Original Article

Melatonin prevents the dysbiosis of intestinal microbiota in sleep-restricted mice by improving oxidative stress and inhibiting inflammation

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Abstract

Background: Intestinal inflammation caused by sleep restriction (SR) threatens human health. However, radical cure of intestinal inflammatory conditions is considerably difficult. This study focuses on the effect of melatonin on SR-induced intestinal inflammation and microbiota imbalance in mice.

Methods: We successfully established a water platform to induce long-term SR in mice for 28 days with or without melatonin supplementation. The SR-induced oxidative stress and inflammatory changes were evaluated in plasma and jejunum tissue samples using *in vitro* assays. Additionally, changes in the intestinal microbiota were explored using high-throughput sequencing of the 16S rRNA gene.

Results: After 20 h of chronic sleep restriction for 28 consecutive days, plasma melatonin was significantly reduced by 48.91% ($P < 0.05$), while GLU, NE, and CORT were significantly increased (34.32%–90.28%, $P < 0.05$). The activities of antioxidant enzymes (SOD, GSH-Px, and CAT) and T-AOC in intestinal tissues of SR mice were decreased (17.02%–40.92%, $P < 0.05$), while the content of MDA was increased (15.12%, $P = 0.0089$). The levels of pro-inflammatory cytokines (IL-6 and TNF-α) ware increased (65.27%–123.26%, $P < 0.05$), while the levels of anti-inflammatory cytokines (IL-10 and IFN-γ) were decreased (26.53%–60.41%, $P < 0.05$). High-throughput pyrosequencing of 16S rRNA from jejunum samples demonstrated an overall increase in the number of OTUs (30.68%, $P = 0.015$). The α-diversity (Shannon, ACE and Chao1) of jejunum was increased (28.18%–48.95%, $P < 0.05$), and the β-diversity (PCoA and NMDS) was significantly different from that of the control group ($P = 0.001$). Furthermore, the prevalences of Helicobacter and Clostridium were higher, whereas that of Bacteroidetes and Lactobacillus were lower in SR mice than in controls ($P < 0.05$). However, melatonin supplementation reversed the SR-induced changes and improved oxidative stress, inflammatory response, and microbiota dysbiosis in the jejunum, and there was not significant difference compared with the control group ($P > 0.05$).

Conclusions: Melatonin prevents the dysbiosis of intestinal microbiota in SR mice by improving oxidative stress and inhibiting inflammation. Our results may provide a theoretical basis for conducting clinical research on insufficient sleep leading to intestinal health in humans and hence facilitate a better understanding of the role of melatonin.

Keywords: Intestinal inflammation, intestinal microbiota, melatonin, oxidative stress, sleep restriction

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INTRODUCTION

Modern lifestyles, with extended working hours and commute time, lead to increased short sleep durations. Sleep time was found to be minimal while work time was maximal in the age group of 45–54 years.[1] Sufficient sleep is important for maintaining physical and mental health, yet 28.5% of the adults receive less than 6 h of sleep per day.[2] The Youth Risk Behavior Survey found that 72.7% of the students reported <8 h of sleep on an average during school nights.[3] A significant negative effect of sleep restriction has been observed on cognitive processing across cognitive domains for executive functioning, sustained attention and long-term memory,[4] endocrine functions, and metabolic and inflammatory responses.[5] Furthermore, sleep restriction increases the risk of developing cardiovascular and metabolic disorders,[6] intestinal mucosal injury, and microbiota dysbiosis.[7] In contrast, sufficient sleep may help reduce the risk of obesity.[8] There has been a marked increase in the number of studies describing changes in gastrointestinal functioning during sleep and their specific applications for clinicians.[9] Thus, disturbances in natural sleep and sleep deprivation lead to various visceral disorders.[5] Sleep loss can be divided into reduced sleep duration and reduced sleep efficiency. During the COVID-19 pandemic, both sleep duration and sleep efficiency were found to be independently associated with frequent nightmares among the frontline medical workers.[10] Reduced sleep duration can be classified into acutely sleep-deprived (SD) and chronically sleep-restricted (SR) conditions. Both SD and SR can lead to a pro-inflammatory state in the absence of overt infection or injury.[11,12] SD has been reported to induce intestinal barrier dysfunction,[7] resulting in oxidative stress and intestinal mucosal injury.[13] However, SR but not SD mice show morphological signs of microglial activation and enhanced microglial phagocytosis of synaptic elements, which may predispose the brain to further damage.[14] Although SR resembles sleep loss more than acute sleep disturbance, the effect of SR on the intestinal microbiota composition has not been fully understood.[5] Simultaneously, it is of potential clinical significance to study the role of MT in preventing SR-induced intestinal microbiota imbalance and inflammation.[15,16]

MATERIALS AND METHODS

Animals and treatments

Thirty-six male CD1 mice (8 weeks old; Vital River Laboratory Animal Technology Co. Ltd., Beijing, China) were housed under conventional conditions, at a temperature of 21°C ± 1°C and relative humidity of 50% ± 10% with 14-h light: 10-h dark cycle (with lights on at 7:00 am). All mice had free access to food and water. After acclimatization for 1 week, the mice were randomly divided into three groups: sleep restriction group (SR), SR + melatonin supplement group (SR + MT), and non-sleep-restricted control group (CON). All the experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the Animal Welfare Committee of the Agricultural Research Organization, China Agricultural University (Approval No. AW18079102-2).

Sleep restriction in mice was induced for 28 days by using a modified multiple platform water bath every day from 12:00 pm to 8:00 am of the next day, and the daily sleep time was set from 8:00 am to 12:00 pm. Briefly, the platforms were placed in a water tank and the mice from SR or SR + MT groups were placed in the water bath. Every mouse in the water bath could move from one platform to another by jumping. The water was filled up to 4 cm in the water bath. When mice reached the rapid eye movement stage, the paradoxical phase of sleep, muscle atonia caused mice to fall into the water. The mice then woke up and tried to climb up the platform to avoid drowning. Throughout the experiment, clean water was maintained in the water tank and was changed daily.

Based on a previous study,[17] mice in the SR + MT group were supplemented with 10^{-5} mol/L MT (M5250; Sigma, St. Louis, MO, USA) through drinking water. The MT was dissolved in absolute ethanol and diluted in water to a final concentration. Mice in SR and CON groups were vehicle treated.

All mice were euthanized under anesthesia using 2% pentobarbital sodium (2.5 mL/kg) following the completion of the experiment at 8:00 am on day 29. Blood was collected via tail snipping and blood glucose (GLU) level was measured using the MediSense® Precision® Xtra glucometer and MediSense® Precision® Plus stripes (Abbott Diagnostics, Wiesbaden, Germany). Plasma, jejunal tissue, and jejunal contents were harvested immediately and stored at −80°C.

Enzyme-linked immunosorbent assay (ELISA)

Plasma samples were used for the detection of MT, corticosterone (CORT), and norepinephrine (NE) levels, whereas, jejunum samples were used for the detection of inflammatory factors (IL-6, TNF-α, IL-10, and IFN-γ) by using a competitive ELISA assay (USCN Life Science, Inc., Wuhan, China). All the assays were performed in triplicate and according to the manufacturer's instructions.

Measurements of antioxidant activity and lipid peroxidation

The jejunum segments were immediately homogenized, and clarified lysates were obtained by centrifugation (200 × g for 10 min) at 4°C. The tissue extracts were stored at −80°C for antioxidant activity analysis. Commercially available kits (Nanjing Jiancheng Co. Ltd., Nanjing, China) were used for measuring glutathione peroxidase (GSH-Px), catalase (CAT), and superoxide dismutase (SOD) activities, total antioxidant capability (T-AOC), and malondialdehyde (MDA) contents using colorimetric based methods. The absorbance of GSH-Px, CAT, SOD, T-AOC, and MDA was measured at 593, 340, 520, 450, and 532-nm wavelengths, and the activities of GSH-Px were expressed as mU/mg. The activities of CAT, SOD, and T-AOC were expressed as U/mg, and the activities of MDA were expressed as μmol/mg. All the assays were performed in triplicate.

Microbial sequencing

Bacterial genomic DNA was isolated from the frozen jejunum contents by using a QIAamp DNA Stool Mini Kit (Qiagen Co. Ltd., Germany) according to the manufacturer's instructions, sequenced using high-throughput sequencing, and the microflora detection was performed using BMKCloud (www.biocloud.net, Beijing, China). Briefly, DNA samples were PCR-amplified using bar-coded primers flanking the V3-V4 region of the 16S rRNA gene. The amplification was performed using a thermocycler under the following conditions: 1 cycle at 95°C for 5 min followed by 25 cycles at 95°C for 30 s, 50°C for 30 s, and 72°C for 40 s, and a final extension step at 72°C for 7 min. High-throughput pyrosequencing of the PCR amplicons was performed using Illumina MiSeq platform and paired-end reads were obtained. The paired-end reads were excluded from further analysis if they did not match a 12-base Golay barcode with one error, failed to overlap with each other by 35 bases, overlapped region differed by more than 15% or had more than three base calls of <Q20. The operational taxonomic units (OTUs) were generated by clustering the reads at 97% similarity (USEARCH, version 10.0). The OTU sequences were used for further analysis, including calculation of α diversity measures, such as Shannon, Chao1, ACE, and Simpson indices. Furthermore, Quantitative Insights Into Microbial Ecology (QIIME) was used for the identification of key OTUs, principal components analysis (PCA), principal coordinate analysis (PCoA), and nonmetric multidimensional scaling (NMDS) heat maps for β diversity analysis. In addition, linear discriminant analysis (LDA) effect size (LEfSe) was performed to quantify the biomarkers in each group.

Statistical analyses

Statistical analyses were performed using SPSS 10.0 statistical software (SPSS, Inc., Chicago, IL, USA) and data are expressed as the mean ± standard error (SE). Differences between groups were analyzed using one-way ANOVA followed by Tukey post hoc test. The differences were considered statistically significant at $P < 0.05$.

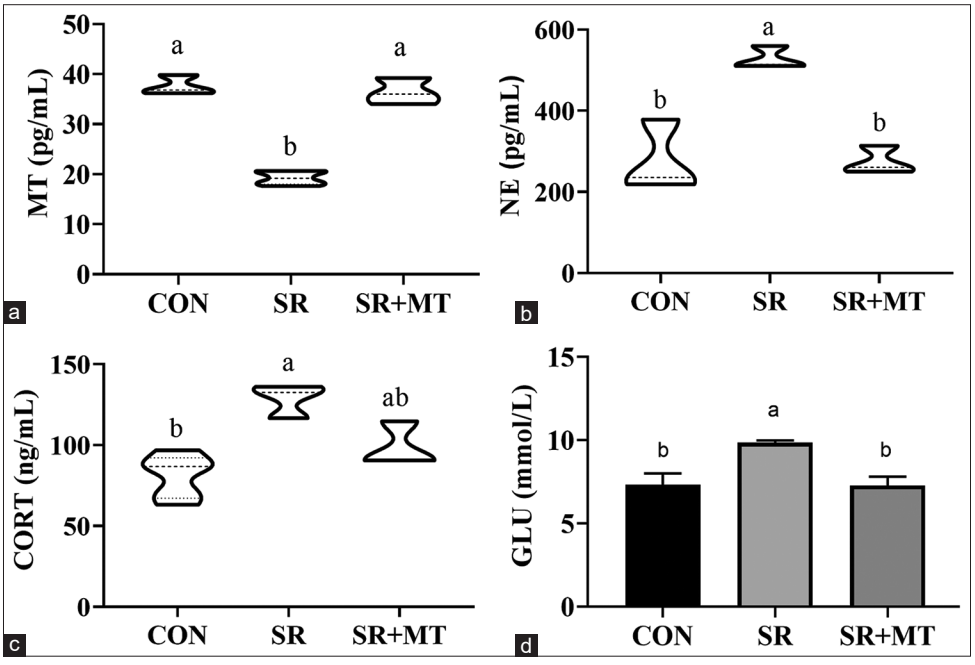

Figure 1: Effects of melatonin on the secretion of stress hormones and blood glucose in mice. Plasma MT (a), NE (b), CORT (c), and GLU (d) in the CON, SR, and SR + MT groups. Values are means ± SE. $P < 0.05$ as significant difference, marked with different lowercase letters.

RESULTS

SR decreased MT level and increased NE, CORT, and glucose levels

The plasma MT level was significantly decreased by 48.91% ($P < 0.05$) in the SR group (19.21 pg/mL) compared to that in the CON group (37.61 pg/mL) [Figure 1a]. Furthermore, plasma NE, CORT, and GLU levels were found to be significantly increased by 90.28% ($P = 0.004$), 58.33% ($P = 0.003$), and 34.32% ($P = 0.012$) in the SR group compared to that in the CON group, respectively [Figure 1b–1d]. However, no significant difference in MT, NE, CORT, or GLU levels were observed between the SR + MT and CON group mice ($P > 0.05$).

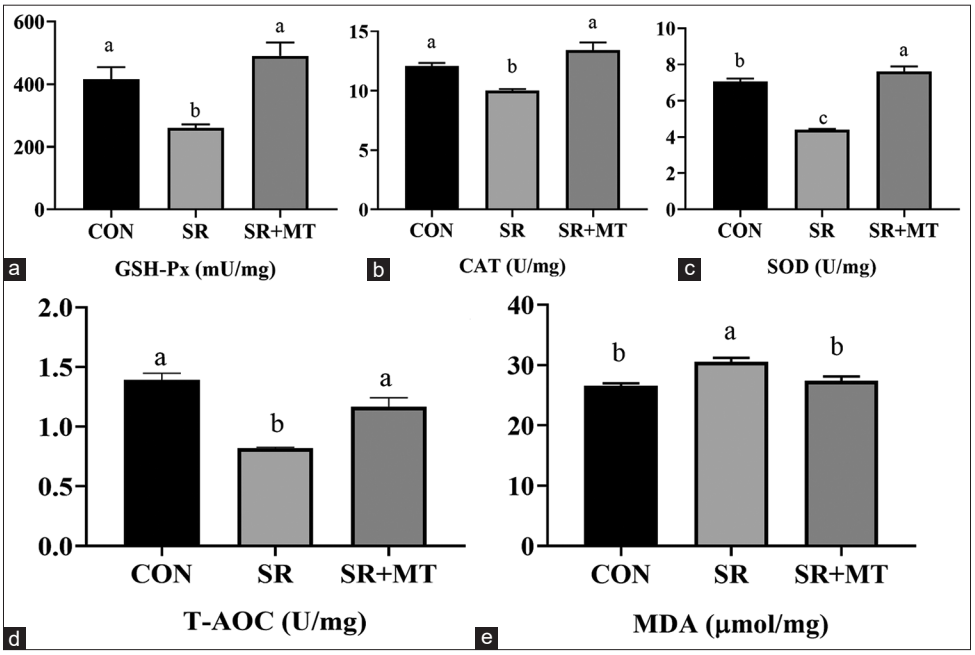

Figure 2: Effects of melatonin on five antioxidant parameters in the jejunum of mice. T-AOC (a), MDA (b), GSH-Px (c), CAT (d), and SOD (e) in the CON, SR, and SR + MT groups. Values are means ± SE. $P < 0.05$ as significant difference, marked with different lowercase letters.

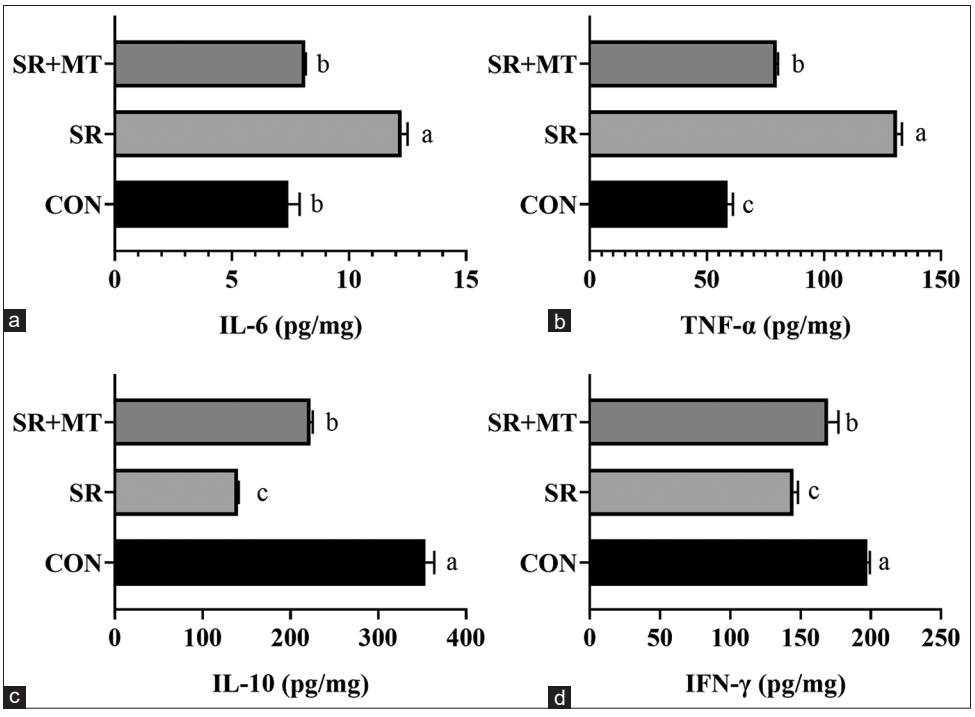

Figure 3: Effects of melatonin on the secretion of cytokines in the jejunum of mice. IL-6 (a), TNF-α (b), IL-10 (c), and IFN-γ (d) in the CON, SR, and SR + MT groups. Values are means ± SE. $P < 0.05$ as significant difference, marked with different lowercase letters.

MT improved SR-induced intestinal oxidative stress

We then examined the activities of antioxidant enzymes (GSH-Px, CAT, and SOD), T-AOC, and MDA content in the jejunum. The levels of antioxidant enzymes were significantly reduced by 37.42% (GSH-Px, $P = 0.060$), 17.02% (CAT, $P = 0.032$), 37.74% (SOD, $P < 0.05$), and that of T-AOC by 40.92% ($P = 0.001$) in the SR group compared to that in the CON group [Figure 2a–2d]. In addition, a significant increase in the levels of MDA, an end product of lipid peroxidation was observed (15.12%, $P = 0.009$) in the SR group compared to that in the CON group [Figure 2e]. However, supplementation of SR mice with MT reversed the stimulatory effects of SR, which was evident from the insignificant difference in T-AOC, MDA, GSH-Px, and CAT levels between the SR + MT and CON groups ($P > 0.05$). In contrast, SOD level was found to be significantly increased by 7.79% ($P = 0.029$) in the SR + MT group compared to that in the CON group.

MT improved SR-induced intestinal inflammation response

Consistent with the reduction in MT content, there was an increase in pro-inflammatory cytokines, including IL-6 and TNF-α levels by 65.27% [$P < 0.05$, Figure 3a] and 123.26% [$P < 0.05$, Figure 3b] and decrease in anti-inflammatory cytokines, including IL-10 and IFN-γ levels by 69.41% [$P < 0.05$, Figure 3c] and 26.53% [$P < 0.05$, Figure 3d] in the jejunum of SR mice compared to that in CON mice, respectively. However, MT supplementation attenuated the stimulatory effects of SR on inflammatory factors. The IL-6 and TNF-α levels were shown to be decreased by 33.63% ($P < 0.05$) and 39.25% ($P < 0.05$), while that of IL-10 and IFN-γ were found to be increased by 59.05% ($P < 0.05$) and 16.98% ($P < 0.05$) in the SR + MT group compared to that in the SR group.

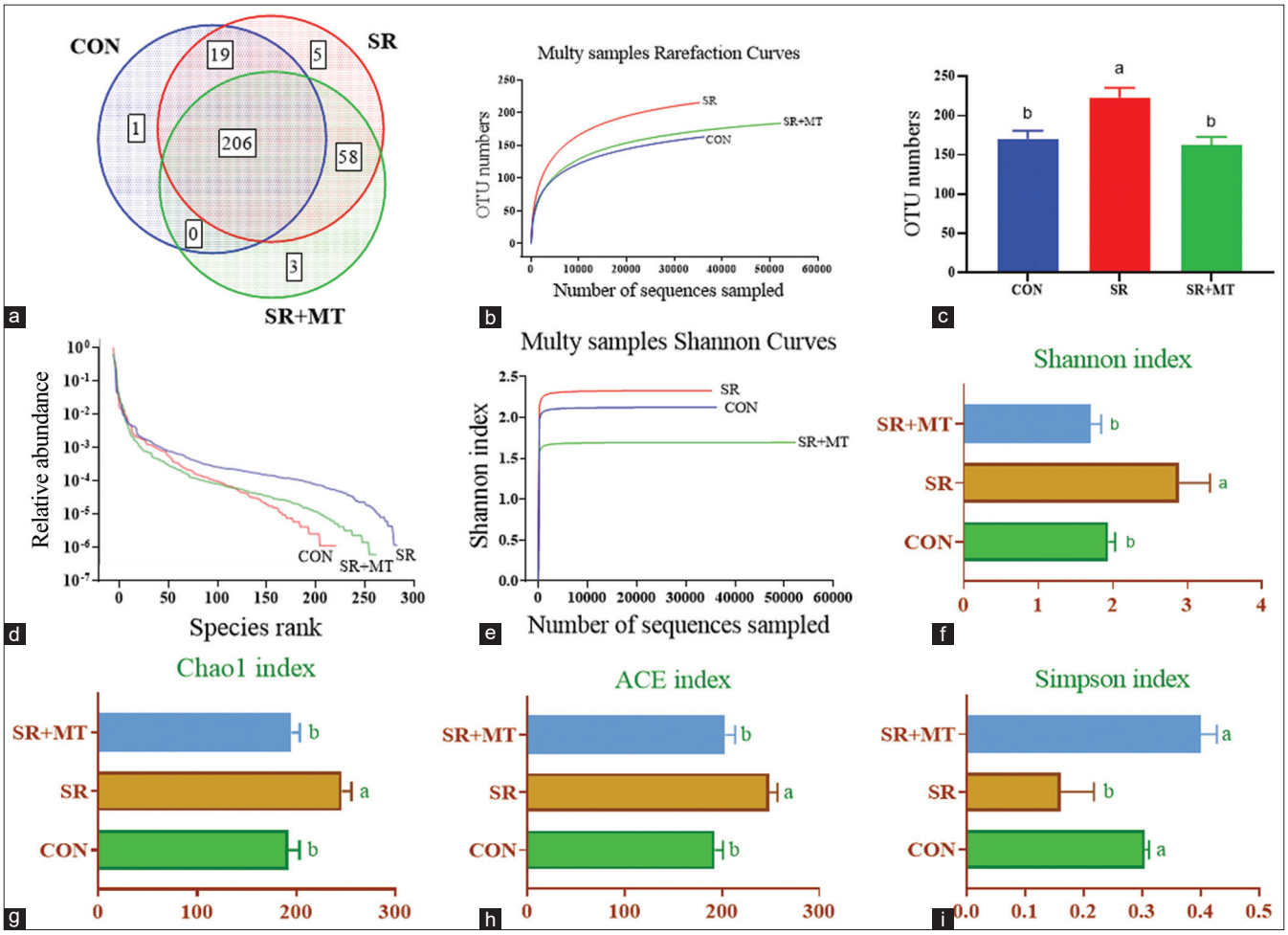

Figure 4: The OTU number and diversity response of the jejunal microbiota to melatonin in the mice. (a) Venn diagram showing the number of OTUs. (b) The multy samples rarefaction curves showing the number of OTUs grows with the number of sequences sampled. (c) The statistics of OTU number for the CON, SR, and SR+MT groups. (d) Relative abundance of the species rank curves is used to explain both the richness and evenness of the species contained. (e) The multy samples rarefaction curves showing the Shannon index grows with the number of sequences sampled. Shannon index (f), Chao1 index (g), ACE index (h), and Simpson (i) index in the CON, SR, and SR + MT groups. Values are means SE. $P < 0.05$ as significant difference, marked with different lowercase letters

MT improved SR-induced intestinal microbiota disorder

High-throughput pyrosequencing of the 16S rRNA gene was used to determine the effect of SR and MT supplementation on gut microbiota. At 97% similarity, all the filtered reads were clustered into OTUs [Figure 4a–4c]. The number of OTUs were significantly higher (30.68%, $P = 0.015$) in the SR group than in the CON group [Figure 4c]. However, no statistically significant difference was observed in the number of OTUs between the mice of SR group supplemented with MT and CON group. The α-diversity analysis showed that the richness and evenness of the jejunum microbiota were significantly higher in the SR group mice [Figure 4d]. For example, Shannon, Chao1, and ACE indices were significantly increased by 48.95% [$P = 0.038$, Figure 4e, 4f], 28.18% [$P = 0.011$, Figure 4g], and 29.04% [$P = 0.004$, Figure 4h], whereas, the Simpson index was shown to be markedly decreased by 47.14% [$P = 0.037$, Figure 4i]. However, in SR mice supplemented with MT, the values of all the α-diversity indices were similar to that of the CON group, with no statistically significant difference between both the groups ($P > 0.05$). The β-diversity analysis presented distinct clustering of the jejunum samples from all the three groups [Figure 5a–5c], indicating their distinct microbiota composition. The results showed a significant increase in the dispersion of jejunum microbiota following SR, suggesting a decrease in the homogeneity of jejunum microbiota. Further, to identify specific bacterial taxa associated with SR and MT supplementation, we employed the LEfSe method [Figure 5d]. The ternary phase diagram shows the relative abundance and relationship of different species across the three groups [Figure 5e]. The heat-map of the redundancy analysis (RDA)-identified key OTUs identifying the most differentially abundant taxon in jejunum microbiota in response to melatonin supplementation [Figure 6a], and representative bacterial taxon information of genus, family, and phylum as shown [Figure 6b]. Further analysis demonstrated that the relative abundances of Bacteroidetes [Figure 7a] and

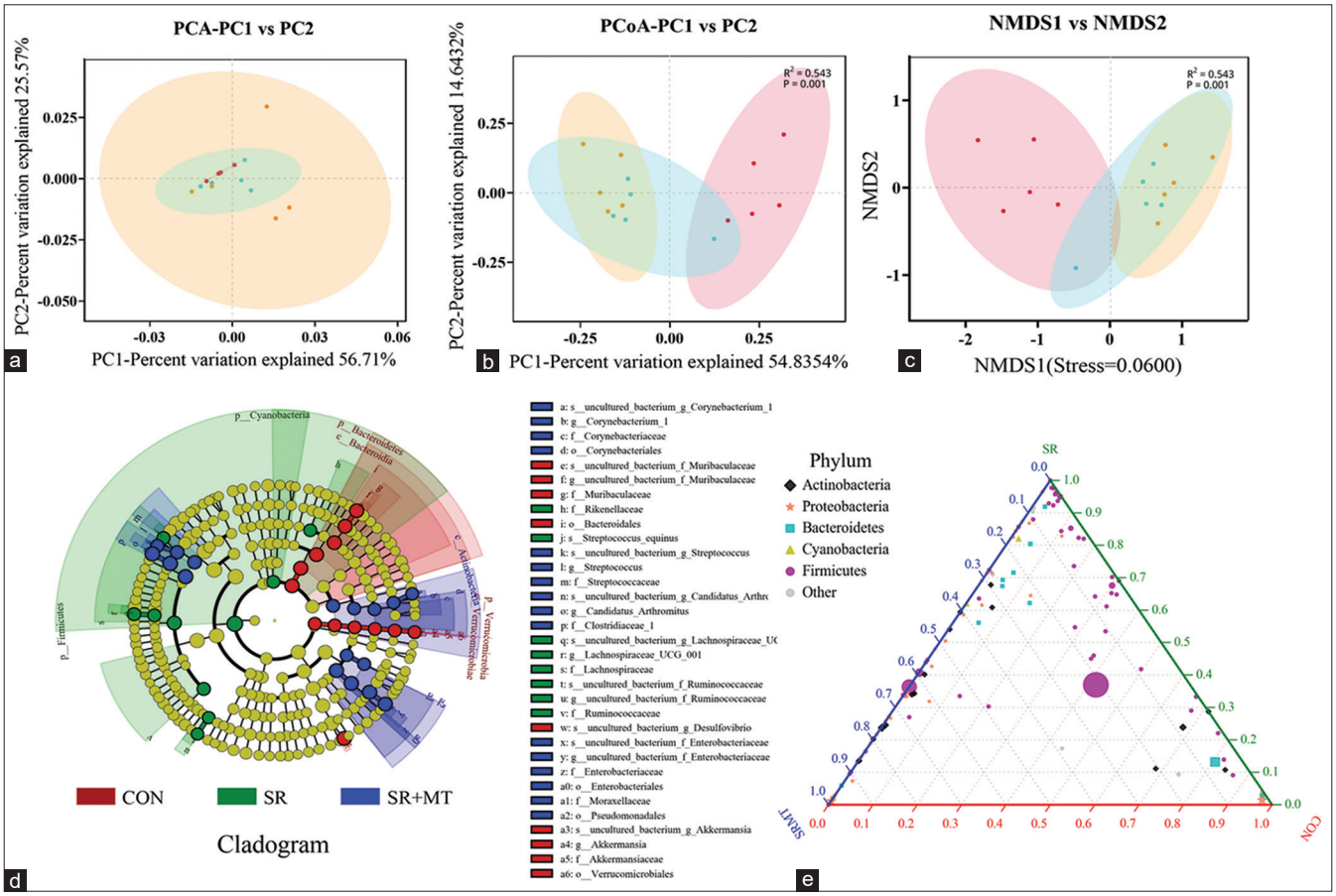

Figure 5: The diversity (a-c) and composition of the microbiota (d-e) after melatonin supplementation in the jejunum of mice. The diversity shows the dispersion of each sample in the CON, SR and SR+MT groups. (A) Principal component analysis (PCA), (b) PCoA score plot, and (c) nonmetric multidimensional scaling (NMDS) score plot based on the Bray–Curtis score plot based on the OTUs in the jejunum. (d) Taxonomic cladogram obtained from LEfSe sequence analysis in the jejunum, and the biomarker taxa are highlighted by colored circles and shaded areas. (e) Ternary phase diagram.

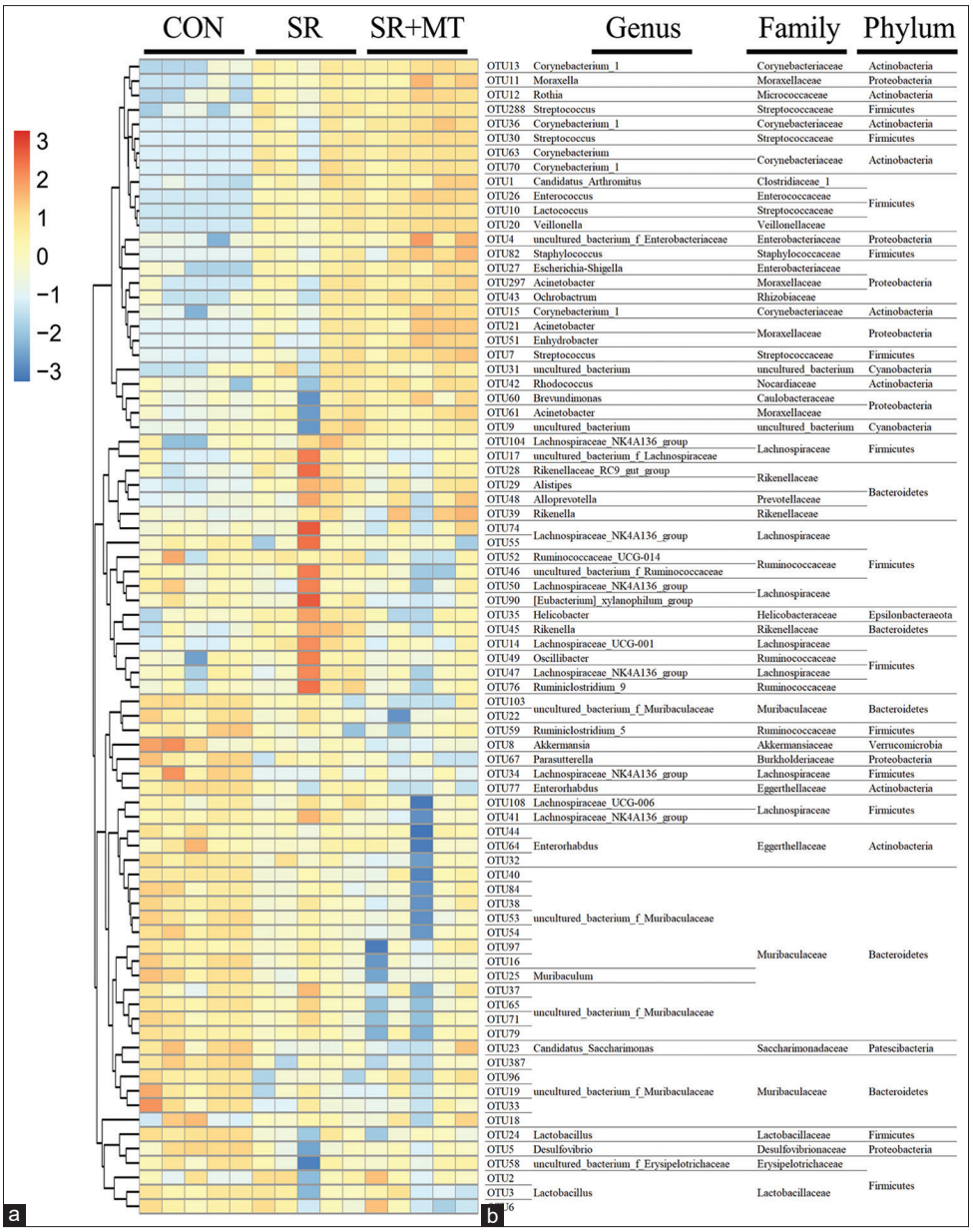

Figure 6: The heatmap of the redundancy analysis (RDA)-identified key OTUs signifying the most differentially abundant taxon in jejunum microbiota in response to melatonin supplementation. (a) Heatmap showing the relative abundance of the RDA-identified 80 key OTUs that were significantly altered by melatonin in SR mice. (b) Representative bacterial taxon information (genus, family, and phylum) of OTUs from (a) are shown.

Lactobacillus [Figure 7b] were reduced, whereas that of Firmicutes [Figure 7c], Helicobacter [Figure 7d], and Clostridium [Figure 7e] and the difference in abundance between Firmicutes and Bacteroidetes [Figure 7f] were found to be increased. However, MT supplementation reversed these patterns.

DISCUSSION

In this study, we successfully established a water platform to achieve long-term sleep restriction in mice, which slept for 4 h per day for 28 days, as well as set the control and MT supplementation groups. The results showed a significant decline in MT and a significant increase in NE, CORT, and GLU levels in the SR group. However, in SR mice, supplemented with 20 mg/kg of MT, the MT, NE, CORT, and GLU levels were close to the normal range. These results indicate that our model was successful and similar to that of the others.[14]

Using high-throughput pyrosequencing of 16S rRNA gene, we found that SR induced dysbiosis of the intestinal microbiota. The number of OTUs and α and β diversity indices of the jejunal samples in SR mice were increased. Furthermore, the changes in the bacterial community composition were characterized by a higher abundance

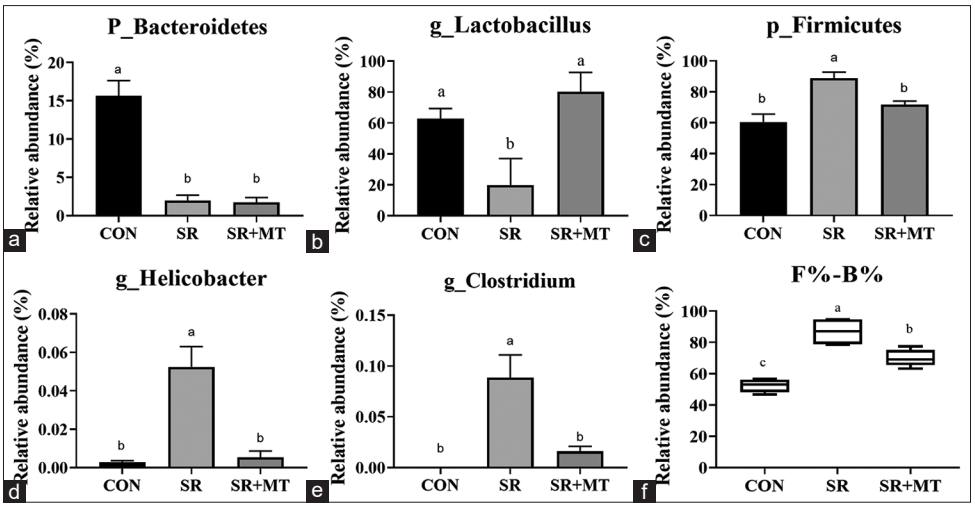

Figure 7: Relative abundance of the jejunal microbiota after melatonin supplementation in mice. Relative abundance of Bacteroidetes (a), Lactobacillus (b), Firmicutes (c), Helicobacter (d), Clostridium (e), and F%-B% (f) in the jejunum microbiota based on the LefSe results of the CON, SR, and SR + MT groups. Values are means ± SE. $P < 0.05$ as significant difference, marked with different lowercase letters,

of harmful bacteria (Helicobacter and Clostridium) and lower abundance of beneficial bacteria (Bacteroidetes and Lactobacillus) in the jejunum of SR-induced mice. Consistent with our findings, a low abundance of Bacteroidetes and Lactobacillus has been reported in mice with chronic sleep disruption.[18] Furthermore, Helicobacter is considered associated with enterohepatic diseases.[19] Interestingly, a recent study reported similar alterations in the microbiota composition following short-term exposure of mice to SD, including an increase in the abundance of Bacteroides.[13] The ratio of Firmicutes and Bacteroidetes is an important indicator of structural modifications of gut microbiota.[20,21] Our study revealed a larger difference in the abundances of Firmicutes and Bacteroidetes in the SR group, indicating the SR-induced imbalance in the intestinal microbiota. In contrast, MT supplementation reversed the SR-induced changes and improved the microbiota dysbiosis in the jejunum, which was in agreement with previous reports.[7,13] Thus, we conclude that MT reverses the intestinal microbiota dysbiosis in SR mice.

Furthermore, our results demonstrated the exact role of MT in the alleviation of SR-induced intestinal microbiota disorders. MT supplementation altered SR-induced intestinal oxidative stress and secretion of intestinal inflammatory cytokines. Sleep is generally thought to be a function of the central nervous system. Moreover, studies on sleep deprivation have demonstrated that sleep deficit affects other physiological systems as well.[22] In addition, oxidative stress and inflammation have been implicated in sleep deprivation-related disorders.[23] Free radicals generated during oxidative stress may promote lipid peroxidation, oxidize proteins, cause DNA strand breaks, and result in activation and release of pro-inflammatory cytokines.[24] Furthermore, sleep deprivation-induced oxidative stress results in activation of the NF-κB pathway, which in turn leads to inflammation and ultimately small intestinal mucosal damage, affecting the absorption of nutrients.[7] Additionally, a few inflammatory factors, such as TNF, IL-6, and IL-10, may affect the intestinal environment, in turn affecting the distribution of intestinal microbiota.[7,25] Sleep restriction has been reported to elevate the pro-inflammatory factors, resulting in changes in the gut microbiome, which in turn drives metabolic and cognitive changes.[26,27] Correlation analysis showed direct association between oxidative stress and the gut microbiota. MT, the master regulator of oxidative stress and inflammation, has been shown to improve oxidative stress and inhibit inflammation.[28-30] These results strongly indicate a link between MT and intestinal microbiota. Therefore, MT may have reversed the dysbiosis of the intestinal microbiota by improving oxidative stress and inhibiting inflammation in SR mice. Poor sleep quality is common in modern society,[1-3] and intestinal inflammation caused by sleep restriction threatens human health. However, radical cure of intestinal inflammatory conditions is difficult. Our finding may provide a theoretical basis for conducting clinical research on insufficient sleep leading to intestinal health in humans and hence facilitate a better understanding of the role of melatonin.

In this study, we investigated the effect of melatonin on SR-induced intestinal inflammation and microbiota imbalance in mice. Although we found that melatonin can prevent the dysbiosis of intestinal microbiota in SR mice by improving oxidative stress and inhibiting inflammation,

additional studies as to how melatonin modulates the SR-induced oxidative stress and inflammatory response in the intestine, as well as interaction between intestinal flora and host in the SR mice, are still required.

In conclusion, our results help to understand the mechanisms underlying the effect of MT on intestinal inflammation and microbiota in SR mice. Moreover, the results support the primary effect of antioxidant function disruption. Our findings show that MT may be used as a probiotic agent to reverse SR-induced imbalance in intestinal homeostasis, as well as facilitate a better understanding of the mechanisms associated with the various beneficial effects of MT.

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Conflicts of interest

There are no conflicts of interest.

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