oxidative effects via the 5-lipoxygenase pathway.[62] Therefore, we speculated that decreased LPC content in the hippocampi of depressed mice might be related to increased oxidative stress and reduced cholesterol acyltransferase activity. In contrast, SFS, RSFS and WPC supplementation could inhibit oxidative stress and increase lecithin cholesterol acyltransferase activity to increase LPC concentration.

LPE is synthesized via the hydrolysis of phosphatidylethanolamine (PE) by phospholipase A_1, which has good antibacterial and antioxidant capabilities. Here, the LPE (C16:0, C18:0, C18:1) content was decreased in stressed mice, suggesting an alteration in the mitochondrial fatty acid metabolic pathway. A possible reason is that oxidative stress reduced phospholipase activity to inhibit the hydrolysis of PE. Treatment with SFS and RSFS could increase LPE concentrations in stressed mice and alleviate phospholipid metabolism disruption. This outcome is consistent with a previous study which suggested that LPEs and LPCs were significantly decreased in patients with bipolar disorder.[63] We speculated that SFS exerted a remarkable effect by improving the phospholipase activity and antioxidant capacity of LPE.

Nucleotide metabolism. Phosphocholine is a derivative of choline and represents the main storage form of choline in cytoplasm. Compared with normal mice, phosphocholine levels were considerably increased in stressed animals. A reduction of choline has been observed in the plasma of patients with depression.[64] After SFS, RSFS and WPC intervention, phosphocholine showed a tendency to return to normal levels, suggesting that SFS could modulate dysfunction of nucleotide metabolism caused by depression.

Increased levels of L-dihydroorotic acid were observed in stressed mice relative to normal mice. A possible reason is that long-term stress inhibited dihydroorotate dehydrogenase (DHODH) activity, causing L-dihydroorotate acid accumulation in the hippocampus. SFS and WPC restored L-dihydroorotic acid levels, indicating a possible protective mechanism to increase DHODH activity and promote uridine monophosphate synthesis.

High 6-methylmercaptopurine (6-MMP) levels are related to hepatotoxicity and myelotoxicity, and excessive 6-MMP production may lead to undesirable side effects and impact efficacy.[65] Results showed that 6-MMP content in stressed mice was significantly elevated, while SFS, RSFS and WPC administration significantly decreased 6-MMP by regulating 6-MP metabolism. Some patients with inflammatory bowel disease exhibited dose-limiting preferential 6-MMP production, characterized by the overproduction of 6-MMP.[66] Therefore, SFS might exert beneficial effects on depression by regulating the preferential metabolism of 6-MP to 6-TG.

Amino acid metabolism. Amino acids are one of the most basic substances in the body and are closely related to the life activities of organisms. Lys and Leu, which are basic amino acids, cannot be synthesized by animals. Lys plays an important role in improving the function of the immune and central nervous systems.[67] Lys and its intermediates are significantly decreased in patients with depression.[68] In this study, L-Lys levels in the RSFS group were significantly increased compared to those in stressed mice, showing that Lys metabolism was affected after RSFS treatment. The decrease in Lys also reflected the loss of appetite and intestinal flora in CUMS-treated mice. In addition, compared with the stressed mice, L-Leu was remarkably elevated in the WPC group. The reduction in Leu levels was considered to be associated with an insufficient diet while under physical stress, which led to weight loss in CUMS-treated mice.[69]

Glycometabolism. Considerably increased levels of D-fructose and 6-phosphogluconic acid trisodium salt were observed in stressed mice, indicating glucose metabolism dysfunction. Glucose metabolism is considered a factor in depression research, and may be affected by the abnormal secretion of depression-related hormones.[70] Insufficient 5-HT and NE in the brain are related to hyperglycemia.[71] However, the concentrations of 6-phosphogluconic acid trisodium salt returned to the normal levels after SFS, RSFS, and WPC supplementation.

In general, we found that SFS was comparable to RSFS in preventing depression-like symptoms, and there were no significant differences between them. CGA can reduce the digestibility value of SFS, but can also induce neuroprotection and promote the release of 5-HT through enhancing synapsin I expression. The antidepressant activity of SFS might counteract limitation. However, the specific underlying mechanisms need to be explored further.

Conclusions

In conclusion, we found that SFS significantly alleviated depression-like symptoms in stressed mice. This antidepressant-like activity might be due to increased levels of aromatic amino acids and monoamine neurotransmitters, improvements in oxidative stress and inflammation, and the regulation of metabolic pathways, including those associated

with lipid, nucleotide, and amino acid metabolism. However, it is challenging to utilize SFS as the main food protein source in reality. Studies have shown that a Trp-rich hydrolyzed protein source is more efficacious than intact protein or pure Trp in terms of increasing brain Trp and 5-HT function. Therefore, we focused on the identification of Trp-rich hydrolysate components of sunflower seeds and developed corresponding food ingredients in small amounts for ingestion to ameliorate depression.

The major limitations and future perspectives of this study include: 1) the hippocampus in mice is small and insufficient to measure all the indicators, so we only selected a few important parameters to assess the hippocampus, and then combined the inflammation and oxidative stress values in the plasma and cortex to comprehensively evaluate the preventive effect of SFS protein on depression. 2) The experimental period of this study was relatively short, whereas depression might be a short-term state or a long-term feature. Thus, we extended the experimental period in a second batch to observe the depression-alleviating effect of SFS at different time periods. 3) The preliminary aim of our experiment was to explore the antidepressant-like effects of SFS in mice. The extraction process of SFS-derived proteins needs to be optimized to elevate protein purity and to ensure that the Trp is not destroyed.

Conflicts of interest

There are no conflicts to declare.

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Figure legend

Fig. 1 Schedule of the animal experiment. CUMS, chronic unpredictable mild stress; SPT, sucrose preference test; FST, forced swimming test; OFT, open field test.

Fig. 2 Effects of deoiled sunflower seeds (SFS) on sucrose preference, locomotor activity, and anxiety-like behaviors. A) Sucrose preference. B) Immobility time in forced swimming test (FST). C) Total distance, central distance travelled, entries to center and time spent in center of open field test (OFT). (n = 10/group. Values in each column without a common lowercase letter are significantly different ($P < 0.05$). The letter is the same, indicating no significant difference ($P > 0.05$)).

Fig. 3 Effects of deoiled sunflower seeds (SFS) on oxidative stress in cortex. A) Glutathione peroxidase (GSH-Px) activity. B) Superoxide dismutase (SOD) activity. C) Catalase (CAT) activity. D) Malondialdehyde (MDA) level. E) Reactive oxygen species (ROS) level. (n = 10/group. Values in each column without a common lowercase letter are significantly different ($P < 0.05$). The letter is the same, indicating no significant difference ($P > 0.05$)).

Fig. 4 Effects of deoiled sunflower seeds (SFS) on inflammatory factors in plasma. A) IL-1 level. B) IL-6 level. C) TNF-α level. (n = 10/group. Values in each column without a common lowercase letter are significantly different ($P < 0.05$). The letter is the same, indicating no significant difference ($P > 0.05$)).

Fig. 5 Effects of deoiled sunflower seeds (SFS) on amino acid levels in plasma. A) Amino acid levels. B) Tryptophan/large neutral amino acids (Trp/LNAAs) ratio. (n = 10/group. Values in each column without a common lowercase letter are significantly different ($P < 0.05$). The letter is the same, indicating no significant difference ($P > 0.05$)).

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Fig. 6 Effects of deoiled sunflower seeds (SFS) on neurotransmitters in hippocampus. A) 5-HT level. B) DA level. C) NE level. D) Ach level. E) BDNF level. (n = 10/group. Values in each column without a common lowercase letter are significantly different ($P < 0.05$). The letter is the same, indicating no significant difference ($P > 0.05$)).

Fig. 7 Effects of deoiled sunflower seeds (SFS) on mRNA expression in hippocampus. A) Brain-derived neurotrophic factor (*BDNF*) mRNA expression level. B) Glucocorticoid receptor (*GR*) mRNA expression level. C) Indoleamine 2,3-dioxygenase (*IDO*) mRNA expression level. D) Interleukin 1β (*IL-1β*) mRNA expression level. E) Interleukin 6 (*IL-6*) mRNA expression level. (n = 10/group, Values in each column without a common lowercase letter are significantly different ($P < 0.05$). The letter is the same, indicating no significant difference ($P > 0.05$)).

Fig. 8 Extensive targeted metabolome and multivariate statistical analysis in hippocampus. A) Principal component analysis (PCA) plot ($R^2X = 0.895$, Q2 = 0.7). B) Partial least squares discriminant analysis (PLS-DA) plot ($R^2X = 0.984$, $R^2Y = 0.993$, $Q^2 = 0.582$). C) PLS-DA validation plot. D, F, H and J) Orthogonal partial least squares discriminant analysis (OPLS-DA) plot (D: $R^2X = 0.647$, $R^2Y = 0.978$, $Q^2 = 0.83$, F: $R^2X = 0.675$, $R^2Y = 0.958$, $Q^2 = 0.825$, H: $R^2X = 0.707$, $R^2Y = 0.932$, $Q^2 = 0.708$, J: $R^2X = 0.618$, $R^2Y = 0.995$, $Q^2 = 0.722$). E, G, I and K) S-plot, (n=5/group).

Fig. 9 Summary of differential metabolite pathway analysis by MetaboAnalyst. (a. Aminoacyl-tRNA biosynthesis; b. Glycerophospholipid metabolism; c. Valine, leucine and isoleucine biosynthesis; d. Biotin metabolism; e. Amino sugar and nucleotide sugar metabolism). B) PLS biplot describing the correlation among all variables, including the metabolite variation (X variables), biochemical indicators (Y variables) and the grouping or cluster (observation).

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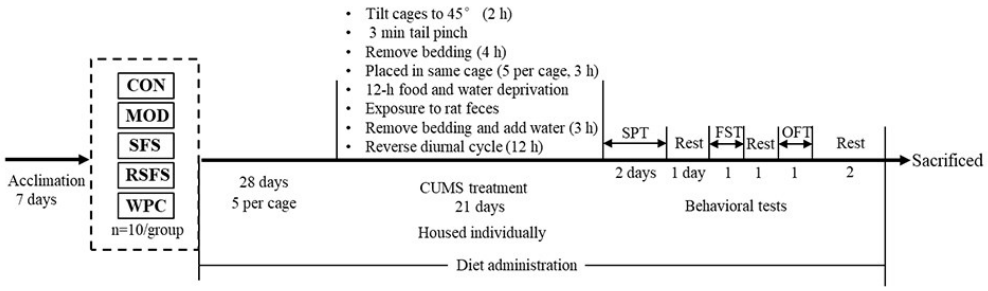

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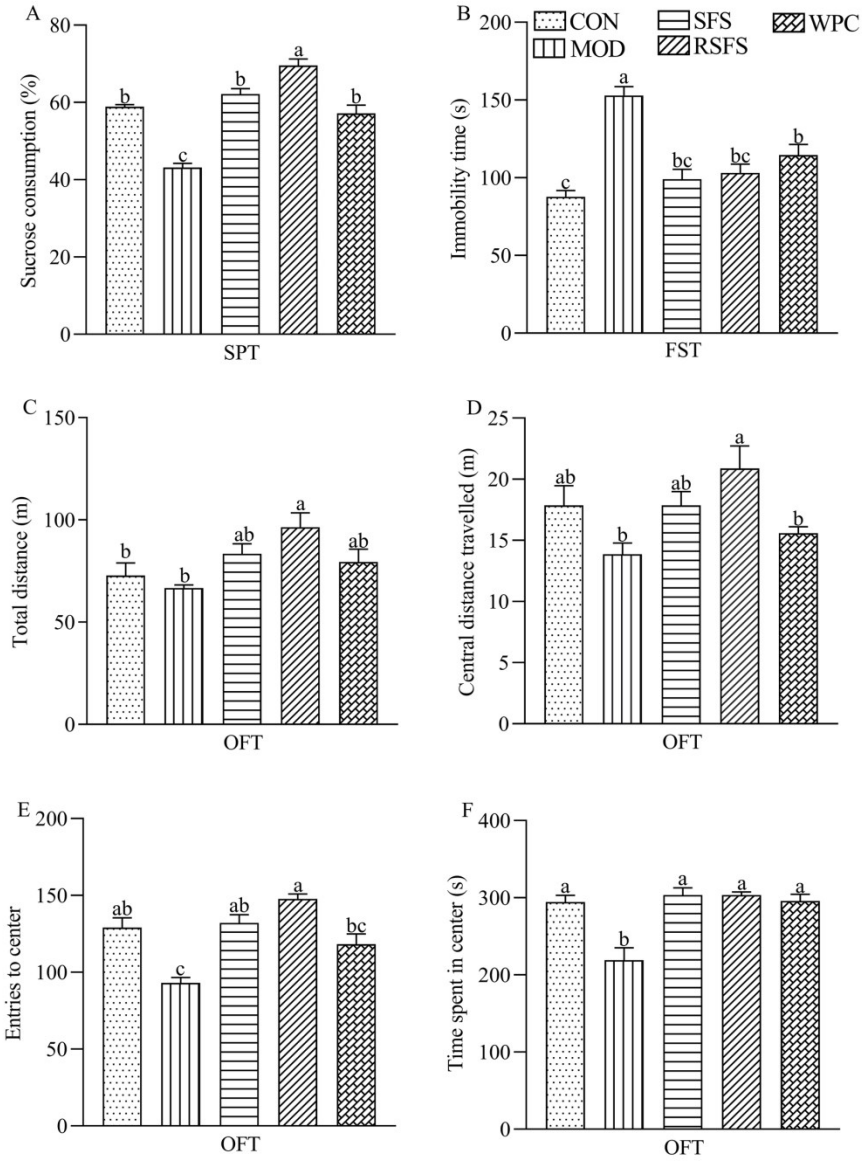

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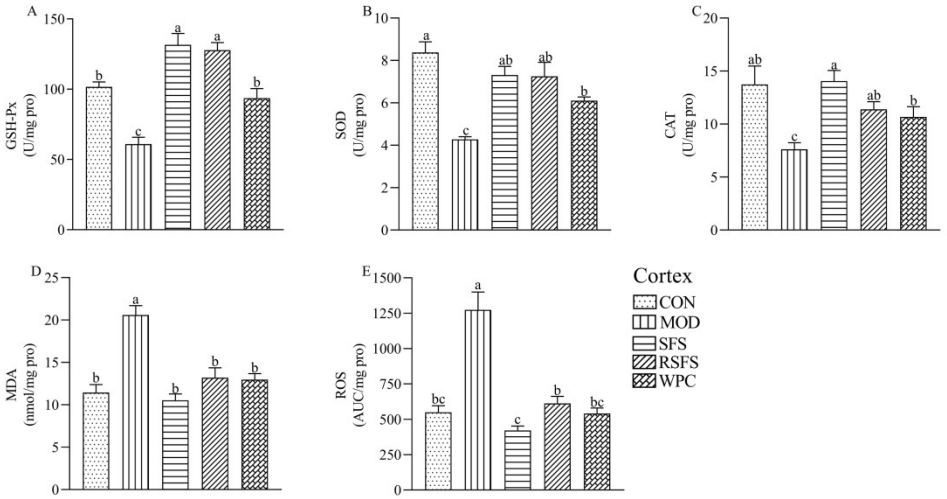

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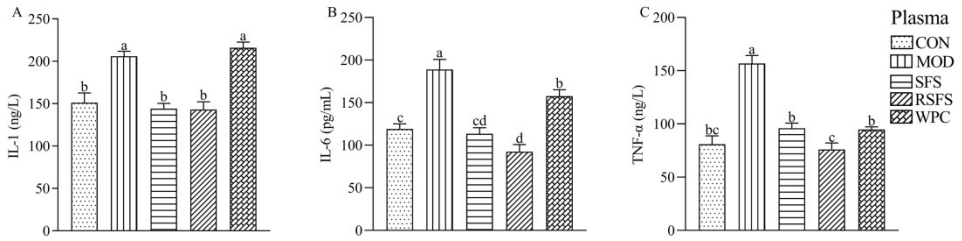

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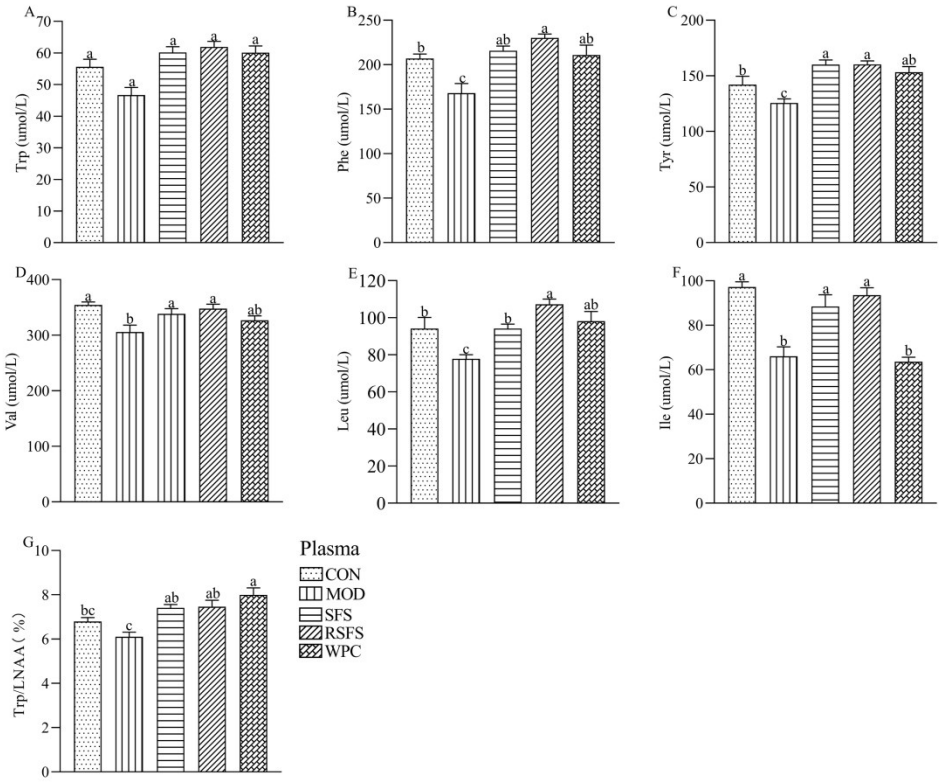

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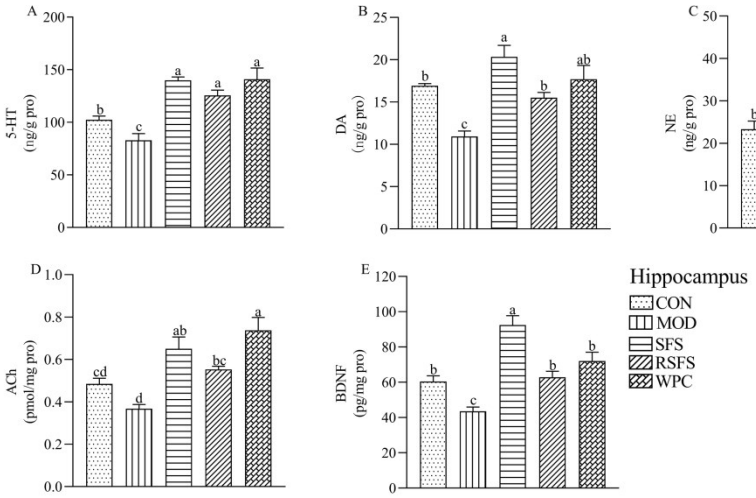

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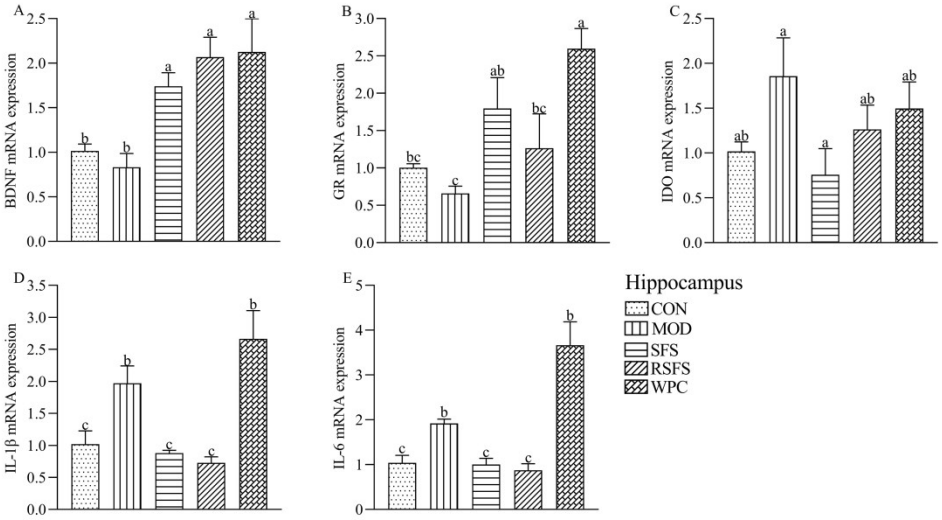

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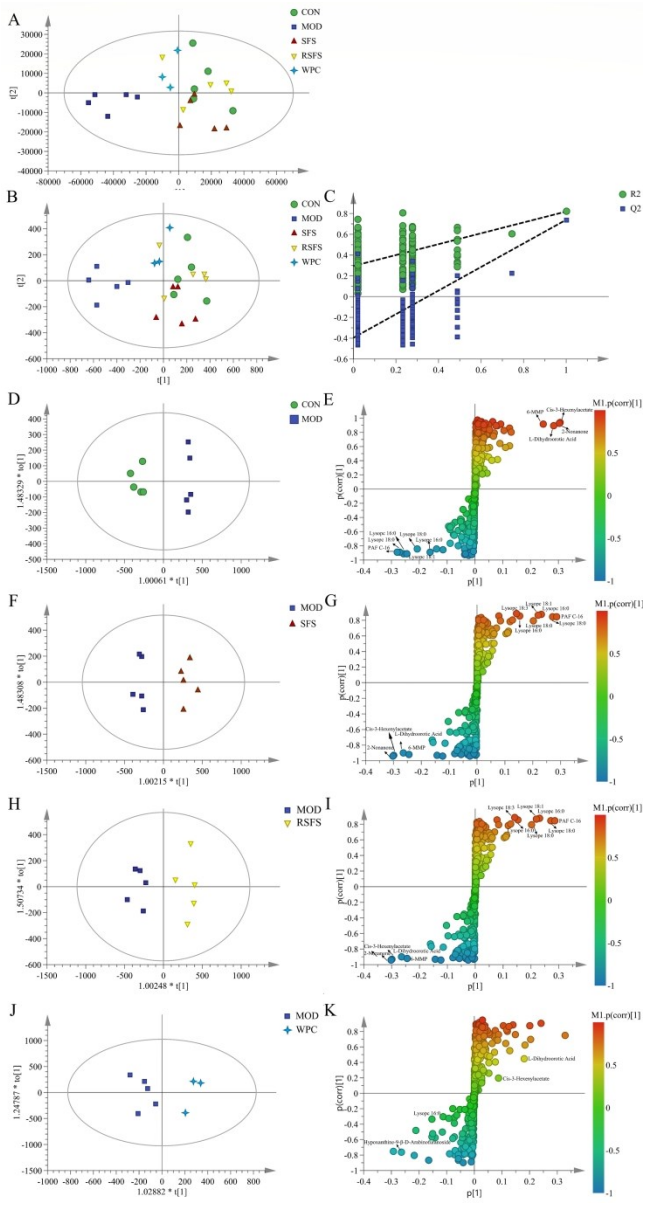

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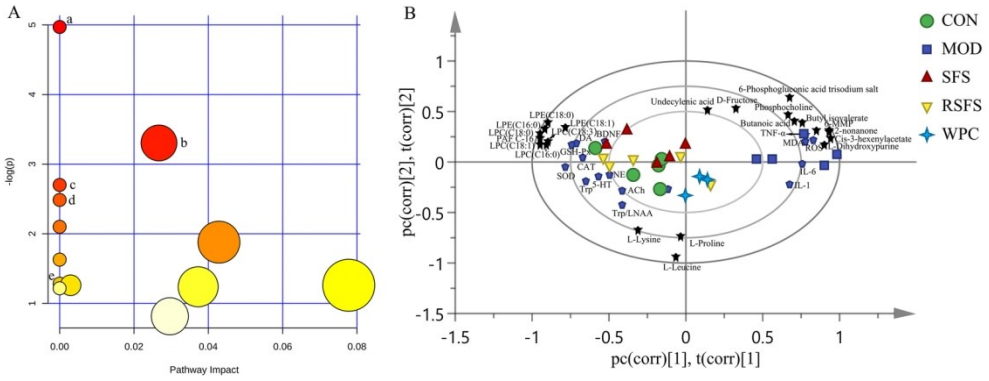

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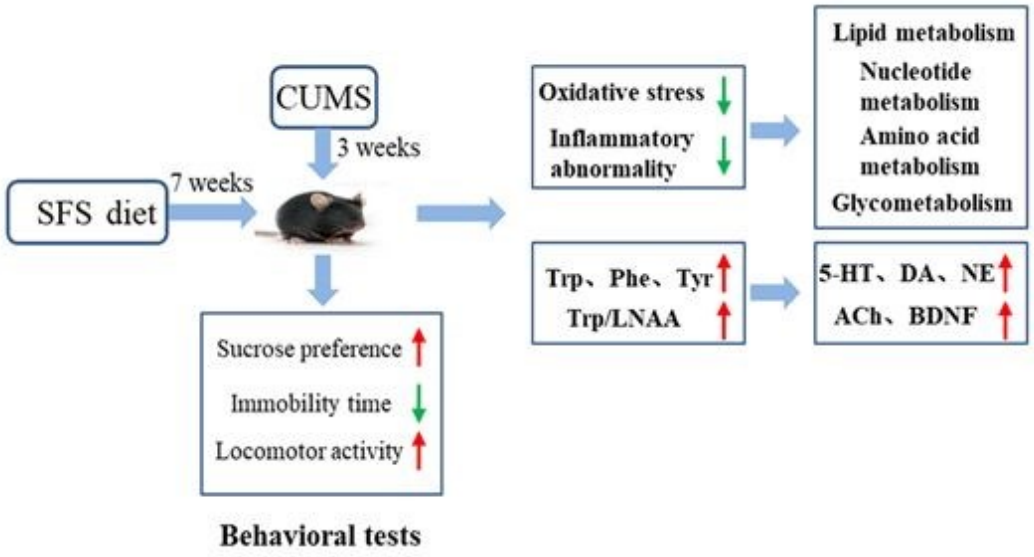

- SFS intake reversed responsiveness to anhedonia and anxiety-like behavior in stressed mice
- SFS increased aromatic amino acids and neurotransmitters in stressed mice
- SFS improved oxidative stress and inflammatory abnormalities in stressed mice
- SFS may improve depression by modulating lipid metabolism, nucleotide metabolism and amino acid metabolism.