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Effects of black soybean seed coat (BSSC) crude extract on the immune regulation, gut microbiota, and brain function of mice with sleep deprivation

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ABSTRACT

Circadian rhythm disruption induces oxidative stress in the brain and gut, causing chronic inflammation and immune imbalance. Using the Lafayette sleep fragmentation device to simulate sleep deprivation, sleep disruption was induced in 6-week-old male C57Bl/6J mice for a duration of 28 days. These results showed administration of BSSC crude extract could significantly differentiate helper T cells into Th1, reduce Th2/Th1 ratio, and improve eosinophil and basophil counts due to sleep deprivation. Furthermore, the crude extract notably improved reduced gut microbial abundance and enhanced gut barrier integrity caused by sleep deprivation. Gene expression analysis showed BSSC crude extract mitigated dysregulation in rhythmic and serotonin-related genes, reducing inflammatory gene expression in the brain. In conclusion, BSSC crude extract has the potential to address sleep disturbance-induced immune imbalance, reduced gut microbial abundance, altered gut microbiota, and brain gene disruption.

1. Introduction

According to a 2022 survey conducted by the Ministry of Labor in Taiwan, approximately 11 % of workers are engaged in nighttime employment, leading to disrupted circadian rhythms and sleep-wake cycles. Sleep deprivation can result in fatigue, cognitive impairments, anxiety, and depression (Siengsukon et al., 2018). In addition, sleep deprivation negatively impacts memory and attention, leading to decreased work performance and increased error rates, as well as an elevated risk of accidents (Lim and Dinges, 2010). Sleep deprivation can also led to obesity, cancer (Kettner et al., 2016; Papantoniou et al., 2018), metabolic syndrome, and cardiovascular diseases (Gangwisch, 2014). Therefore, addressing sleep deprivation and its associated physical and psychological health issues is an urgent health concern.

Stress and circadian rhythms are recognized as significant factors influencing gut microbiota composition (Cerdá et al., 2016). Metabolites produced by gut bacteria, such as SCFAs, can signal to the brain through various pathways, regulating brain function and metabolism (Nguyen et al., 2015). Additionally, many mental health disorders such as depression, anxiety, and schizophrenia, as well as increased gut permeability, have been linked to dysbiosis or imbalances in gut microbiota (Misiak et al., 2020). El et al. (2020) pointed out that significant differences in gut microbiota composition between sleep-deprived and regular sleepers (El et al., 2020).

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Black soybean (*Glycine max*) is a common food crop in the genus *Glycine* in the Fabaceae family. Black soybeans are rich in nutritional components such as proteins, minerals, vitamins, and abundant anthocyanins (Fetriyuna, 2015). Research demonstrated that black soybeans are enrich in flavonoids and phenolic compounds, which can prevent oxidative damage (Takahashi et al., 2005), metabolic syndrome (Kusunoki et al., 2015), neurodegenerative diseases (González-Sarrías et al., 2017), and regulate neuroinflammation (Micek et al., 2022). Furthermore, black soybeans exhibit superior antioxidant activity than yellow soybeans (Jeng et al., 2010; Kumar et al., 2010), attributed to the higher content of anthocyanins in their seed coats (Correa et al., 2010; Kim et al., 2008). Due to black soybeans possessing biological activities that can mitigate oxidative damage, reduce neurodegeneration, regulate neuroinflammation, this study investigates the effect of black soybean seed coat (BSSC) crude extract on stress, disrupted gut barrier function, dysbiosis, inflammation, and circadian rhythm disturbances of mice with sleep deprivation.

2. Materials and methods

2.1. The extraction, identification, and quantification of the major compounds from BSSC crude extracts

Different varieties of the black soybeans (Tainan 3, Tainan 5, Tainan 8, Tainan 9, Kaohsiung 7) were obtained from Taiwan, Council of Agriculture. The BSSC was collected and extracted by 50 % ethanol for 7 days. Then, the resultant solution was filtered and evaporated to dryness using a rotavapor under reduced pressure at 45 °C, and kept in stored at −80 °C for analysis. Different varieties of the BSSC crude extracts were analyzed using UHPLC (Ultra performance liquid chromatography; UltiMate 3000 Rapid Separation Dual System, Thermo Fisher Scientific) system coupled with high resolution mass spectrometer (Orbitrap Fusion Lumos Tribrid mass spectrometer, Thermo Fisher Scientific, San Jose, CA, USA) in positive ion detection mode. The solid phase was an ACQUITY UPLC BEH C_{18} column (1.7 μm, 50 × 2.1 mm; Waters, MA, USA). For the mobile phase, the elution conditions were 0–1.0 min of 5 % A (acetonitrile + 0.1 % formic acid) to B (H_2O + 0.1 % formic acid); 1.0–11.0 min of 5–100 % A to B (linear gradient); 11.0–13.0 min of 100 % A to B; 13.0–13.2 min of 100–5 % A to B (linear gradient); 13.2–15.0 min of 5 % A. The column oven temperature was 40 °C and the flow rate was 0.4 mL/min. The mass data were converted to mzXML format and uploaded to Global Natural Products Social Molecular Networking (GNPS, https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp). The molecular networking analysis was conducted according to the GNPS documentation.

2.2. Animals and procedures

C57BL/6J mice, aged 5 weeks, were procured from the National Laboratory Animal Center in Taipei, Taiwan. These mice were housed and cared for in the Laboratory Animal Center of National Chung Hsing University, where they were kept under stable environmental conditions, including a 12-hour light/dark cycle, a temperature of 23 ± 2 °C, and a relative humidity of 60 ± 10 %. They were provided with a standard laboratory diet and had unrestricted access to water. All experimental procedures involving animals were thoroughly examined and approved by the Institutional Animal Care and Use Committee (IACUC) of National Chung Hsing University (approval number: 110–089). The mice were acclimated to the environment and diet for 1 weeks prior to SD. A total of 18 mice were randomly assigned to the control (CN), chronic sleep deprivation (CSD) and CSD with BSSC (CSDBB) groups (n = 6 per group). In the present study, the CSD was applied to the CSD and CSDBB group for 28 day-CSD. Body weight and food intake were recorded. After the completion of 28 days, fecal samples were collected immediately, and subsequent the behavior tests were carried out. At the end of the experiment, all mice were euthanized to obtain samples, including the blood, brain tissue (hippocampus, PFC, and cerebral cortex) and proximal colon. Blood samples were collected via *retro*-orbital bleeding using microhematocrit capillary tubes containing heparin sodium. Whole blood (300 μL) was used for white blood cell classification and sent to a certified laboratory (Accuspeedy Medical Laboratory, Tainan, Taiwan) for analysis.

2.3. Sleep deprivation

In the present study, the sleep fragmentation (Lafayette Instrument Company, Lafayette, IN, USA) was applied to 28 day-CSD. The detailed protocol and device specifications were previously described (Cabrera-Aguilera et al., 2020) with slight modified. Sleep disruptions were triggered using a nearly silent mechanical motor fitted with a horizontal bar sweeping just above the cage floor, spanning from one side to the other, within the standard mouse laboratory enclosure. This automated system, devoid of human interference, reduced stress for the animals. The intervals between each sweep were set at 2 min during the mice's sleep period, which ranged from 8 a.m. to 6p.m., and this regimen persisted for 28 consecutive days.

2.4. Stress and endotoxin markers in plasma

Dexamethasone is an exogenous steroid that can negatively feedback on the pituitary gland, thereby inhibiting the secretion of adrenocortical hormone. Therefore, an intraperitoneal injection of dexamethasone is used to measure corticosterone levels, assessing the function of the hypothalamic–pituitary–adrenal (HPA) axis. The steps of the experiment are as follows: One day before the test (Day 18), an intraperitoneal injection of saline is administered, and blood is collected from the cheek to obtain mouse plasma (100 μL) as the blank group one hour later. On Day 19, an intraperitoneal injection of dexamethasone is given, and blood is collected from the cheek one hour later to obtain mouse plasma (100 μL) as the test group. The Corticosterone ELISA kit is used to measure the corticosterone content in the plasma. The stress marker corticosterone was assessed using the Corticosterone AssayMax enzyme-linked immunosorbent assay (ELISA) Kit (Assaypro, Saint Charles, MO, USA). The formula for corticosterone suppression efficiency is: 100 − ([Corticosterone] after DEX/[Corticosterone] after saline) * 100.

The endotoxin marker LPS was detected using ToxinSensor Chromogenic LAL Endotoxin Assay Kit (Cat# L00350, GenScript, Piscataway, NJ). The ELISA procedures were carried out in accordance with the manufacturer's provided instructions.

2.5. Behavior test

2.5.1. Open field test (OFT)

The OFT method has been previously described (Seibenhener and Wooten, 2015). This assessment was carried out after 27 day-CSD. Each mouse was individually placed in an acrylic enclosure measuring 50 cm × 50 cm × 38 cm, with a central 30 cm × 30 cm area, and allowed to explore for 15 min. The bouts in central area (times) within the first 5 min and the distance traveled within 15 min were automatically measured and quantized using CleverSys software (TopScan Lite, CleverSys, USA) to assess anxiety and locomotor activity. To eliminate any potential cues from previous animals, the apparatus was cleaned with 75 % ethanol before each evaluation.

2.5.2. Elevated plus maze (EPM)

The EPM was conducted following previously established procedures (Walf and Frye, 2007). This assessment was carried out after 27 day-CSD. The EPM apparatus for the mice consists of two opposing open arms (measuring 35 cm × 5 cm) intersected by a central platform, as well as two opposing closed arms (measuring 35 cm × 5 cm) with tall walls (20 cm), elevated 40 cm above the floor. The animals were individually placed at the center of the EPM with their heads oriented

toward one of the open arms. The 10-minute test session was recorded on video for subsequent analysis. The number of entries into the open arms (open/open + closed) were automatically quantified by CleverSys software. The apparatus was cleaned with 75 % ethanol in preparation for the next session.

2.5.3. Novel object recognition test (NORT)

The NORT was performed as previously described (Lueptow, 2017) and completed over 3 days (i.e., habituation, familiarization, and testing). On the habituation day, the mice were placed in the box for 10 min. On the familiarization day (24 h after habituation), the mice were allowed to explore two identical objects (round plastic box: 8 cm diameter × 4.5 cm height) for 10 min. On the testing day (24 h after familiarization), one of the familiar objects was replaced with a novel object (toy building block: 6.2 cm length × 6.2 cm width × 4.2 cm height) for 10 min. The time utilized for the exploration of both objects on the testing day was recorded manually, and the discrimination index (novel – familiar/novel + familiar) was calculated.

2.5.4. T maze test (TM)

The TM was conducted following a previously described protocol (Yadang et al., 2020). This assessment was carried out after 28 day-CSD. The T-shaped maze consisted of a start arm (16.5 cm in length, 10 cm in width, and 28 cm in height) and two choice arms (10 cm in length, 48.2 cm in width, and 28 cm in height). During the habituation phase, the animals were placed in the T-maze for a 5-minute period to become familiar with the apparatus. The first arm chosen by the animal was noted as the preferred arm, and the other as the discriminated arm. In the acquisition phase, the discriminated arm chosen by the mouse was closed off, and food was placed in the preferred arm. The mouse was positioned in the starting arm and allowed to move to the open arm. This procedure was done for 5 min for each mouse. The time spent to the preferred arm were automatically recorded and analyzed using CleverSys software. The maze was cleaned with 75 % ethanol to prepare it for the subsequent session.

2.6. Flow cytometry immunoassay

All antibodies used in this study were sourced from isolated naive CD4 + T cells obtained from the spleen of mice, as described by Yang et al. (2018). Spleen tissues from each experimental mouse were fragmented into small pieces and mechanically disrupted to extract the cells. A single-cell suspension was prepared using 200-mesh screens for filtration. The labeling, permeabilization, and fixation steps were conducted following the instructions provided in the kit, which included MS FITC Rat anti-mouse CD4 (Clone GK1.5, Biolegend, San Diego, CA), MS PE anti-mouse NK 1.1 (Clone GK1.5, Biolegend, San Diego, CA), MS PE anti-mouse IFN-γ antibody (Clone XMG1.2, Biolegend, San Diego, CA), and MS PE anti-mouse IL-4 antibody (Clone PC61, BD Biosciences, San Jose, CA). The final analysis was carried out using a Beckman Coulter FC500 cytometer (Beckman Coulter, Brea, CA, U.S.A.), and data analysis was performed using FC500 flow cytometry analysis software CXP.

2.7. Western blotting analysis

Protein expressions in the proximal colon were assessed using Western blotting following a previous protocol (Ho et al., 2019). Membranes were incubated with primary antibodies (ZO-1, Occludin, Claudin-1, and β-actin) on a shaker at 4 °C for 12 h. In this study, the primary antibodies used were anti-ZO-1 (Cat# 21773–1-AP, 1:1000, Proteintech), anti-Occludin (Cat# 27260, 1:1000, Proteintech), anti-Claudin-1 (Cat# AF0127, 1:1000, Affinity), and anti-β-actin (Cat# 4970S, 1:3000, Cell Signaling). Immunoreactive bands were visualized using enhanced chemiluminescence (ECL) for routine immunoblotting. The relative protein expressions were quantified by densitometry using Image J software (Wayne Rasband, Madison, WI, USA). The values were then normalized to β-actin levels in the proximal colon and expressed as fold increases.

2.8. Fecal SCFA analysis

Fecal samples weighing 40 mg were initially collected and stored in 400 μL of water containing 0.5 % phosphoric acid. These samples were promptly frozen at −80 °C after collection. Upon thawing, the fecal samples were homogenized and then subjected to centrifugation at 14,000 rpm for 15 min, with the supernatant collected for further analysis. Subsequently, an equal volume of ethyl acetate was added to the supernatant, and the solution was vigorously mixed using a vortex for 2 min, followed by another centrifugation at 14,000 rpm for 15 min. The organic phase was isolated and preserved at −80 °C until further analysis. Gas chromatography was performed following previously established protocols (Tung et al., 2021). Identification of the short-chain fatty acids (SCFAs) relied on comparing the retention times with those of standard compounds (acetic acid, propionic acid, and butanoic acid).

2.9. Fecal microbiota analysis

The amplification and library construction of the 16S rRNA gene followed the protocols recommended by Illumina (https://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html). In brief, we utilized universal primers 341F (forward primer; 5′-CCTACgggNggCWgCAg-3′) and 805R (reverse primer; 5′-gACTACHCggg-TATCTAATCC-3′), which contained Illumina overhang adapter sequences in both the forward and reverse primers. These primers were employed to amplify the V3-V4 region of the bacterial 16S rRNA gene through polymerase chain reaction (PCR) with a limited number of cycles. Subsequently, the Nextera XT Index kit from Illumina Inc. in San Diego, CA, USA, was used to attach Illumina sequencing adapters and dual-index barcodes to the amplicons. We assessed the quantity and quality of the sequenced library using a Qsep100 Analyzer from BiOptic Inc. in Taipei, Taiwan. For more detailed procedures, information on the database, validation, and analysis, the reference is recommended to Tung et al. (2021).

2.10. RNA extraction and real-time quantitative PCR (qPCR)

RNA samples extracted from the brain regions, including the cerebral cortex, hippocampus, and prefrontal cortex (PFC), of each mouse were preserved in RNA Save (Biological Industries, Kibbutz Beit Haemek, Israel) and stored at −80 °C until further analysis. These samples were extracted using the EZ-RNA II total RNA isolation kit from Biological Industries. Subsequently, 1 μg of RNA per 20 μL of reaction volume was reverse transcribed into cDNA using the ToolsQuant II Fast RT Kit (TOOLS, Taipei, Taiwan). The quantitative PCR (qPCR) reactions included TOOLS 2X SYBR qPCR Mix (TOOLS) and were evaluated in a LightCycler® 480 (Roche, Basel, Switzerland). The cDNA template was analyzed for various genes, including circadian genes (*Bmal-1*、*Clock*、*Per1*、*Per2*、*Cry1* and *Cry2*), inflammatory genes (*Tnf-α*、*Il-6* and *Ifn-γ*), and serotonin-related genes (*Ido-1*、*Tph-2*、*5-Htr1a* and *Slc6a4*). The relative mRNA expression levels of each target gene were calculated based on the threshold cycle (Ct) values and were normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

2.11. Statistical analysis

Statistical analysis was carried out using the GraphPad Prism software version 6.0 (GraphPad Software, San Diego, CA, USA). Data were subjected to analysis using the Mann-Whitney *U* test, a non-parametric statistical test. All results are presented as the mean ± standard error of the mean (SEM). P-values less than 0.05 were considered indicative of statistically significant differences.

3. Results

3.1. Analysis of different varieties of BSSC crude extracts using UHPLC-MS/MS

To understand the metabolomic profiles of different varieties of BSSC crude extracts, we performed molecular networking analysis using GNPS. As shown in Fig. 1A, 1414 nodes (a node represents a precursor ion of a metabolite) and 31 molecular clusters (connected nodes > 3) were found in the molecular networking analysis. Because flavonoids could might be a preventive factor for sleep deprivation (Wang et al., 2022). Therefore, we focused on the screening of flavonoid components in BSSC crude extracts (Fig. 1B). Among these nodes, the top 10 flavonoid compounds based on signal intensity are identified as follows: cyanidin-3-*O*-glucoside, 6″-*O*-malonyldaidzin, 6-*O*-malonylglycitin, genistein-7-glucoside, delphinidin-3-*O*-glucoside, daidzein-7-*O*-glucoside, glycitin, petunidin-3-*O*-glucoside, daidzin-7-sulfate, and 6″-*O*-malonylgenistin according to the previous studies (Lin et al., 2020; Lin et al., 2023; Zhang et al., 2018) (Table 1). According to the results of the flavonoid group analysis, there are differences in the flavonoid contents among different varieties of BSSC crude extracts. Among these, Taiwan 9 exhibits the highest intensity in the top-ranked compound, cyanidin-3-*O*-glucoside, compared to other BSSC crude extracts. Consequently, we selected Taiwan 9 as the experimental strain to evaluate the effect of BSSC crude extract on immune regulation, gut microbiota composition, and brain function in a mouse model subjected to sleep deprivation.

3.2. Effect of BSSC crude extract on body weight, food intake, and stress hormones in mice with sleep fragmentation-induced sleep deprivation

As shown in Fig. 2A, during the course of the experiment, the body weight of mice in the CN group, CSD group, and CSDBB group all exhibited normal and gradual increases, and there were no significant differences in the changes in final body weight among the CN group, CSD group, and CSDBB group (Fig. 2B). On the other hand, there were no differences in food intake among the groups (Fig. 2C), indicating that inducing sleep deprivation through 28 days of fragmented sleep did not affect the body weight and food intake of mice. Fig. 2D showed the stress indicator corticosterone levels in mice, after 19 days of induced sleep deprivation followed by intraperitoneal injection of dexamethasone, indicated that mice in the CSD group fails to suppress HPA axis activity. However, mice in the CSDBB group were able to effectively suppress HPA axis activity. The results indicated that after the administration of BSSC crude extract could significantly increase their ability to regulate corticosterone levels.

3.3. Effect of BSSC crude extract on anxiety, locomotor activity and cognition in mice with sleep fragmentation-induced sleep deprivation

An open-field test was to assess the anxiety and locomotor activity levels of mice. As shown in Fig. 3A, the CSD group exhibited a significantly lower number of entries into the central zone compared to the CN group, indicating higher anxiety levels in the CSD group. However, after supplementation with BSSC crude extract, there were no significant differences compared to the CSD group. In terms of locomotor activity, the total distances traveled by mice in the CN, CSD, and CSDBB groups showed no significant differences (Fig. 3A). In the elevated plus maze test, there were no significant differences in the number of entries into the open arms among the CN group, CSD group, and CSDBB group (Fig. 3B). The novel object recognition test was employed to assess the long-term memory impact of sleep deprivation in mice. The discrimination index showed no significant differences among the CN, CSD, and CSDBB groups (Fig. 3C). Although the CSD group exhibited a declining trend compared to the CN group, and after supplementation with BSSC crude extract, there was a recovery trend similar to the CN group. In addition, the T-maze test was employed to assess the short-term memory effect of sleep deprivation in mice. The total time spent in the CN group, CSD group, and CSDBB group showed no significant differences (Fig. 3D). However, compared to the CN group, the CSD group exhibited an increasing trend in time spent, and after supplementation with BSSC crude extract, the CSDBB group showed a slight reduction in time spent, trending towards the CN group.

3.4. Effect of BSSC crude extract on immunological leukocyte, T helper cells (Th cells) and natural killer (NK) cells in mice with sleep fragmentation-induced sleep deprivation

Immunological leukocyte analysis of mouse blood was analyzed (Fig. 4A), and the results revealed that the CSD group exhibited a significant increase in eosinophils compared to the CN group. However, after supplementation with BSSC crude extract, the CSDBB group showed a declining trend in eosinophils. Regarding basophils, there were no significant differences between the CN group and the CSD group, but after supplementation with BSSC crude extract, the CSDBB

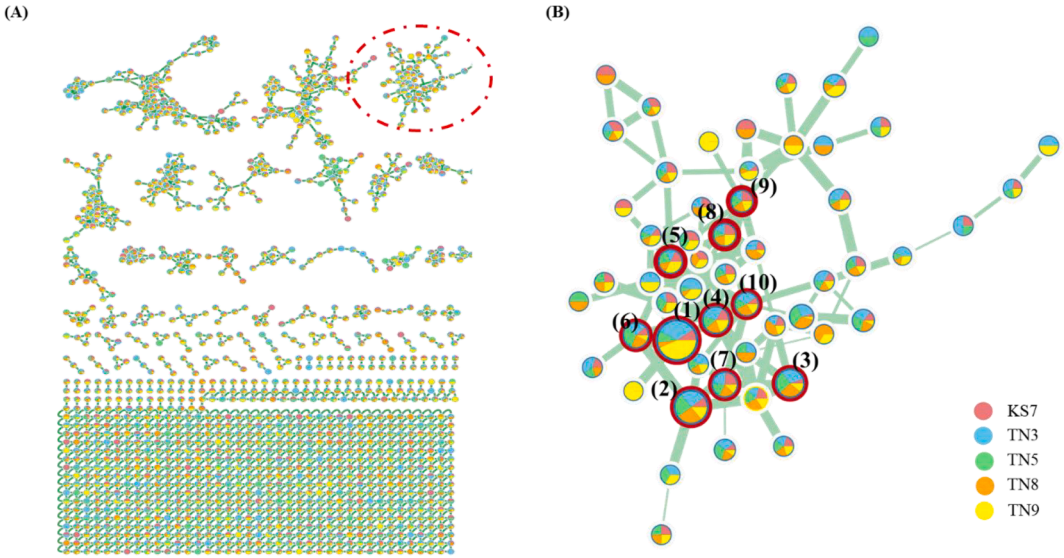

Fig. 1. Analysis of different varieties of black soybean seed coat crude (BSSC) extract using UHPLC-MS/MS. (A) Molecular networking of different varieties of BSSC crude extract. (B) Flavonoid glycosides-related cluster in different varieties of BSSC crude extract.

Table 1
The molecular network of flavonoid composition ranking 1 to 10 in different varieties of black soybean seed coat (BSSC) crude extract.

Rank	Compound name	RT	Parent mass	Intensity (10^8)				
				KS7	TN3	TN5	TN8	TN9
1	Cyanidin-3-*O*-glucoside	3.51	449.108	0.31	2.86	0.62	0.08	4.85
2	6′′-*O*-Malonyldaidzin	4.58	503.118	0.29	1.79	1.81	1.30	0.42
3	6-*O*-Malonylglycitin	4.61	533.129	0.18	0.72	0.55	0.40	0.21
4	Genistein-7-glucoside	4.56	433.118	0.14	0.30	0.20	0.16	0.17
5	Delphinidin-3-*O*-glucoside	4.44	465.103	0.27	0.23	0.31	0.21	0.30
6	Daidzein-7-*O*-glucoside	4.09	417.118	0.39	1.50	0.80	0.62	0.24
7	Glycitin	4.16	447.129	0.20	0.66	0.36	0.21	0.13
8	Petunidin-3-*O*-glucoside	5.49	479.118	0.81	0.78	n/a	0.95	0.08
9	Daidzin-7-sulfate	4.42	495.113	0.06	0.04	0.20	0.11	0.13
10	6′′-*O*-Malonylgenistin	4.99	519.113	0.23	0.90	0.70	0.69	0.53

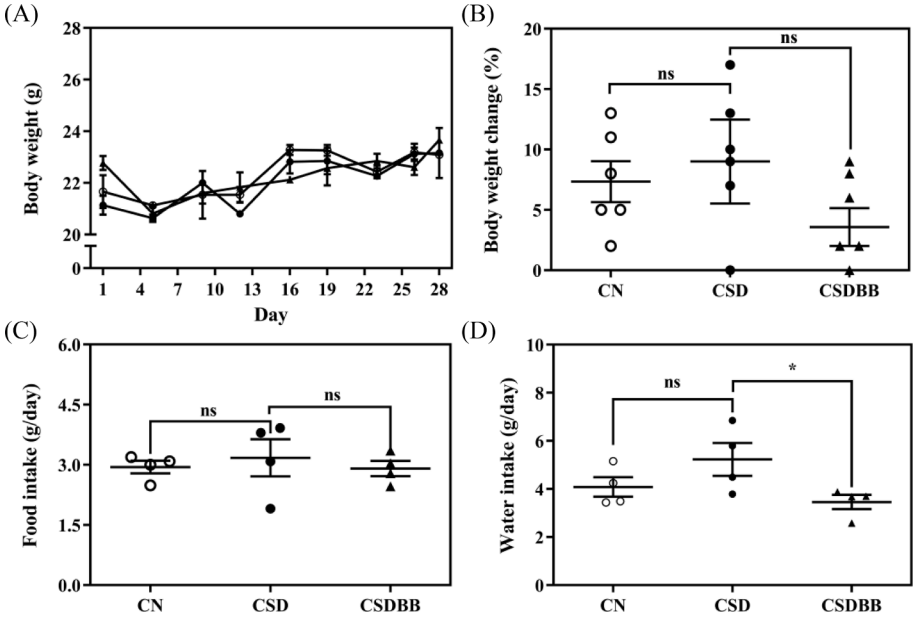

Fig. 2. Effect of black soybean seed coat (BSSC) crude extract on body weight, food intake, and corticosterone regulation in mice with sleep fragmentation-induced sleep deprivation. (A) 28-day growth curve of body weight. (B) 28-day change of body weight. (C) Average of food intake. (D) Dexamethasone suppression of corticosterone CN: Control group; CSD: Chronic sleep deprivation group; CSDBB: Chronic sleep deprivation + 300 mg/kg black soybean extract group. Statistical methods are used by the unpaired one-sided *U* test. Values represent the mean ± SEM ($n = 6$), ns is not significant different; $^{**}p < 0.01$ compared with CSD.

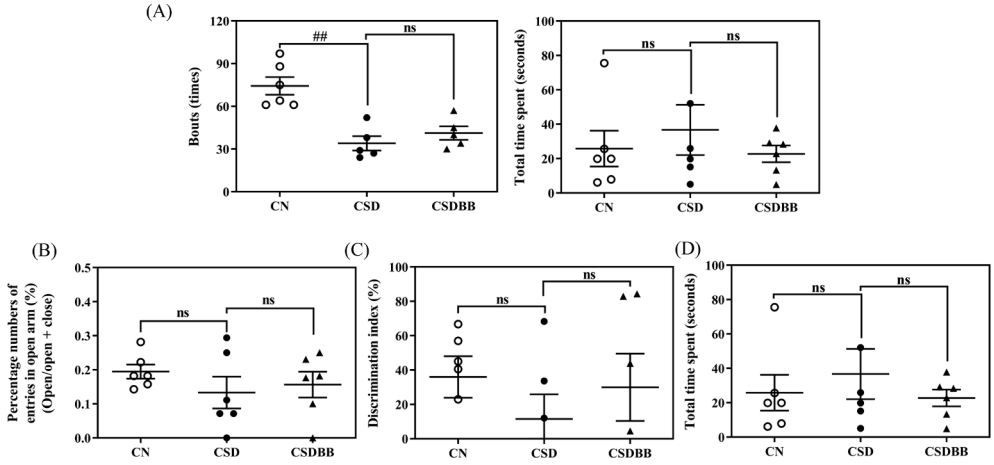

Fig. 3. Effect of black soybean seed coat (BSSC) crude extract on anxiety, locomotor activity, and cognition in mice with sleep fragmentation-induced sleep deprivation. (A) Bouts in center (times) and total distance traveled of open field test. (B) Percentage numbers of entries in open arm of elevated plus maze test. (C) Discrimination index of novel object recognition test. (D) Total time spent of T maze test. CN: Control group; CSD: Chronic sleep deprivation group; CSDBB: Chronic sleep deprivation + 300 mg/kg black soybean extract group. Statistical methods are used by the unpaired one-sided *U* test. Values represent the mean ± SEM (n = 6), ns is not significant different; $^{\#\#}p < 0.01$ compared with CN.

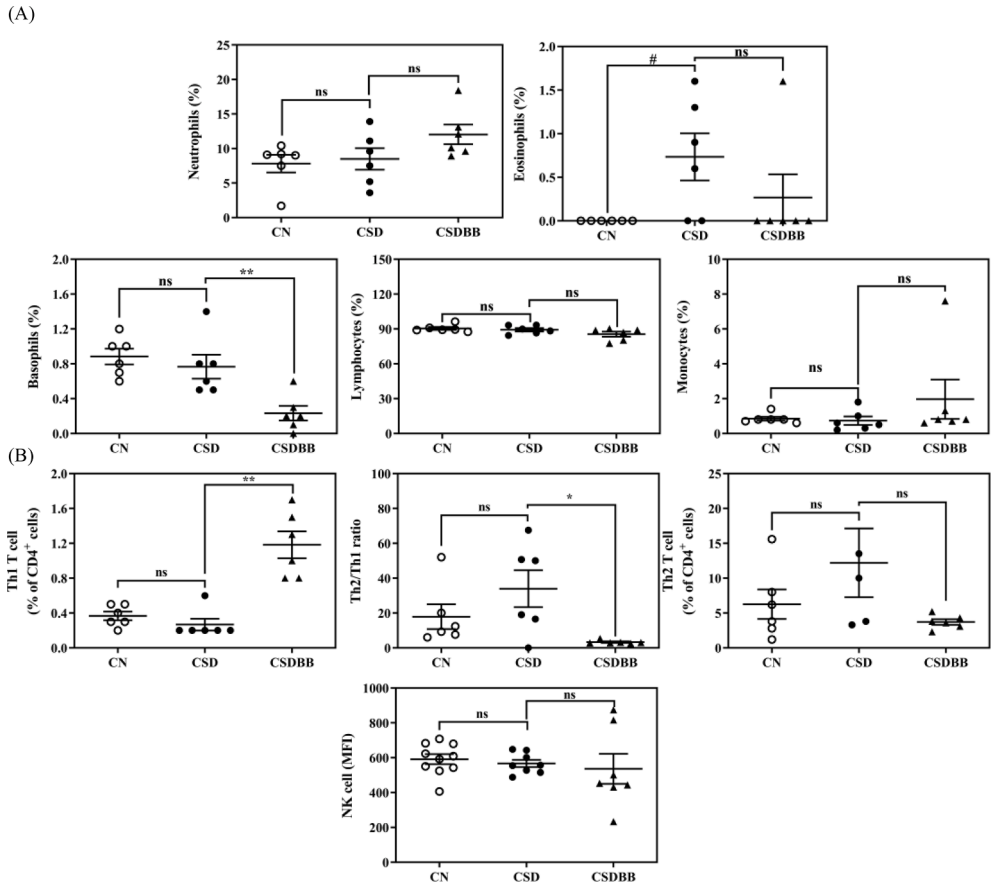

Fig. 4. Effect of black soybean seed coat (BSSC) crude extract on (A) immunological leukocyte, (B) T helper cells (Th cells), and (C) natural killer (NK) cells in mice with sleep fragmentation-induced sleep deprivation. CN: Control group; CSD: Chronic sleep deprivation group; CSDBB: Chronic sleep deprivation + 300 mg/kg black soybean extract group. Statistical methods are used by the unpaired one-sided *U* test. Values represent the mean ± SEM ($n = 6$), ns is not significant different; $^{\#}p <$ 0.05 compared with CN; $^{*}p < 0.05$; $^{**}p < 0.01$ compared with CSD.

group showed a significant decrease. In addition, neutrophils, lymphocytes, and monocytes showed no significant differences among these three groups.

Furthermore, the differentiation of Th cells (Fig. 4B) and NK cells (Fig. 4C) were analyzed. The results indicated that there was no significant difference in Th1 differentiation in the CSD group compared to the CN group. However, after supplementation with BSSC crude extract, the CSDBB group exhibited a significant increase in Th1 differentiation. In the term of Th2 differentiation, there were no significant differences between the CN and CSD groups, but the CSD group showed an increasing trend. However, after supplementation with BSSC crude extract, the CSDBB group exhibited a decrease trend in Th2 differentiation, approaching the levels observed in the CN group. Regarding the Th2/Th1 ratio, there were no significant differences between the CN and CSD groups, but the CSD group displayed an increasing trend. However, after supplementation with BSSC crude extract, the CSDBB group showed a significant decrease in the Th2/Th1 ratio. Regarding the analysis of the effect of sleep fragmentation induction on NK cells, the results indicated no significant differences among the CN, CSD, and CSDBB groups.

3.5. Effect of BSSC crude extract on the protein expressions of tight junctions, endotoxin, and fecal short chain fatty acid (SCFA) in mice with sleep fragmentation-induced sleep deprivation

To confirm the effect of sleep fragmentation-induced sleep deprivation in mice on the integrity of tight junction proteins in the proximal colon, this study assessed the protein expression levels of ZO-1, Occludin, and Claudin-1. Additionally, LPS in the blood of mice in the CN group, CSD group, and CSDBB group was to verify whether sleep fragmentation results in damage to tight junction proteins and an increase in blood LPS levels. As shown in 5A, the CSD group of mice did not exhibit significant differences in the protein expression levels of ZO-1, Occludin, and Claudin-1 in the proximal colon compared to the CN group, although there was a decreasing trend observed for ZO-1. However, after supplementation with BSSC crude extract, the CSDBB group of mice showed a significant increase in protein expression levels of ZO-1 and Claudin-1 in the proximal colon, while there were no significant differences in Occludin. Moreover, there were no significant differences in serum LPS levels among the CN, CSD, and CSDBB groups (Fig. 5B).

The CSD group of mice showed significantly lower faecal acetic acid and total SCFA concentrations than the CN group. However, CSDBB group could increase acetic acid and total SCFA concentrations compared to the CSD group. Furthermore, propionic acid and butyric acid did not exhibit significant differences among the CN, CSD, and CSDBB groups (Fig. 5C).

3.6. Effect of BSSC crude extract on the compositions of gut microbiota in mice with sleep fragmentation-induced sleep deprivation

As shown in Fig. 6A, the CSD group exhibited a significant decrease in species richness of gut microbiota as indicated by the Observed index and Chao1 index compared to the CN group. But no significant difference was observed between Shannon index and Simpson index among the CN, CSD, and CSDBB groups. Therefore, supplementation with BSSC crude extract in the CSDBB group resulted in a significant recovery of

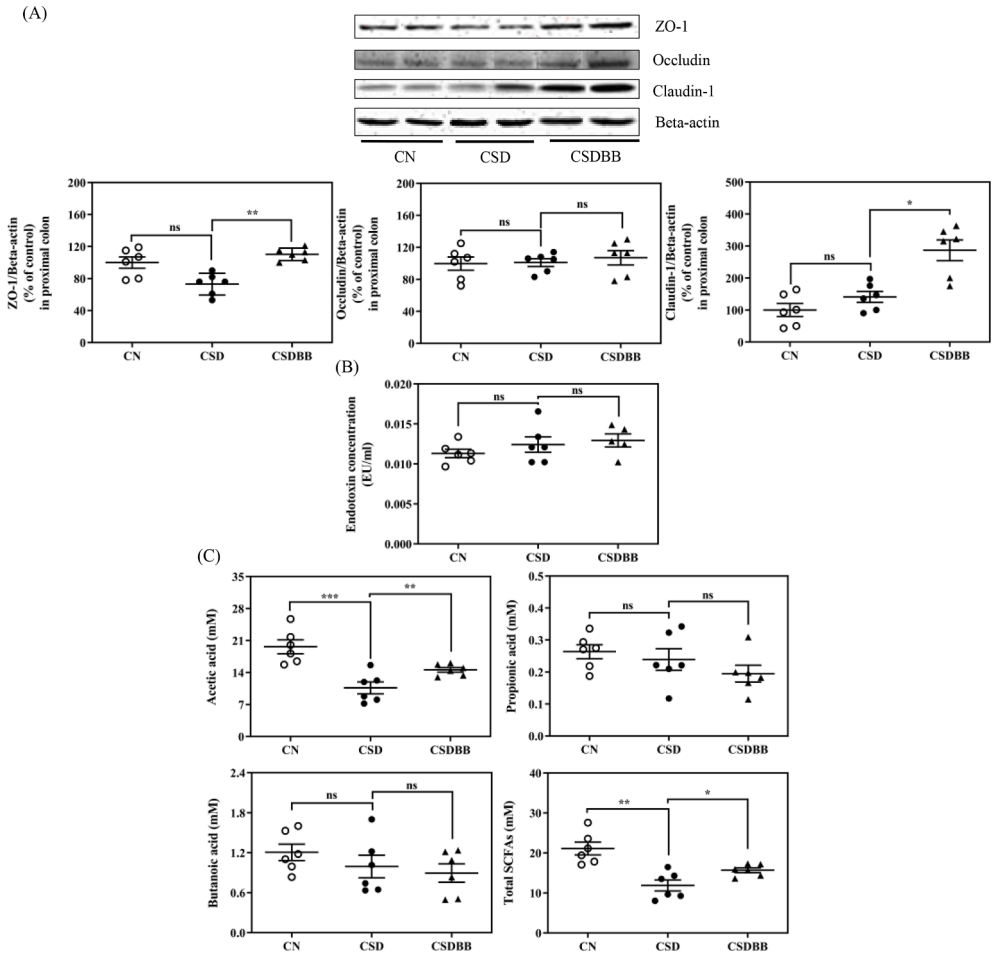

Fig. 5. Effect of black soybean seed coat (BSSC) crude extract on (A) the protein expressions of tight junctions in colon, (B) serum lipopolysaccharide, and (C) short chain fatty acids with sleep fragmentation-induced sleep deprivation. CN: Control group; CSD: Chronic sleep deprivation group; CSDBB: Chronic sleep deprivation + 300 mg/kg black soybean extract group. Statistical methods are used by the unpaired one-sided *U* test. Values represent the mean ± SEM ($n = 6$), ns is not significant different; $^{*}p < 0.05$; $^{**}p < 0.01$ compared with CSD.

gut microbiota species richness. In beta-diversity, there was a high degree of divergence between the CN group and CSD group in the principal coordinate analysis based on Bray-Curtis distance (Fig. 6B). After supplementation with BSSC crude extract, the gut bacterial communities of the CSDBB group were significantly affected and tended to recover towards the gut bacterial communities observed in the CN group.

As shown in Fig. 6C, the phylum-level results indicated that the gut microbiota of CN, CSD, and CSDBB groups are primarily composed of the phyla Firmicutes (CN group: 64.7 %; CSD group: 53.8 %; CSDBB group: 60.8 %) and Bacteroidota (CN group: 31.2 %; CSD group: 38.2 %; CSDBB group: 31.0 %). Additionally, in all three groups of mice, the proportions of the phylum Deferribacterota are as follows: CN group 4.05 %, CSD group 3.40 %, CSDBB group 8.15 %; the phylum Proteobacteria: CN group 0.04 %, CSD group 0.01 %, CSDBB group 0.06 %; and the phylum Actinobacteriota: CN group 0.05 %, CSD group 0.07 %, CSDBB group 0.01 %.

As shown in Fig. 6D, at the family level, the results indicated that compared to CN group, CSD group showed an increase in the proportions of the families Aerococcaceae, Butyricicoccaceae and Muribaculaceae, while CSDBB group exhibited a restoration of the proportion of the family Aerococcaceae to levels observed in CN group. Compared to CSD group, CSDBB group show an increase in the proportions of the families Deferribacteraceae, Enterobacteriaceae, Tannerellaceae, and UCG-010, whereas the proportions of Aerococcaceae, Eggerthellaceae, and Streptococcaceae decrease.

In CN group, the predominant genera are *Candidatus_Arthromitus*, *Candidatus_Soleaferrea*, *GCA-900066575*, *Mucispirillum*, *Muribaculum*, *NK4A214_group*, and *UCG-005*. On the other hand, CSD group were characterized by a dominant genus, *Butyricicoccus*. Furthermore, our analysis revealed that supplementation with BSSC crude extract led to a significant recovery in the relative abundance of *NK4A214_group* and *UCG-005* in CSDBB group (Fig. 6E).

3.7. Effect of BSSC crude extract on brain clock, inflammation, and serotonin-related gene expressions in mice with sleep fragmentation-induced sleep deprivation

Regarding the circadian rhythm genes of the cerebral cortex, the CSD group exhibits an upward trend in the expression of *Per2* compared to the CN group. However, after supplementation with BSSC crude extract, there is a significant reduction in the expression of *Per2*, leading to a restoration of normal circadian rhythm (Fig. 7A). In terms of inflammatory genes, BSSC crude extract effectively reduces the expression of the inflammatory gene *Tnf-α*, thereby suppressing brain inflammation (Fig. 7A). Regarding serotonin-related genes, compared to the CN group, the CSD group exhibits a significant increase in the expression levels of the *Tph-2* and *5-Htr1a*. However, supplementation with BSSC crude extract significantly reduces the expression of *Tph-2*.

In the hippocampus, the CSD group exhibits increased expression of the circadian rhythm gene *Per1*, leading to disrupted circadian rhythms. However, following supplementation with BSSC crude extract, the CSDBB group demonstrates a tendency toward restoring *Per1* expression

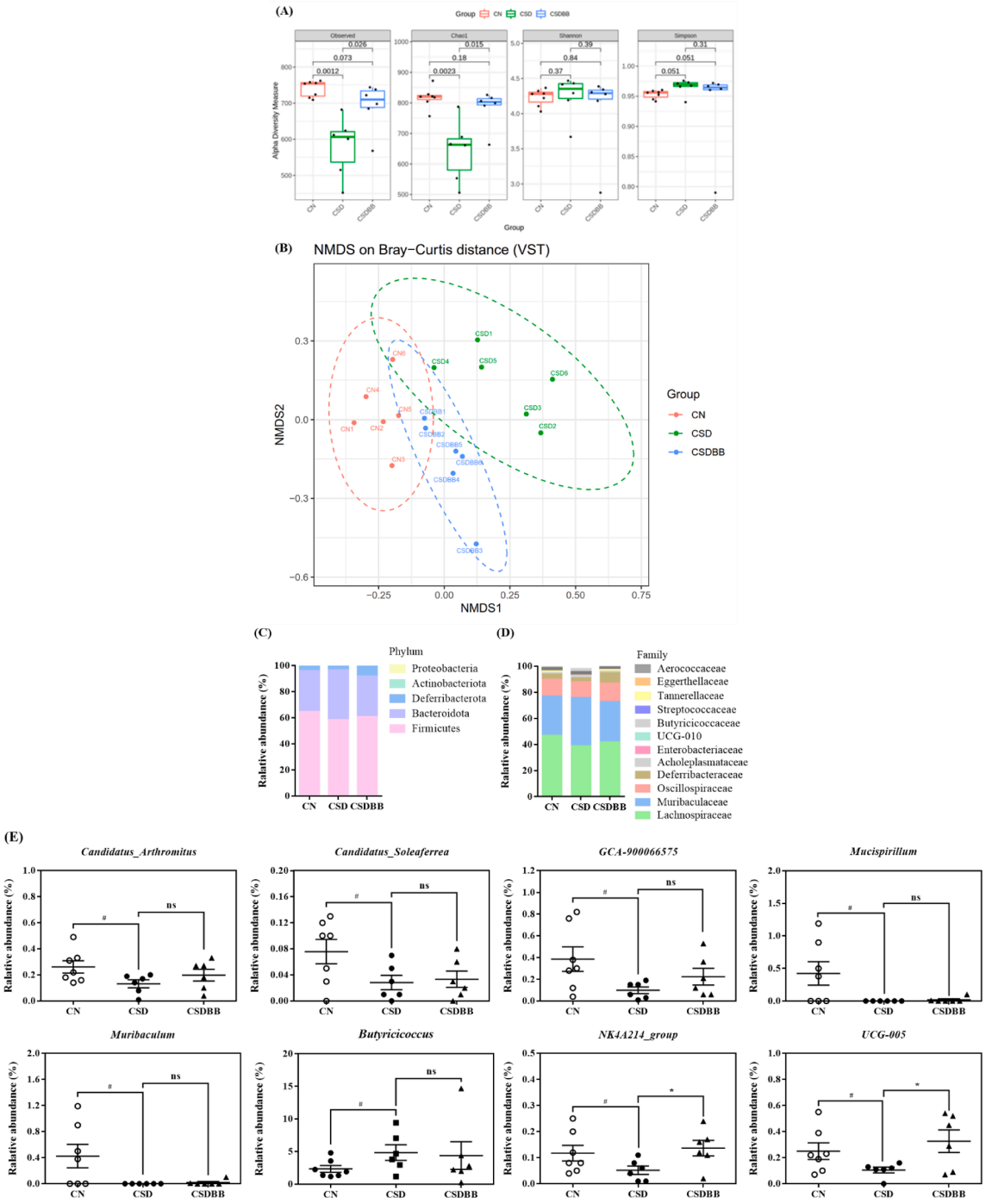

Fig. 6. Effect of black soybean seed coat (BSSC) crude extract on the diversity of gut microbiota in the faeces of mice with sleep fragmentation-induced sleep deprivation. (A) Alpha diversity of the observed, Chao1, Shannon and Simpson indices. (B) Beta diversity on Bray-Curtis distance. Relative abundances of microbiota species at the (C) phylum level and (D) family level. (E) The most differentially abundant species enriched in the microbiota of CN and CSD groups. CN: Control group; CSD: Chronic sleep deprivation group; CSDBB: Chronic sleep deprivation + 300 mg/kg black soybean extract group. Statistical methods are used by the unpaired one-sided *U* test. Values represent the mean ± SEM ($n = 6$), ns is not significant different; $^{\#}p < 0.05$ compared with CN; $^{*}p < 0.05$ compared with CSD.

to normal levels (Fig. 7B). Among inflammatory genes, the CSD group displays significantly higher levels of the *Ifn-γ* compared to the CN group, but supplementation with BSSC crude extract results in a significant reduction in *Ifn-γ* expression (Fig. 7B). Regarding serotonin-related genes, compared to the CN group, the CSD group shows increased expression of *Ido-1*, *Tph-2*, and *5-Htr1a*. However, supplementation with BSSC crude extract significantly restores the expression of *Ido-1* and *Tph-2* to normal levels, with a trend towards the restoration of *5-Htr1a* (Fig. 7B).

In the prefrontal cortex, the CSD group exhibits higher expression levels of circadian rhythm genes *Per1*, *Per2*, and *Cry1* compared to the CN group, indicating disrupted diurnal rhythm in the prefrontal cortex due to sleep deprivation. However, supplementation with BSSC crude extract results in a significant restoration of the expression levels of *Per1* and *Per2*, leading to a return to normal circadian rhythm (Fig. 7C). In terms of inflammatory genes, the CSD group shows significantly higher expression of the *Il-6* compared to the CN group. But after supplementation with BSSC crude extract, there is a significant reduction in the

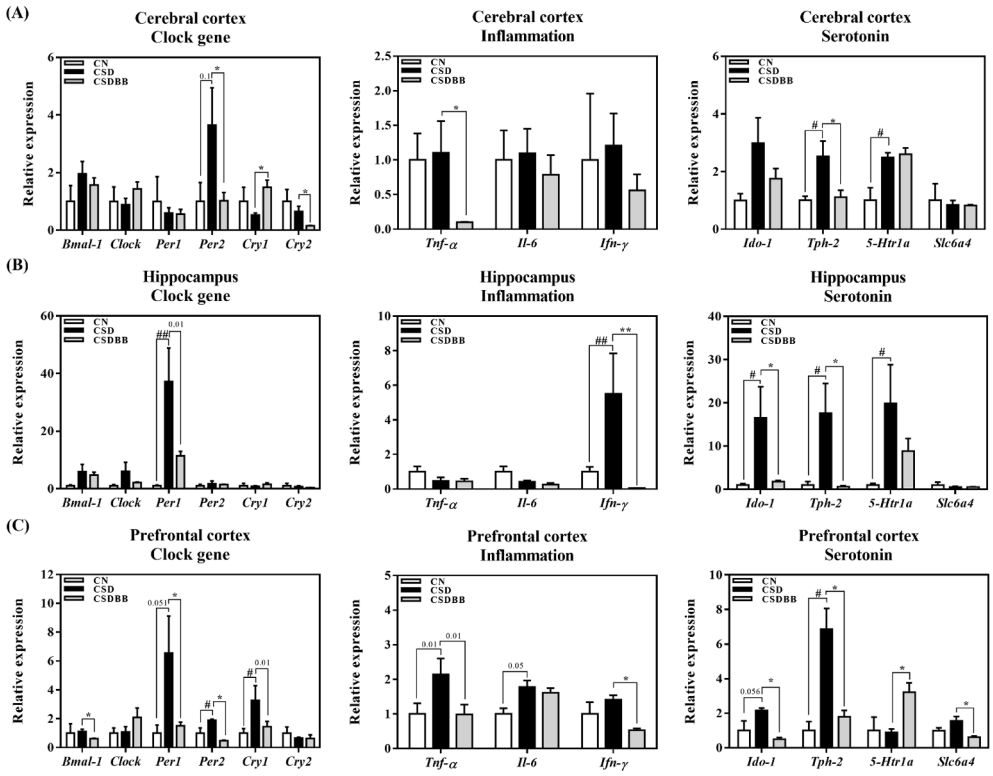

Fig. 7. Effect of black soybean seed coat (BSSC) crude extract on circadian genes, inflammatory genes, serotonin related genes of (A) cerebral cortex, (B) hippocampus, and (C) prefrontal cortex in mice with sleep fragmentation-induced sleep deprivation. CN: Control group; CSD: Chronic sleep deprivation group; CSDBB: Chronic sleep deprivation + 300 mg/kg black soybean extract group. Statistical methods are used by the unpaired one-sided *U* test. Values represent the mean ± SEM ($n = 6$), ns is not significant different; $^{\#}p < 0.05$ compared with CN; $^{\#\#}p < 0.01$ compared with CN; $^{*}p < 0.05$ compared with CSD; $^{**}p < 0.01$ compared with CSD.

expression of the *Ifn-γ* (Fig. 7B). Among serotonin-related genes, compared to the CN group, the CSD group exhibits a significant increase in *Tph-2* expression. However, supplementation with BSSC crude extract significantly restores the expression of *Tph-2* to normal levels. Additionally, crude extract increases the expression of *5-Htr1a* while reducing the expression of *Ido-1* and *Slc6a4*.

4. Discussion

Black soybean has long been one of the most important agricultural products in the international market. Due to the high content of anthocyanins, black soybeans have demonstrated various biological activities such as mitigating oxidative damage (Takahashi et al., 2005), reducing neurodegeneration (González-Sarrías et al., 2017), and modulating neuroinflammation (Micek et al., 2022). Studies showed that under conditions of chronic sleep deprivation, there is an imbalance in the host's oxidative and antioxidative dynamics, leading to lipid peroxidation, DNA damage, and cell death (Gopalakrishnan et al., 2004). In this study, a Lafayette Sleep Deprivation Chamber (Model 80391) was used to induce sleep disruption in mice. The study evaluated how sleep deprivation and the administration of BSSC crude extract affected the behavior of mice in relation to anxiety and short-term and long-term memory. Additionally, the study also examined the stress hormone level, LPS levels, and immune system-related markers, and the levels of SCFAs and the compositions of gut microbiota in feces. Furthermore, the tight junction proteins in the proximal colon as well as the mRNA expression levels of circadian rhythm-, inflammatory-, and serotonin-related genes in various brain regions (cerebral cortex, hippocampus, and prefrontal cortex) were analyzed to assess the efficacy of BSSC crude extract in ameliorating the negative effects of sleep deprivation.

The main components of BSSC were analyzed using UHPLC-MS/MS, and 10 flavonoid compounds were ranked by signal intensity: cyanidin-3-*O*-glucoside, 6′′-*O*-malonyldaidzin, 6-*O*-malonylglycitin, genistein-7-glucoside, delphinidin-3-*O*-glucoside, daidzein-7-*O*-glucoside, glycitin, petunidin-3-*O*-glucoside, daidzin-7-sulfate, 6″-*O*-malonylgenistin. Additionally, cyanidin-3-*O*-glucoside in the black soybean Tainan 9 exhibited higher signal intensity compared to other black soybean varieties. Hence, we have chosen the black soybean Tainan 9 to assess the effect of crude extracts from BSSC on immune modulation, neuropsychological function, and gut microbiota in mice with sleep deprivation. Lee et al. (2009) found that the main components of soybean seed are catechin-cyanidin-3-*O*-glucoside, delphinidin-3-*O*-galactoside, delphinidin-3-*O*-glucoside, cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, pelargonidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, and cyanidin. Lin et al. (2020) showed that the major components of soybean seed are cyanidin-3-*O*-glucoside, delphinidin-3-*O*-galactoside, delphinidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, myricetin, quercetin, and rutin. Lin et al. (2023) showed that the major components of soybean seed are daidzin, genistin, glycitin, daidzein, genistein, glycitein, malonyl daidzin, malonyl genistin, malonyl glycitin, acetyl daidzin, acetyl glycitin, and acetyl genistin. In our UHPLC-MS/MS analysis, we have identified compounds that align with those reported in previous related studies. Previous evidence demonstrates that a bioactive dietary polyphenol preparation (BDPP) protects against SD-mediated cognitive impairment. This protection involves mechanisms such as the phosphorylation of the mammalian target of rapamycin complex 1 (mTORC1) and its direct downstream targets, including the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase β-1 (p70S6K) (Frolinger et al., 2018). Further in vitro studies have identified brain bioavailable phenolic metabolites derived from BDPP metabolism as key factors that attenuate SD-mediated memory impairments (Frolinger et al., 2018). High-throughput bioavailability studies

revealed that specific polyphenol metabolites, such as cyanidin-3′-*O*-glucoside, can reverse changes in mTOR and p70S6K phosphorylation in primary cortico-hippocampal neuronal cultures. They also counteract the effects of 4EGI-1, a specific inhibitor of the eIF4E-eIF4G interaction, thereby rescuing 4E-BP1 phosphorylation (Frolinger et al., 2018). It is clear from the literature that cyanidin-3′-*O*-glucoside, a major component of BSSC, significantly ameliorates the negative effects of SD through the phosphorylation of key proteins in the mTOR pathway, crucial for cognitive functions.

In the study, the 28-day model of sleep deprivation induced by sleep fragmentation, there were no significant changes in mouse body weight and food intake. However, the CSD group did not significantly change stress regulation function. On the other hand, mice in the CSDBB group were able to effectively suppress corticosterone level. The results indicated that after the administration of BSSC crude extract could significantly improve their ability to regulate corticosterone levels. Previous study showed that exposure to stress stimuli activates the HPA axis, leading to the release of corticosterone to regulate physiological processes (such as blood glucose and the immune system) (Taves et al., 2011). However, prolonged exposure to stress can lead to dysfunction of the HPA axis and sustained release of corticosterone, resulting in dysfunction of the HPA axis and the development of mental disorders (such as depression) (Brown et al., 2004) and metabolic diseases (such as obesity, diabetes, hypertension, and ischemic heart disease) (Martocchia et al., 2016). Therefore, administration of BSSC crude extract significantly enhanced stress regulation function, which may contribute to maintaining endocrine and metabolic functions within the mice.

The anxiety levels of mice were evaluated using an open-field test and the elevated plus maze test. In the CSD group, there was a significantly lower number of entries into the central zone compared to the CN group, indicating higher anxiety levels in the CSD group. However, following supplementation with BSSC crude extract were no significant differences in anxiety levels compared with the CSD group. As for locomotor activity, the total distances traveled by mice in the CN group, CSD group, and CSDBB group did not exhibit any significant differences. Sakamoto et al. (2020) indicated that mice subjected to 7 days of acute sleep deprivation significantly spend less time and decrease distance traveled in the central zone compared with normal mice, indicating increased anxiety and decreased activity under conditions of sleep deprivation-induced stress. Vasciaveo et al. (2023) found that 24-hour sleep fragmentation inducted Alzheimer's disease mice for 30 days resulted in decreased time spent in the center of the open-field maze, indicating increased anxiety levels in the mice. In the present study, the CSD group did not exhibit decreased activity levels. The lack of significant differences in activity levels within the CSD group, compared to findings in the literature, could potentially be attributed to the possibility that the sleep fragmentation imposed on the mice may have facilitated compensatory sleep, thereby resulting in less noticeable differences in activity levels..

In the elevated plus maze test, there were no notable variations in the number of entries into the open arms among the CN, CSD, and CSDBB groups. In previous animal studies, following long-term chronic sleep deprivation, mice showed a significant decrease in the time spent in and entries into the open arms of the elevated plus maze, indicating the presence of anxiety-like behaviors (Tai et al., 2020). Similarly, under conditions of acute sleep deprivation, mice exhibited a significant decrease in the time spent in and entries into the open arms of the elevated plus maze (Liu et al., 2022). In the current study, the anxiety levels of mice in the CSD group did not show a significant increase. The limited disparity in anxiety levels within the CSD group, as opposed to findings in the existing literature, could potentially be ascribed to the prior studies employing long-term chronic sleep deprivation or acute sleep deprivation models for inducing mice. These models might have exposed the mice to more intense stress when contrasted with the 28-day sleep fragmentation-induced sleep deprivation model utilized in this experiment.

The novel object recognition test was utilized to evaluate the long-term memory effects of sleep disruptions in mice. The Discrimination index did not reveal any significant differences among the CN group, CSD group, and CSDBB group. However, in comparison to the CN group, the CSD group displayed a declining trend in performance, and following supplementation with BSSC crude extract, a recovery trend similar to the CN group was observed. Previous research has indicated that mice subjected to sleep fragmentation for 4 weeks showed a significantly lower discrimination index in the novel object recognition test compared to the CN group (Puech et al., 2022). If sleep fragmentation was extended to 16 weeks, the difference in the discrimination index between the CN and CSD groups became even more significant than that observed in the CN and CSD groups subjected to shorter-term sleep fragmentation (4 weeks), suggesting that sleep fragmentation may impair object recognition memory in mice (Puech et al., 2022).

Additionally, the T-maze test was employed to assess the short-term memory effects of sleep deprivation in mice. The total time spent in the CN, CSD, and CSDBB groups exhibited no significant differences. Nevertheless, when compared to the CN group, the CSD group demonstrated an increasing trend in time spent. Conversely, after supplementation with BSSC crude extract, the CSDBB group displayed a reduction in time spent, moving towards the performance level of the CN group. Previous studies showed that disrupting mouse sleep for short durations (4 hrs/day) or extended periods (8 hrs/day) did not significantly affect the time spent by mice in the T-maze but showed an increasing trend in time spent. These results suggest that sleep disruption may have potential negative effects on spatial learning and memory (Xie et al., 2015). Therefore, supplementing with BSSC crude extract has the potential to mitigate the negative effects of sleep deprivation on spatial learning and memory.

Previous pointed out that in acute sleep-deprived mice after 72 h, there was a significant decrease in neutrophils and a significant increase in lymphocytes and eosinophils, leading to increased susceptibility to infections, allergies, and inflammatory responses (Ige, 2020). In the study, sleep fragmentation-induced mice exhibited an increase in eosinophils, which could potentially lead to allergic diseases, autoimmune conditions, and infectious diseases (Simon & Simon, 2007). However, after supplementation with BSSC crude extract, the CSDBB group showed a declining trend in eosinophils and a significant decrease in basophils. This suggests that BSSC crude extract may have immunomodulatory effects, improve allergies, and suppress inflammation.

Exposure to stress may lead to the inhibition of Th1 cells and the activation of Th2 cells, potentially accelerating the development of infectious or allergic diseases (Daynes & Araneo, 1989). Additionally, clinical trials demonstrated that after 5 days of acute sleep deprivation, subjects tend to exhibit Th2 differentiation in their T helper cells (Axelsson et al., 2013). In terms of Th2 differentiation, there were no significant differences between the CN and CSD groups, but the CSD group showed an increasing trend. However, after supplementation with BSSC crude extract, the CSDBB group exhibited a decreasing trend in Th2 differentiation, approaching the levels observed in the CN group. Regarding the Th2/Th1 ratio, there were no significant differences between the CN and CSD groups, but the CSD group displayed an increasing trend. However, after supplementation with BSSC crude extract, the CSDBB group showed a significant decrease in the Th2/Th1 ratio. Tanaka et al. (2011) found that T helper cell differentiation toward Th1 could be increased through Toll-like receptors (*TLR2* and *TLR4*). In mice with *Tlr2* and *Tlr4* genes removed, supplementation with BSSC crude extract led to an increase in Th1 cells, preventing allergic diseases in the mice. In this study, BSSC crude extract also led to a significant increase in Th1 cells, indicating that BSSC crude extract has a immunomodulatory effect.

The CSD group of mice did not display significant differences in the protein expression levels of ZO-1, Occludin, and Claudin-1 in the proximal colon when compared to the CN group, although a decreasing trend was observed for ZO-1. However, following supplementation with BSSC

crude extract, the CSDBB group of mice exhibited a significant increase in protein expression levels of ZO-1 and Claudin-1 in the proximal colon, while no significant differences were noted for Occludin. Previous study found that the protein expression levels of ZO-1, Occludin, and Claudin-1 in mice significantly decreased under short-term acute sleep deprivation (3 days), possibly due to oxidative stress induced by sleep deprivation, leading to systemic inflammation and ultimately affecting the intestinal mucosal barrier (Gao et al., 2020). Lai et al (2022) showed that under conditions of chronic sleep deprivation, there were significant decreases in the intestinal tight junction proteins ZO-1, Occludin, and Claudin-1 in mice, accompanied by a significant increase in intestinal-derived LPS in the body. But in the study, the results suggest that the duration of sleep fragmentation-induced sleep deprivation in mice may not be sufficient to impact tight junction proteins and LPS. However, after supplementation with BSSC crude extract, there was a significant increase in the tight junction proteins ZO-1 and Claudin-1, suggesting that BSSC crude extract supplementation may increase the expression of intestinal tight junction proteins.

In the further, we analyzed the genus abundance of gut microbiota composition among CN, CSD, and CSDBB groups. The CSD group of mice exhibited a notable reduction in the species richness of gut microbiota. Intriguingly, administration of BSSC crude extract could led to a significant restoration of the species richness of gut microbiota. In addition, a substantial divergence between the CN group and CSD group based on the principal coordinate analysis with the Bray-Curtis distance. Following the supplementation of BSSC crude extract, the gut microbiota composition in the CSDBB group exhibited significant alterations and displayed a tendency toward recovery, approaching the gut microbiota composition observed in the CN group. Previous studies showed that the richness of gut microbiota in individuals with insomnia is significantly lower than that in normal individuals (Liu et al., 2019).

The predominant genera of CN group included *Candidatus_Arthromitus*, *Candidatus_Soleaferrea*, *GCA-900066575*, *Mucispirillum*, *Muribaculum*, *NK4A214_group*, and *UCG-005*. On the other hand, CSD group was characterized by a dominant genus, *Butyricicoccus*. Furthermore, our analysis revealed that supplementation with BSSC crude extract led to a significant recovery in the relative abundance of *NK4A214_group* and *UCG-005* in CSDBB mice. Bolotin et al. (2014) found that *Candidatus_Arthromitus* is a symbiotic bacterium necessary for the induction of steady-state innate and adaptive immune responses in the gut of mice after birth. Cai et al. (2020) pointed out that the abundance of *Candidatus_Soleaferrea* is significantly reduced in diabetic mice compared to normal mice, and increased *Candidatus_Soleaferrea* can help maintain gut barrier integrity. Guo et al. (2023) found that increasing the abundance of *GCA-900066575* significantly increases the concentration of SCFAs in feces, while reducing endotoxin levels in mice. Herp et al. (2021) found that *Mucispirillum* is associated with intestinal mucus and gut barrier stability, protecting the gut from the invasion of harmful microorganisms. Yamane et al. (2021) reported that the relative abundance of *Muribaculum* is negatively correlated with pro-inflammatory cytokines and positively correlated with tight junction protein expression in the gut, contributing to gut barrier maintenance. Calderón-Pérez et al. (2020) observed in clinical research that *NK4A214_group* has a higher abundance in normotensive hosts compared to hypertensive patients. Couch et al. (2021) found that *UCG-005* has a significantly reduced abundance in obese mice induced by a high-fat diet and can alleviate type 2 diabetes and insulin resistance. Additionally, it also could produce butyric acid, enhancing gut barrier integrity (Flint et al., 2012). In this study, sleep disturbances induced sleep deprivation in mice, and the analysis of gut microbiota imbalance revealed that sleep deprivation can lead to reduced abundance of genera related to gut mucosal and barrier integrity, SCFAs content, and regulation of blood pressure and blood sugar. However, supplementation with BSSC crude extract can enhance *NK4A214_group* and *UCG-005*, which are associated with maintaining gut barrier integrity and regulating blood pressure and blood sugar. In previous studies, anthocyanins have been shown to promote the proliferation of healthy anaerobic bacterial populations while inhibiting pathogenic varieties (Hidalgo et al., 2012), and they can also reduce the occurrence of gut-related diseases such as gut inflammation and colorectal cancer (Turroni et al., 2017).

In the study, we found sleep deprivation lead to the dysregulation of rhythmic genes and serotonin related genes, ultimately resulting in an upregulation of inflammatory gene, which contributes to brain inflammation. However, administering the crude extract of BSSC, several noteworthy changes in gene expression were observed in different brain regions. In the cerebral cortex, the expression levels of *Per2*, *Cry2*, and *Tph-2* were reduced, accompanied by a decrease in *Tnf-α* gene expression. In the hippocampus, the BSSC crude extract led to a reduction in the expression of *Ido-1* and *Tph-2*, as well as a decrease in *Ifn-γ* expression. Similarly, in the prefrontal cortex, the BSSC crude extract downregulated the expressions of *Bmal-1*, *Per1*, *Per2*, *Ido-1*, *Tph-2*, and *Slc6a4*, while concurrently increasing the expression of the *5-Htr1a* and reducing the expression of *Ifn-γ*. These observations suggested that the administration of BSSC crude extract could reduce alterations in the brain clock, inflammation, and serotonin-related gene expressions caused by sleep fragmentation-induced sleep deprivation within these brain regions.

The overexpression of *Per1* in mice leads to a faster circadian rhythm in the wake-sleep cycle compared to normal mice, resulting in reduced sleep duration. Additionally, these mice exhibit altered eating behavior, impacting metabolism, and disrupting circadian rhythms in peripheral tissues, leading to peripheral tissue rhythm disturbances (Liu et al., 2014). On the other hand, clinical studies involving *Per2* showed that some individuals with depression display overexpression of *Per2*, making emotional regulation overly sensitive and prone to symptoms of depression and anxiety (Partonen et al., 2007). Regarding inflammatory genes, in mice subjected to chronic sleep deprivation, symptoms of hypersomnia, anxiety, and depression emerge, accompanied by a significant increase in the expression levels of TNF-α and its receptor TNFR1 (Tumor necrosis factor receptor 1) protein in the brain. However, upon deletion of the Tnfr gene, the symptoms of hypersomnia, anxiety, and depression disappear. Therefore, the upregulation of the pro-inflammatory gene TNF-α in the brain is associated with the occurrence of anxiety and depression in mice (Ramesh et al., 2012). In studies related to sleep and *Ifn-γ*, it is shown that sleep deprivation patients exhibit a significant increase in the expression of *Ifn-γ*, influenced by the levels of corticosterone in their receptors (Irwin & Opp, 2017). Regarding serotonin, previous study showed that overexpression of *Ido-1* may produce an immunosuppressive effect. Elevated *Ido-1* gene expression increases the ratio of tryptophan conversion to kynurenine, potentially inhibiting the activity of immune cells such as T cells and natural killer cells, weakening the immune response (Mellor et al., 2004). Furthermore, it can accumulate in the brain, causing damage to neurons (Marx et al., 2021). Chon et al. (1996) also indicated that the production of the pro-inflammatory cytokine IFN-γ induces *Ido-1* expression (Chon et al., 1996). On the other hand, *Tph-2* expression correlates positively with *5-Htr1a* expression (Invernizzi, 2007). Additionally, previous studies found that when mice undergo chronic stress and experience brain damage, the expression of the *5-Htr1a* in the brain increases, causing heightened emotional sensitivity and negative effects on sleep, mood, and behavior (Richardson-Jones et al., 2010). *Slc6a4* regulates serotonin transport, recycling serotonin from the synaptic cleft back into neurons to maintain neurotransmission balance (Murphy & Lesch, 2008). Reduced *Slc6a4* expression increases serotonin utilization and may reduce anxiety and depression (MacGillivray et al., 2010). Hence, BSSC crude extract may potentially ameliorate sleep deprivation by addressing dysregulation in rhythmic genes and serotonin-related genes, leading to decrease inflammatory gene expression. Consequently, it holds the potential for regulating emotions.

The limitation of the study is that the current experimental design cannot verify whether the effects of BSSC crude extract due to sleep deprivation are caused by gut microbiota imbalance. To determine

whether BSSC crude extract alleviates certain side effects associated with sleep deprivation through the gut microbiota, it is necessary to conduct experiments involving germ-free animals or fecal transplantation.

5. Conclusions

The administration of BSSC crude extract has the potential to address various issues associated with sleep deprivation. It can restore the regulation of the HPA axis, promote the differentiation of helper T cells into Th1 cells, thereby reducing the Th2/Th1 ratio, and improve eosinophil and basophil counts. Additionally, the crude extract notably enhances gut barrier integrity and increases the richness of gut microbiota observed in mice with sleep deprivation. Furthermore, the BSSC crude extract enhances the abundance of *NK4A214_group* and *UCG-005*, leading to a tendency for the composition of gut microbiota to recover toward that of the mice with normal sleep. Therefore, BSSC crude extract has the potential to improve sleep deprivation by addressing dysregulation in rhythmic genes and serotonin-related genes, which in turn leads to decrease expression of inflammatory genes. In summary, BSSC crude extract showed promise in addressing immune imbalances, restoring the richness of gut microbiota, normalizing gut microbiota, and alleviating disruptions in brain gene expression (specifically related to rhythmic, inflammatory, and serotonin-related genes) associated with sleep deprivation.

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Ethics statement

All experimental procedures involving animals were thoroughly examined and approved by the IACUC of National Chung Hsing University, and the protocol guidelines 110–089 were approved by the ethics committee of the IACUC. The current study was carried out in compliance with the ARRIVE guidelines.

Availability of data and materials

The datasets used and analyzed in this study are available from the corresponding author upon reasonable request.

Consent for publication

Not applicable.

CRediT authorship contribution statement

Chun-Liang Tung: Resources, Conceptualization. **Jyh-Horng Wu:** Supervision, Resources, Conceptualization. **Hung-Chang Chang:** Investigation, Data curation. **Jin-Wei Xu:** Investigation. **Yu-Chen S.H. Yang:** Investigation, Data curation. **Changwei W. Wu:** Resources. **Yu-Tang Tung:** Writing – review & editing, Writing – original draft, Supervision, Resources, Data curation, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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