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Regulation of Sleep and Circadian Rhythms by *S*-Adenosylmethionine-Producing Probiotics

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ABSTRACT

Current first-line medications for sleep disorders often overlap with antidepressants, yet their effectiveness remains suboptimal, highlighting the urgent need for novel therapeutic strategies based on new mechanisms. Our study initially identified a significant reduction in serum *S*-adenosylmethionine (SAM) levels in insomnia patients through a cross-sectional analysis, suggesting SAM as a potential biomarker and therapeutic target for sleep disorders. We then screened 60 gut strains across seven species and identified a high-SAM-producing probiotic, *Lactobacillus helveticus* CCFM1320, which improved cognitive and memory impairments caused by sleep deprivation in mice. Mechanistically, CCFM1320 enhances the methylation of *N*-acetylserotonin, a precursor of melatonin synthesis, normalizing the expression of downstream circadian rhythm genes. A subsequent four-week placebo-controlled clinical trial demonstrated that CCFM1320 significantly improved sleep quality in patients with sleep disorders, as evidenced by reduced Pittsburgh sleep quality index (PSQI) scores, lower serum cortisol levels, and decreased prevalence of pathogenic species in the gut. Probiotic treatment also elevated the abundance of SAM synthesis and metabolism-related enzyme genes in the gut microbiome, likely due to the introduction of high-SAM-producing probiotics and subsequent SAM accumulation. This study presents a promising probiotic-based strategy for managing sleep disorders, offering a potential non-pharmacological treatment alternative.

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1. Introduction

Sleep is essential for overall health, influencing cognitive abilities, emotional stability, and psychological well-being, as well as cardiovascular and metabolic functions [1]. Insomnia, a widespread sleep disorder, has become increasingly common, particularly in the aftermath of the coronavirus disease 2019 (COVID-19) pandemic. A global study involving 57 298 participants reported a short-term insomnia prevalence of 11.3%, with rates varying between 2.3% and 25.5% across different regions [2]. Women, younger individuals, and residents of Brazil, Canada, Norway, Poland, the United States, and the United Kingdom exhibited higher prevalence rates, while Asian countries reported lower figures [3]. Sleep deprivation contributes to systemic inflammation, metabolic dysfunction, cognitive decline, and heightened risks of mental health disorders, severely affecting physical well-being [4]. Insomnia encompasses all sleep continuity disorders caused by environmental changes, lifestyle factors, or physical and mental health conditions [5]. Consequently, identifying effective treatment strategies is crucial.

The gut–brain axis is increasingly acknowledged for its influence on mental health and neurological disorders. Alterations in gut microbiota composition, or dysbiosis, have been associated with numerous mental health conditions, including Alzheimer's

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disease, autism, depression, Parkinson's disease, schizophrenia, and sleep disturbances [6,7]. In individuals with insomnia, the gut microbiota exhibits reduced diversity, diminished populations of anaerobic and short-chain fatty acid (SCFA)-producing bacteria, and an elevated presence of pathogenic species compared to healthy controls [8]. Animal study [9] with germ-free mice has demonstrated that the absence of gut microbiota protects against inflammation and cognitive deficits triggered by sleep deprivation. Conversely, transferring gut microbiota from sleep-deprived human donors to germ-free mice induces cognitive impairment and inflammation in both peripheral and central nervous systems. These findings [10] have sparked growing interest in modulating gut microbiota through dietary strategies, including the administration of probiotics and prebiotics, to support mental health. Clinical evidence [11] suggests that probiotics may enhance sleep quality. For instance, a ten-day treatment with 25 mg of heat-inactivated *Lactobacillus brevis* SBC8803 significantly increased delta wave activity on electroencephalograms (EEG) in patients with insomnia. Additionally, strains such as *Lactobacillus gasseri* CP2305 and *Bifidobacterium breve* CCFM1025 have been shown to improve sleep by modulating the hypothalamic–pituitary–adrenal (HPA) axis [12,13]. Despite these promising outcomes, the precise molecular mechanisms through which probiotics enhance sleep remain largely unexplored.

This study aims to: ① identify serum biomarkers associated with gut microbiota and sleep in individuals with insomnia; ② screen and characterize novel probiotics with potential insomnia-alleviating properties, along with their underlying mechanisms of action; and ③ assess the clinical efficacy of these probiotics in mitigating insomnia, offering innovative perspectives and strategies for non-pharmacological insomnia treatment.

2. Methods

2.1. Cross-sectional study

Inclusion criteria: participants aged 18 to 65 years, including individuals diagnosed with insomnia and healthy volunteers without insomnia. Exclusion criteria: ① current use of hypnotic medications; ② engagement in night shift work or maintenance of an irregular lifestyle; ③ history of alcohol dependence; ④ diagnosis of other mental disorders; ⑤ pregnancy, lactation, or plans for pregnancy (applicable to both men and women).

Volunteers were categorized into control and insomnia groups based on Pittsburgh sleep quality index (PSQI) [14], with a PSQI > 7 indicating membership in the insomnia group. The evaluation was performed by hospital physicians. The Athens insomnia scale (AIS) was also employed as a supplementary tool for sleep quality assessment [15]. Blood samples were collected from participants for metabolomics analysis and cortisol level measurement.

2.2. Serum metabolomics analysis

Serum metabolites were analyzed using liquid chromatography–tandem mass spectrometry (LC–MS/MS) with detailed methodology previously reported [15]. Mass spectrometry utilized electrospray ionization (ESI) in both positive and negative ion modes, employing a data-dependent acquisition method (full-scan MS/data-dependent-MS/MS). Data processing and statistical analysis were conducted using Metabo Analyst 5.0 (McGill University, Canada).

2.3. Measurement of S-adenosylmethionine production by bacteria

Bacterial strains used in this study were isolated from fresh feces of healthy individuals. Detailed strain information is available in Table S1 in Appendix A. Bacterial suspension was streaked onto de man, rogosa, and sharpe (MRS) solid medium and incubated anaerobically at 37 °C for 24 h. Single colonies were then transferred to a 5 mL liquid MRS medium and cultured under the same conditions for 18 h to obtain seed cultures for further fermentation to produce *S*-adenosylmethionine (SAM).

The fermentation broth was adjusted to a bacterial concentration of 5×10^9 CFU·L^{-1}. Cell-free supernatant was obtained by centrifugation at 6000*g* (*g* = gravitational acceleration, 9.8 m·s^{-2}) for 20 min, followed by freeze-drying 3 mL of the supernatant. The dried material was reconstituted in 200 μL of high-performance liquid chromatography (HPLC)-grade water and filtered through a 0.22 μm membrane. SAM levels in the fermentation broth were quantified using HPLC under the following conditions, adapted from a previously reported method [16].

2.4. Animal experiment design

Male C57BL/6J mice, aged nine weeks and weighing 20–24 g, were used in the study. A total of 32 mice were randomly assigned to four groups (n = 8 per group): healthy control (HC) group; sleep deprivation (SD) group; high SAM-producing *Lactobacillus helveticus* (*L. helveticus*) (Lh-SAM-H) group, treated with strain CCFM1320; and low SAM-producing *L. helveticus* (Lh-SAM-L) group, treated with strain DSCAB11L3. The experimental design is illustrated in Fig. S1 (a) in Appendix A.

Except for the HC group, all other mice were subjected to SD, which lasted from 12:30 to 08:30 (the second day) daily, followed by a 4 h rest period in their home cages before starting a new sleep deprivation session. Sleep deprivation was performed using a modified multiple platform water environment method (MMPM) [17]. The experimental chamber dimensions were 40 cm (length) × 30 cm (width) × 20 cm (height). The model group chambers were equipped with 20 small platforms, each with a diameter of 3 cm and a height of 4 cm, spaced 4 cm apart. The presence of multiple platforms prevented the mice from entering deep sleep, as a decrease in muscle tone could lead to the mouse's head touching the water or falling into the water, causing a sudden awakening. The HC group chambers were equipped with two large platforms, each with a diameter of 15 cm and a height of 4 cm. Water was added to the chambers to a height of approximately 3 cm. Sufficient food and water bottles were placed on top of the chambers, allowing the mice to access food and water freely while standing on the platforms, ensuring adequate hydration and nutrition during sleep deprivation.

Mice in the HC and SD groups were gavaged with 200 μL of saline daily, while the Lh-SAM-H and Lh-SAM-L groups received 5×10^9 CFU of *L. helveticus* CCFM1320 or DSCAB11L3, respectively, for 14 days. Behavioral tests began on day 23 (Fig. S1(a)). On day 28, mice were euthanized, and organs were collected for analysis.

2.5. Behavioral experiments

Open field test: The experimental mice were placed in the center of an open area measuring 40 cm × 40 cm, and timing and recording commenced immediately. After 6 min, the recording was stopped. The movement trajectory, time spent moving, and movement speed of the mice in the open field were recorded to analyze their spontaneous activity and exploratory behavior. Novel object recognition test: This test involved two phases, including adaptation and testing. During adaptation, two objects were placed at opposite ends of a chamber, and mice were allowed to explore for 10 min. After a 24 h interval, one object was replaced with a novel object, and the mice were allowed to explore again for 10 min. The recognition and discrimination indices are calculated as recognition index = (time exploring novel object)/(time

exploring novel object + time exploring familiar object) × 100%. Discrimination index = (time exploring novel object − time exploring familiar object)/(time exploring novel object + time exploring familiar object) × 100%.

Y-maze test: The Y-maze had three arms, including labeled start, novel, and other. In stage one, the novel arm was blocked, and mice explored the start and other arms for 5 min. After a 4 h interval, stage two began, with the block removed from the novel arm, allowing exploration of all three arms for 5 min. The total number of entries into each arm and the duration spent in each arm were recorded.

2.6. Quantification of 5-HT metabolites in mouse striatum

An HPLC-fluorescence detection system was used to quantify 5-hydroxytryptamine (5-HT), *N*-acetylserotonin (NAS), and melatonin in the mouse frontal cortex. Tissue samples were homogenized and protein was precipitated with 5% perchloric acid. After centrifugation (6000*g* for 15 min at 4 °C), the supernatant was filtered through a 0.22 μm membrane. Chromatography was performed using a Waters Atlantis T3 column (Waters, USA) as described in a previous study [18].

2.7. Real-time quantitative PCR (RT-qPCR)

Total RNA from the hypothalamus and striatum of mice was extracted using TRIzol reagent (Invitrogen, USA). An appropriate amount of brain tissue (20–30 mg) was homogenized in 1 mL TRIzol with two enzyme-inactivated magnetic beads, and total RNA was extracted. The RNA was then purified using chloroform, isopropanol, and 75% ethanol sequentially. After quantifying the total RNA concentration, a reverse transcription reaction was set up using a reverse transcription kit to generate complementary DNA (cDNA) from 1 μg of total RNA. After reverse transcription, the obtained cDNA was diluted to an appropriate concentration with 80 μL of diethyl pyrocarbonate (DEPC) water as the template for subsequent reverse transcription quantitative polymerase chain reaction (RT-qPCR). Table S2 in Appendix A lists the sequences of the primers required for the experiment. *Gapdh* was used as the reference gene.

2.8. Western blot

Total protein was extracted from the mouse hypothalamus using radio immunoprecipitation assay lysate (RIPA) buffer (Beyotime, China) with protease inhibitors. Electrophoresis was performed at 100 V using a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separating gel to separate proteins, and a 5% SDS-PAGE stacking gel was used to load the samples into the separating gel. Proteins from the gel were transferred onto a 0.22 μm polyvinylidene fluoride (PVDF) membrane using a transfer buffer and 100 V voltage. The membrane was then blocked with 5% skim milk for 1 h and subsequently incubated with a series of primary antibodies (β-actin monoclonal antibody, Proteintech, China, Catalog Number: 66009-1-Ig, 1:20000; MTNR1A Rabbit pAb, Abclonal, China, A13030, 1:1000; MT2A Rabbit pAb, Abclonal, A22103, 1:500; cyclic adenosine monophosphate (cAMP)-response element-binding protein (CREB) 1 Rabbit pAb, Abclonal, A11064, 1:500; Phospho-CREB1-S133 Rabbit pAb, Abclonal, AP0019, 1:500; and BMAL1 Rabbit mAb, Abclonal, A4714) overnight at 4 °C. The membrane was incubated with IgG-HRP antibody (Absin-abs20002, 1:20000) for 2 h to amplify the signal, followed by conventional chemiluminescent detection. ImageJ software (National Institutes of Health, USA) was used for grayscale scanning analysis to quantify protein expression levels.

2.9. Probiotic intervention trial

Before enrollment (baseline), participants completed the PSQI and AIS questionnaires to assess their sleep quality and eligibility. Participants who met the inclusion criteria completed the questionnaires and provided blood and fecal samples.

A total of 60 volunteers with insomnia were recruited and randomly allocated into three groups: the placebo group, low-dose CCFM1320 intervention (Pro-L) group, and high-dose CCFM1320 intervention (Pro-H) group, with 20 participants in each group. Ultimately, two participants dropped out of the placebo group, and two participants dropped out of the low-dose intervention group, while all participants in the high-dose intervention group completed the trial (Fig. 5(a)). Participants in the placebo group received maltodextrin (2 $g \cdot day^{-1}$), while those in the Pro-L and Pro-H groups received freeze-dried bacterial powder (maltodextrin as the freeze-drying protective agent, 2 $g \cdot day^{-1}$, with bacterial counts of 5×10^6 and 5×10^9 CFU, respectively). Volunteers in all three groups were unable to distinguish whether they were receiving probiotics or placebos based on the appearance or taste of the products. During the intervention period, participants were instructed to avoid consuming probiotic-containing supplements or foods, with no other dietary restrictions. The intervention period with probiotics or placebos lasted for four weeks. All participants provided blood samples once before and once after the intervention.

2.10. 16S ribosomal RNA (rRNA) gene sequencing analysis of fecal microbiota

Bacterial DNA was extracted using the MP Biomedical kit (Cat No. 6570200). The V3–V4 region of the bacterial 16S rDNA was amplified for sequencing on an Illumina MiSeq platform (Illumina, USA). Paired-end reads were quality-filtered and denoised with the divisive amplicon denoising algorithm 2 (DADA2) library, generating an amplicon sequence variant (ASV) table. Alpha diversity was assessed with the Shannon index and observed species, while beta diversity was evaluated using Aitchison distance and permutational multivariate analysis of variance (PERMANOVA), with principal component analysis (PCA) for visualization. Functional metagenomic analysis was conducted using phylogenetic investigation of communities by reconstruction of unobserved states 2 (PICRUSt2), annotating the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologues (KOs).

2.11. Statistical analysis

For the cross-sectional study, an unpaired *t*-test was used to analyze the intergroup differences between control and insomnia. MedCalc (v. 22.023, MedCalc Software Ltd., China) software was employed to conduct receiver operating characteristic curve (ROC) analysis on serum S-adenosylmethionine (SAM) and cortisol (CORT), assessing their diagnostic potential for insomnia. The predicted probability from the logit model based on the serum SAM and CORT, was utilized to construct the ROC curve, evaluating the combined diagnostic potential of the two indicators.

For the animal study, all data underwent normality checks using the Shapiro–Wilk test before further analyses. One-way analysis of variance (ANOVA) and post-hoc Dunnett's multiple comparisons test (against the SD group) were employed. For the probiotic intervention trial, differences before (pre) and after (post) the administration of placebo or probiotics were assessed using paired *t*-tests. Intergroup differences in score changes before and after intervention were compared using one-way ANOVA and post-hoc Dunnett's multiple comparisons test (against the placebo group). For non-normally distributed data, the Mann–Whitney test

was employed for comparisons between two groups, while the Kruskal–Wallis test was used for analyses involving three groups. *p*-values for multiple comparisons were adjusted with family-wise significance. Statistical significance was considered at a 95% confidence interval and a *p*-value of less than 0.05 in all comparisons. All statistical analyses were conducted using Prism 9.0 (GraphPad Software, USA).

3. Results

3.1. Circulating SAM may help alleviate insomnia

A total of 20 insomnia patients and 20 healthy volunteers were recruited for the study. Demographic information (Table S3 in Appendix A) showed no significant differences in age, gender, body mass index (BMI), and educational background between the healthy control group and insomnia patients. In terms of sleep scales, participants in the insomnia group had significantly higher PSQI and AIS scores compared to the healthy control group (Figs. 1(a) and (b)). Metabolomic analysis of serum from both groups revealed significant differences in composition (Fig. S1(b) in Appendix A). Using thresholds of $p < 0.05$, fold change (FC) > 1.5, and variable importance in projection variable importance in projection (VIP) ≥ 1, several metabolites previously reported to be associated with sleep were found to be significantly reduced in insomnia patients, including gamma-aminobutyric acid (GABA) and adenosine (Fig. 1(c)). GABA, an inhibitory neurotransmitter, promotes sleep in the early stages of sleep [19]. However, GABA in the peripheral system cannot cross the blood–brain barrier, limiting its application in drugs or functional foods [20,21]. Adenosine has been reported to activate specific G-protein-coupled receptors, such as A1 and A2A receptors, affecting sleep by increasing or decreasing intracellular cAMP levels [22]. Adenosine can also be converted into SAM through a series of reactions. SAM, as the sole methyl donor in the central nervous system, is involved in various biochemical reactions, including the methylation of DNA, RNA, and proteins, which are crucial for maintaining normal biological rhythms [23,24].

We further measured SAM levels in the serum of patients using LC-MS and found significant statistical differences between the groups (Fig. 1(d)). There was a negative correlation between serum SAM levels and both PSQI and AIS scores, with the latter showing significant statistical differences (Fig. 1(e)). Metabolomic results also revealed that glucocorticoids (including corticosterone and cortisol) were significantly elevated in the serum of insomnia patients, the serum cortisol level was confirmed by absolute quantification using enzyme-linked immunosorbent assay (ELISA) (Fig. 1(f)). Finally, we found that serum SAM levels (area under curve (AUC) = 0.796, $p < 0.001$; Fig. 1(g)) and cortisol levels (AUC = 0.895, $p < 0.001$; Fig. 1(h)) both demonstrated good diagnostic potential for insomnia. A logistic model combining these two indicators further improved diagnostic accuracy (AUC = 0.933, $p < 0.001$; Fig. 1(i)). These results suggest that increasing SAM levels in the blood may help improve insomnia.

3.2. High SAM-producing L. helveticus *CCFM1320 alleviates neurobehavioral abnormalities induced by sleep deprivation in mice*

Research has shown that gut microbiota can synthesize SAM [25]. We assessed the SAM production capability of 60 microbial strains isolated from the human gut, including 36 strains of *Bifidobacterium* and 24 strains of *Lactobacillus*, and found significant inter-strain differences in SAM production (Fig. 2(a)). Among the *Bifidobacterium* strains, *Bifidobacterium longum* (*B. longum*) C7 produced the least SAM, about 25.85 $\mu g \cdot L^{-1}$, while *B. bifidum* L2 had the highest average SAM production, approximately 204.20 $\mu g \cdot L^{-1}$. Among the *Lactobacillus* strains, *L. helveticus* R6 (original strain number DSCAB11L3) had the lowest average SAM production at 36.49 $\mu g \cdot L^{-1}$, while *L. helveticus* R8 (original strain number CCFM1320) had the highest average SAM production at 375.40 $\mu g \cdot L^{-1}$, far exceeding the second highest producer, *L. paracasei* F4 (approximately 183.10 $\mu g \cdot L^{-1}$), and the highest-producing *Bifidobacterium* strain *B. bifidum* L2 (approximately 204.20 $\mu g \cdot L^{-1}$).

We further evaluated the neurobehavioral effects of the high SAM-producing *L. helveticus* CCFM1320 in a sleep deprivation mouse model, using the low SAM-producing *L. helveticus* DSCAB11L3 as a control (experimental design in Fig. S1(b)). The results showed that sleep deprivation significantly reduced the body weight of mice (Fig. 2(b)), but treatment with both *Lactobacillus* strains did not reverse this trend. Administration of CCFM1320 reversed the decrease in SAM levels in feces and serum and the increase in serum corticosterone induced by sleep deprivation, whereas DSCAB11L3 did not show these effects (Figs. 2(c)–(e)). The SAM level enhancement mediated by CCFM1320 also alleviated neurobehavioral abnormalities caused by sleep deprivation, including improved recognition and memory of new objects (Fig. 2(f)), reduced hyperactivity in the open field test (Figs. 2(g) and (h)), and enhanced spatial exploration ability in the Y-maze test (Fig. 2(i)).

3.3. L. helveticus *CCFM1320 modulates brain melatonin synthesis and circadian gene expression*

The synthesis of melatonin (*N*-acetyl-5-methoxytryptamine (MT)) predominantly occurs at night. It involves the conversion of 5-HT to NAS by serotonin *N*-acetyltransferase (AANAT), and subsequently to MT by acetylserotonin *O*-methyltransferase (HIMOT), with SAM providing the methyl group in this step [26,27] (Fig. 3(a)). As a critical neurotransmitter, MT plays a significant role in improving sleep and regulating circadian rhythms [28]. Our results showed that administering CCFM1320 reversed the reduction in striatal SAM and MT levels caused by SD (Figs. 3(b) and (c)), but had no significant effect on 5-HT, NAS, AANAT, and HIMOT levels (Figs. 3(d)–(g)). This indicates that the increase in brain MT levels was contributed by SAM. Considering MT functions as a hormone, we also measured its serum levels and found similar differences between groups (Fig. 3(h)).

Next, we analyzed the transcription of 15 circadian genes in the hypothalamus (Fig. 4(a)). Compared to healthy mice, SD mice showed significantly reduced *Per1* transcription and significantly increased *Per2*, *Per3*, *Cry1*, *Cry2*, *Bmal1*, *Clock*, *Timeless*, *Rev-erbα*, *RORα*, and *Napas2* transcription levels. The transcription levels of *c-fos*, *Ciart*, *DBP*, and *Rev-erbβ* were also elevated but not statistically significant. Probiotic treatment notably reversed the SD-induced transcription abnormalities in *Per2*, *Cry2*, *Bmal1*, *Timeless*, *Rev-erbα*, *RORα*, and *Napas2*. Interestingly, except for *Rev-erbα* and *DBP*, all other circadian genes were significantly correlated with at least one behavioral phenotype, and *Per1*, *Cry1*, and *Bmal1* were significantly correlated with all behavioral phenotypes (Fig. 4(b)). Among these, brain and muscle *arnt-like 1* (*Bmal1*) is a core circadian gene playing a critical regulatory role in biological activities.

Previous research [29] has found that MT, upon binding to its receptors, can activate the cAMP/protein kinase A (PKA) pathway, leading to the phosphorylation and activation of CREB, which subsequently influences *Bmal1* transcription. Given the significant impact of probiotics on MT levels and *Bmal1* gene transcription, we analyzed this pathway at the protein level. The results indicated no significant effects of SD and probiotics on the protein levels of MT's two receptors, MT1 and MT2, as well as the

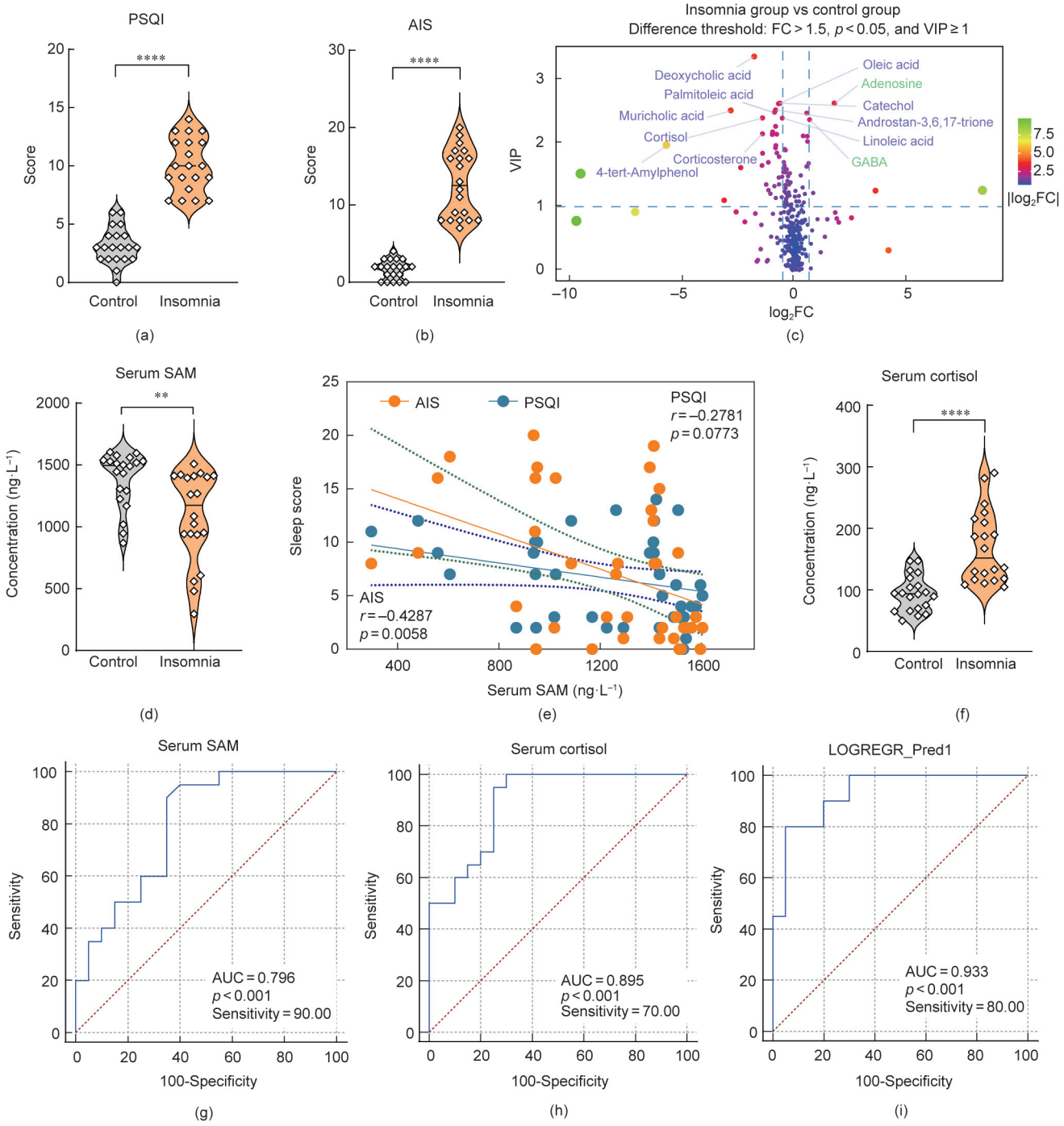

Fig. 1. Correlation of serum levels of SAM and cortisol with sleep quality. (a, b) Comparison of (a) PSQI scores and (b) AIS between individuals with insomnia and healthy controls, n = 20 per group. (c) Volcano plot showing accumulated metabolites significantly different between the control and insomnia group, with metabolites significantly decreased in the insomnia group. Criteria for biomarker screening: FC > 1.5 ($|\log_2 FC| > 0.585$), $p < 0.05$, and VIP (under partial least squares-discriminant analysis) ≥ 1. (d) Serum SAM levels, n = 20 per group. (e) Scatter plot demonstrating a negative correlation between SAM levels in patient serum and their PSQI and AIS scores. (f) Serum cortisol levels, n = 20 per group. (g–i) ROC curves for diagnosing insomnia using (g) serum SAM, (h) cortisol, and (i) the prediction probabilities of log regression (LOGREGR_Pred1) between serum SAM and cortisol. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$, using unpaired t-test. r: Pearson's correlation coefficient.

transcription factor CREB in the hypothalamus. However, probiotics could reverse the SD-induced reduction in phosphorylated-CREB (p-CREB) and Bmal1 protein expression (Figs. 4(c) and (d)). This effect is possibly mediated by the SAM-induced increase in MT levels, but further validation is required using MT receptor knockout mouse models.

3.4. L. helveticus *CCFM1320 as an adjunct therapy for improving sleep quality in insomnia patients*

To further evaluate the clinical efficacy of *L. helveticus* CCFM1320 in improving sleep, we conducted a randomized, placebo-controlled trial involving insomnia patients (Fig. 5 (a)).

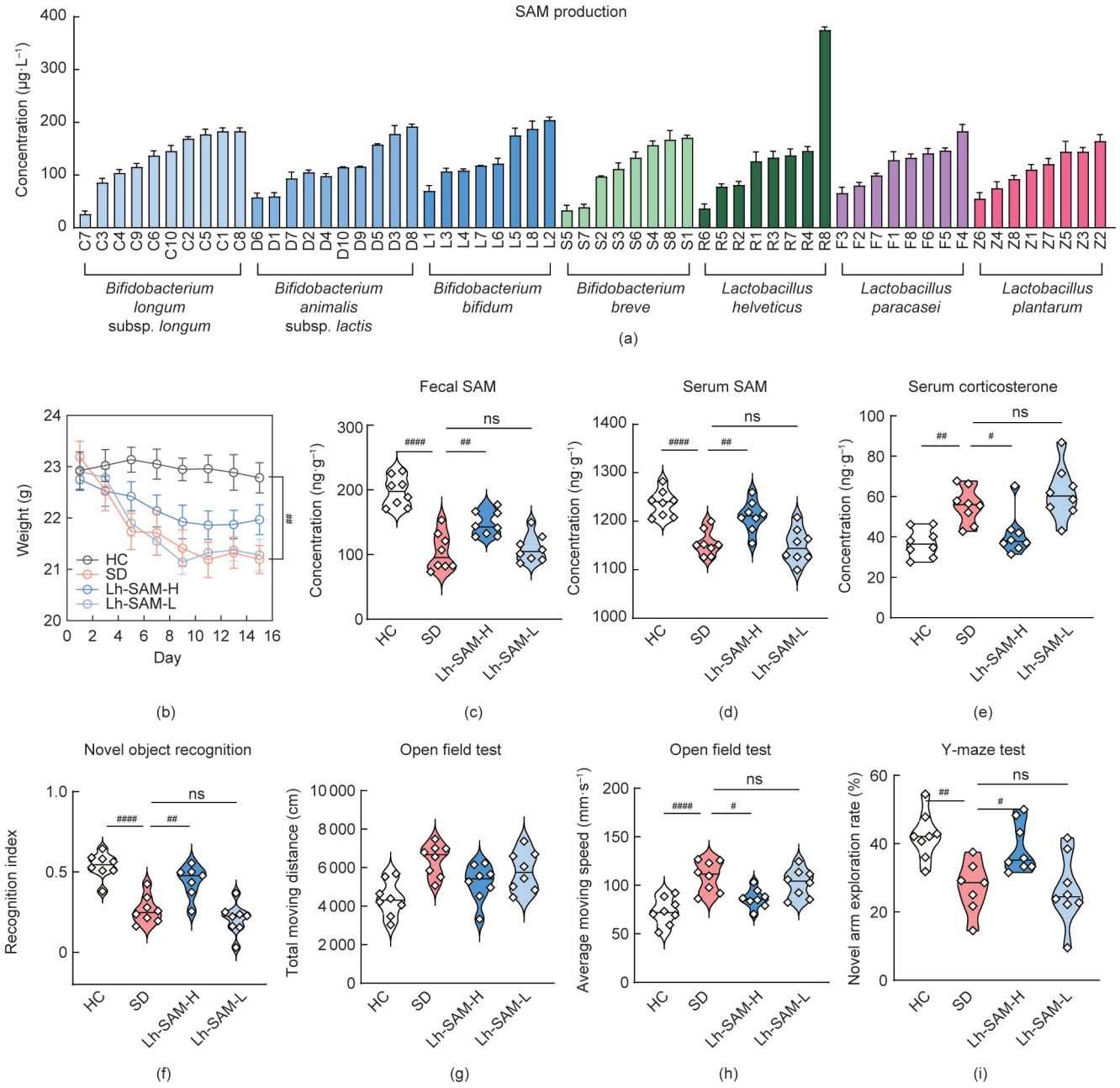

Fig. 2. Treatment with high SAM-producing *L. helveticus* reverses neurobehavioral abnormalities in sleep-deprived mice. (a) HPLC determination of SAM production by 60 gut microorganisms isolated from human feces, including 36 bifidobacteria strains and 24 *Lactobacilli* strains (three replicates per strain). One high SAM-producing strain, *L. helveticus* R8 (complete strain number CCFM1320, referred to as Lh-SAM-H in subsequent figures), and a low SAM-producing control strain (complete strain number DSCAB11L3, referred to as Lh-SAM-L) were identified for further experiments. (b) Weight changes in mice during the two-week probiotic intervention. (c, d) SAM levels in the (c) feces and (d) serum, $n = 8$ per group. (e) Serum corticosterone levels, $n = 8$ per group. (f–i) Behavioral tests: (f) novel object recognition test, (g, h) open field test, and (i) Y-maze test, $n = 8$ per group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$, using one-way ANOVA with Dunnett's *post hoc* multiple comparisons test. ns: no significant.

The probiotic intervention group was divided into two doses: 5×10^6 and 5×10^9 CFU·day^{-1}, to explore the dose–response relationship of the probiotics. All patients were advised to use probiotics (or placebo) as a priority over sleeping pills, and those who took sleeping pills during the trial were excluded from the final analysis (demographic information is presented in Table S4 in Appendix A). The results showed that after four weeks of probiotic consumption, participants had significantly reduced PSQI and AIS scores (Figs. 5(b) and (c)).

We performed an inter-group analysis to evaluate the changes in scale scores before and after treatment, comparing the effects of different probiotic doses. The results showed that the reduction in PSQI scores differed across the three groups, with the Pro-H group exhibiting a greater reduction than the Pro-L group, and this difference was statistically significant compared to the placebo group (Fig. 5(b)). However, no significant differences were observed in the changes in AIS scores among the groups before and after treatment (Fig. 5(c)).

Furthermore, high-dose probiotics significantly increased serum SAM levels and reduced serum cortisol levels compared to low-dose probiotics, with a statistically significant difference observed when compared to the placebo group (Figs. 5(d) and (e)). However, no significant difference was found in serum MT levels between the probiotic and placebo groups (Fig. 5(f)).

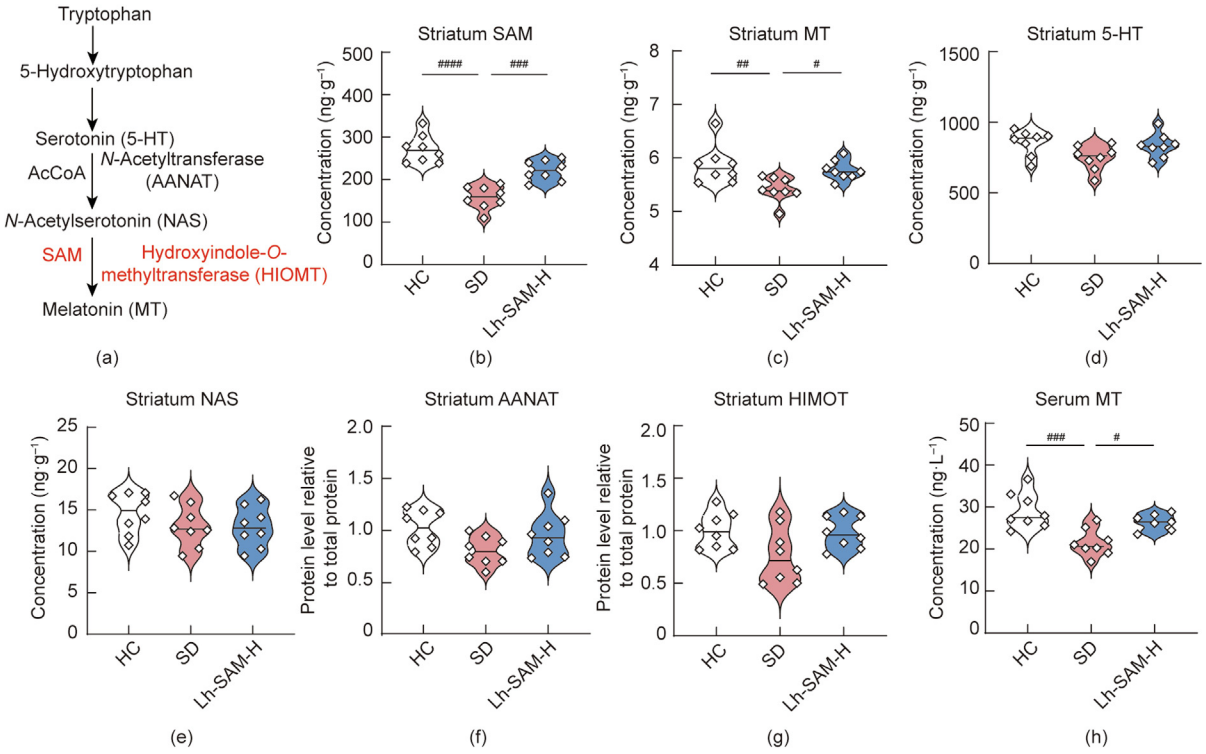

Fig. 3. Effect of high SAM-producing probiotics on MT biosynthesis in the striatum. (a) Current understanding of the MT biosynthetic pathway. AcCoA: acetyl-coenzyme A. (b–e) HPLC determination of the levels of (b) SAM, (c) MT, (d) 5-HT metabolites, and (e) NAS in the striatum, $n = 8$ per group. (f, g) The protein levels of AANAT and HIOMT in the striatum, $n = 8$ per group. (h) Serum MT level, $n = 8$ per group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$, using one-way ANOVA with Dunnett's *post hoc* multiple comparisons test.

3.5. L. helveticus *CCFM1320 alters core gut microbiota structure and SAM synthesis capacity in insomnia patients*

16S amplicon sequencing was performed on the gut microbiota of the Pro-H and placebo groups. Neither probiotic nor placebo intervention resulted in significant changes in gut microbiota α-diversity and β-diversity (Figs. S1(g)–(i)). However, the interventions did impact the composition and abundance of core microbiota species (species detected in ≥ 50% of samples with a relative abundance ≥ 1%). Following placebo treatment, two new core species, *Erysipelotrichaceae* UCG 003 and *Streptococcus*, emerged in the gut. These bacteria are widely reported to be highly associated with host inflammation and infection [30–32]. In contrast, probiotic treatment reduced the number of core gut species from 14 to 10, including the pathogenic bacteria *Streptococcus* and *Collinsella* (Fig. 6(a)) [33]. Linear discriminant analysis (LDA) effect size analysis of differential species before and after placebo and probiotic treatment revealed that probiotic treatment increased the abundance of *Lachnospiraceae* ND3007 (Fig. 6(b)), which has been previously reported to be associated with the development of depression and Parkinson's disease [34,35]. Compared to the placebo-post group, the Pro-H group had higher abundances of *Holdemanella* and *Fournierella*, both of which have been reported to be associated with improved glucose and lipid metabolism and enhanced efficacy of anticancer drugs [36,37]. Lastly, predictive annotation of the gut microbiota was performed, particularly focusing on enzymes related to the SAM synthesis pathway. Four enzymes were annotated in the reported SAM synthesis and metabolic pathways: SAM synthetase (EC:2.5.1.6), DNA (cytosine-5)-methyltransferase (EC:2.1.1.37), SAM decarboxylase (EC:4.1.1.50), and spermidine synthase (EC:2.5.1.16) (Fig. 6(c)). The abundances of these enzymes were significantly increased following probiotic treatment, potentially due to the introduction of high SAM-producing probiotics and the accumulation of SAM in the gut.

4. Discussion

This study investigates the potential of SAM as a biomarker for the clinical diagnosis of insomnia disorders. Additionally, we screened and identified a high-SAM-producing lactic acid bacterium and evaluated its physiological effects on sleep-deprived mice and individuals with sleep disorders. The results demonstrated that the beneficial effects are related to SAM-mediated methylation and MT synthesis, as well as the normalization of circadian rhythm gene expression in the brain.

SAM is the sole methyl donor involved in the methylation of DNA, RNA, and histones. Studies using the drug 3-deazaneplanocin A to simulate methylation defects have shown that alterations in methylation levels can disrupt the biological rhythms of various organisms [38]. In the central nervous system, SAM not only directly participates in MT synthesis but also influences the expression of circadian genes through the regulation of histone and DNA methylation, thereby affecting the biological rhythms of the organism [23,24,26]. In this study, we found significant differences in serum SAM levels between patients with sleep disorders and control groups, indicating its potential for disease diagnosis, especially when combined with cortisol levels. Considering the current mainstream diagnostic methods based on psychological scales, this approach could reduce the subjective errors associated with such evaluations.

Studies have shown that SAM can cross the blood–brain barrier, and its administration either orally or by injection can significantly increase cerebrospinal fluid SAM levels, effectively treating various neuropsychiatric disorders [39,40]. A meta-analysis involving 1522 subjects compared the effects of SAM with placebo as a

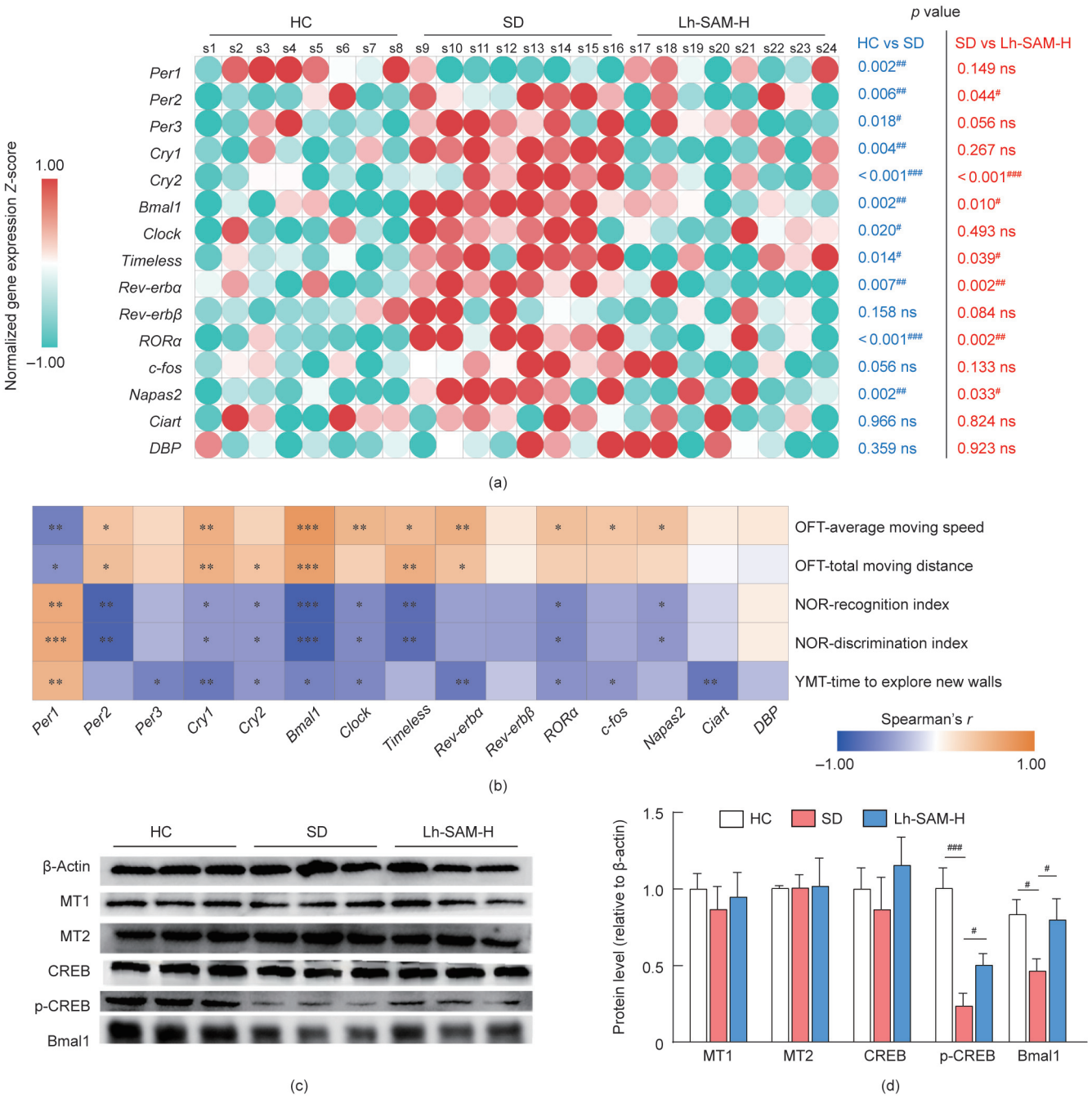

Fig. 4. Effect of probiotics on hypothalamic gene transcription related to circadian rhythms. (a) Messenger RNA (mRNA) levels of 15 circadian rhythm-related genes in the hypothalamus. Z-score: a statistical measurement representing the number of standard deviations a data point is from the mean. (b) Spearman correlation analysis of the expression levels of 15 circadian rhythm-related genes and behavioral phenotypes in mice; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ indicate significant correlations. OFT: open field test; NOR: novel object recognition; YMT: Y-maze test. (c, d) Western blot analysis of the MT-mediated CREB–Bmal1 signaling pathway, with β-actin as the loading control. One-way ANOVA with Dunnett's *post hoc* multiple comparisons test was used to assess differences between groups; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

monotherapy, SAM with imipramine or escitalopram as a monotherapy, and SAM with placebo as an adjunct therapy. The results indicated that SAMe could alleviate depressive symptoms, with effects comparable to imipramine or escitalopram [41]. Oral SAM also improved sleep quality in patients with depression and reduced excessive activation of the HPA axis under stress [42,43]. However, recent research has found that excessive exogenous intake of SAM under physiological conditions can disrupt biological rhythms. Therefore, the safety of SAM as a direct dietary supplement remains controversial.

SAM is widely present in organisms. Here, we found that various gut probiotics, including species from the genera *Lactobacillus* and *Bifidobacterium*, can synthesize SAM. Using probiotics to provide endogenous SAM has several advantages over direct SAM intake. Firstly, probiotics can reside in the human gut for a certain period, creating an endogenous sustained-release effect. This partially avoids the shortcomings of oral administration, such as the rapid clearance of the drug due to its short half-life, the need for intermittent dosing, and the potential risks associated with high plasma drug exposure in a short time [44]. Additionally, as active

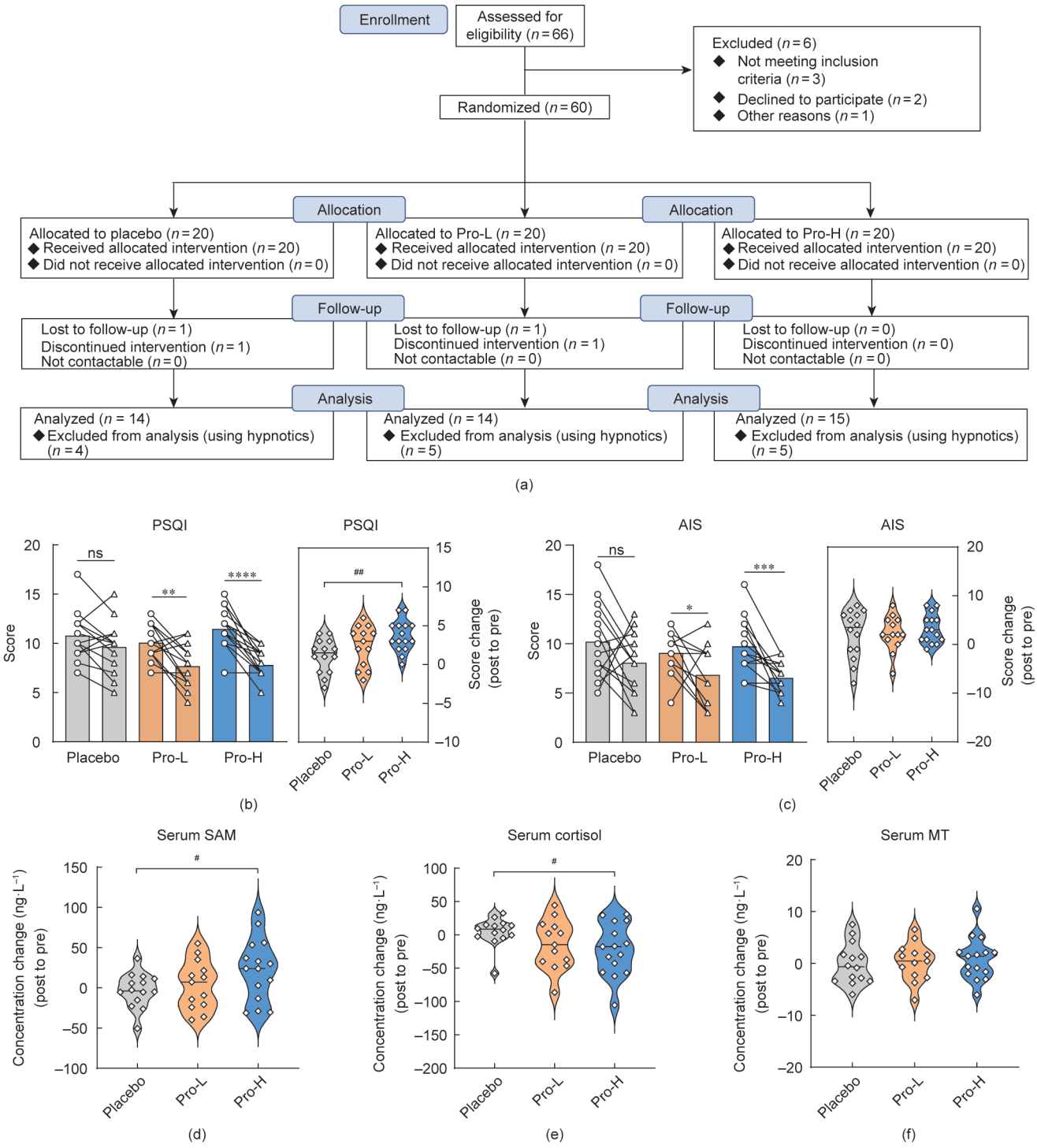

Fig. 5. Clinical evaluation of *Lactobacillus helveticus* CCFM1320 in improving insomnia. (a) Flow chart of patient recruitment and grouping. The number of samples included in the data analysis at the end of the experiment for the placebo, Pro-L, and Pro-H group were 14, 14, and 15, respectively. (b, c) Analysis of changes in (b) PSQI and (c) AIS scores before (marked by a dot) and after (marked by a triangle) the probiotic intervention. A paired *t*-test was used to analyze differences between baseline and post-treatment scores within the same group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$. One-way ANOVA with Dunnett's *post hoc* multiple comparisons test was used to analyze the changes in scores before and after the intervention between groups; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$. (d–f) Analysis of changes in serum levels of (d) SAM, (e) cortisol, and (f) MT before and after treatment between groups.

microorganisms, probiotics can modulate the ecological balance of the gut microbiota, exerting effects through the "microbiota–gut–brain axis" [45]. Notably, gut microbes themselves and their interactions with the gut can produce a significant amount of neurotransmitters and their precursors (including 5-HT, dopamine, acetylcholine, etc.), enabling crosstalk between the enteric nervous system and the central nervous system [46,47]. In our previous animal study [48], we found that the SAM synthesis pathway and GABA degradation pathway in the gut microbiota of sleep-deprived mice were significantly disrupted.

The gut microbiota functions as a complex micro-ecosystem, where the introduction of any exogenous strain does not result

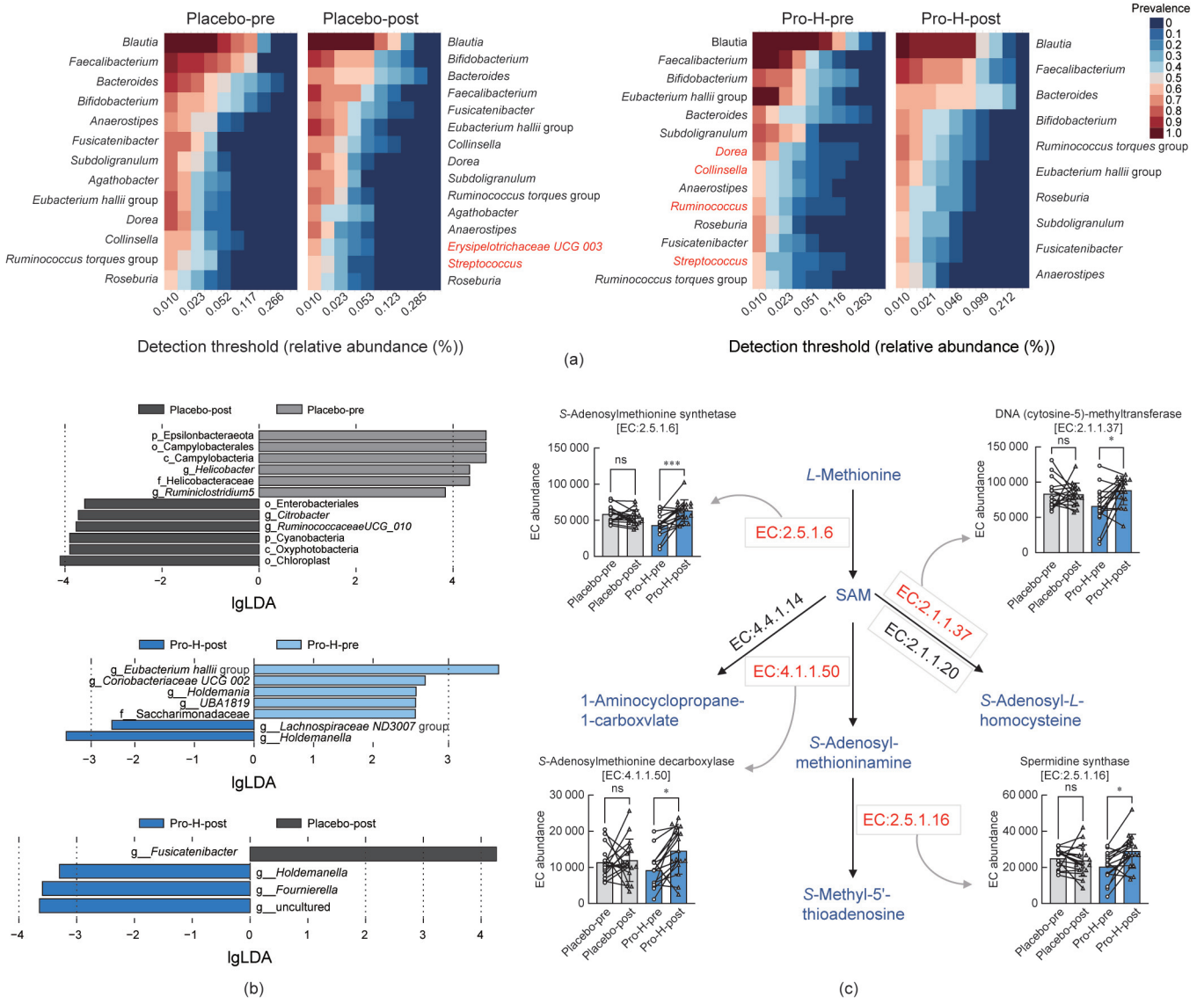

Fig. 6. The impact of *L. helveticus* CCFM1320 on the gut microbiota of insomnia patients. (a) Analysis of core microbiota. The taxa shown are present in over 50% of the population with a relative abundance of 0.01%. (b) Analysis of differential species between groups. LDA effect size (LEfSe) was employed to identify significant microbial taxa ($p < 0.05$ and lgLDA > 2.0 as thresholds). (c) Abundance analysis of enzymes related to SAM synthesis and metabolism pathways. Red labels indicate annotated enzymes. The differences in gene abundance of the enzymes between groups are present.

in a straightforward "additive" effect. If a strain possesses strong colonization ability, the additive effect may be more pronounced; conversely, weaker colonization may lead to rejection by resident microbial species. In this context, we suggest that *L. helveticus* CCFM1320 does not stably colonize the gut. Analysis of the relative abundance of Lactobacillus from our 16S rRNA amplicon sequencing dataset revealed no significant differences between the Pro-H-pre and Pro-H-post groups (Fig. S1(j)). This finding aligns with previous research on other Lactobacillus strains, such as *L. rhamnosus*, *L. reuteri*, and *L. plantarum*, which typically exhibit colonization durations of less than seven days, whereas bifidobacteria can persist for over two weeks [49]. To accurately assess the colonization ability of a strain, it is essential to develop strain-specific primers and utilize RT-qPCR for absolute quantification. Furthermore, considerations must be made for the natural washout time after ceasing intake to evaluate the strain's persistence.

However, this colonization ability does not diminish the effects of probiotics, particularly with continuous consumption. In this human trial, we further validated these findings, revealing that while probiotic administration did not alter overall gut microbiota diversity, it significantly increased the abundance of genes associated with SAM synthesis and metabolic enzymes. These results support the hypothesis that after introducing a high SAM-producing probiotic, its weak colonization ability—evidenced by the lack of significant increase in *Lactobacillus* abundance—led to SAM stimulating metabolic responses and consumption by other microbial species. This is reflected in the increased expression of enzymes such as EC:2.1.1.37, EC:4.4.4.50, and EC:5.5.1.16 within the microbial community. Furthermore, the introduction of probiotics reduced the number of potentially pathogenic species in the core gut microbiota, contributing to improved immune homeostasis and reduced inflammatory responses. Such changes have been shown to reverse neurophysiological abnormalities induced by sleep deprivation, as demonstrated in fecal microbiota transplantation experiments [9]. Overall, we propose that SAM supplementation from continuous probiotic intake, alongside its effects on

cortisol and MT levels, directly contributes to improved sleep, while the reduction of pathogenic species and inflammation acts as a supportive secondary factor.

It is important to acknowledge that this study faces some unresolved issues due to current technical limitations. First, the efficiency of gut microbiota-derived SAM supplementation to the circulatory system and brain requires further validation, particularly through pharmacokinetic studies utilizing isotopic labeling methods. Second, in evaluating the effects of probiotics, we compared changes in scale scores before and after treatment across groups rather than focusing solely on within-group pre- and post-treatment changes. This approach allows for accounting for any natural recovery that might occur without intervention. Unfortunately, statistically significant improvements induced by probiotic intervention were observed only in the PSQI scale scores and serum levels of SAM and cortisol. There were no significant differences in AIS scale scores or serum MT levels, which contrasts with the strong effects noted in animal studies. MT, produced by the pineal gland, exhibits concentration fluctuations according to the circadian rhythm, with higher secretion occurring at night [50,51]. In our trial, most blood samples from patients were collected during the day due to experimental constraints, which do not accurately reflect MT secretion capacity. Conversely, in the mouse experiments, blood was collected during the human daytime, coinciding with the nighttime for mice, resulting in heightened sensitivity and concentration of MT in serum, thereby amplifying the differences observed between groups. This underscores the necessity for future studies to select more appropriate time points, sample sizes, and varying intervention durations for hormone measurement to accurately assess secretion capabilities.

5. Conclusions

In conclusion, our study highlights the potential of SAM as both a biomarker and therapeutic target for sleep disorders, addressing the pressing need for novel treatment strategies. We demonstrated that high SAM-producing probiotics, particularly *L. helveticus* CCFM1320, significantly improve sleep quality and mitigate neurobehavioral impairments associated with sleep deprivation. Mechanistically, these probiotics enhance MT synthesis by promoting the methylation of NAS, thereby normalizing circadian rhythm gene expression. The clinical trial further supports the efficacy of this probiotic intervention, showing substantial improvements in sleep quality and related biomarkers. This research introduces a promising probiotic-based approach for managing sleep disorders, offering a non-pharmacological alternative that leverages gut microbiome modulation to restore healthy sleep patterns.

CRediT authorship contribution statement

Peijun Tian: Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization. **Yuming Lan:** Visualization, Investigation. **Zhiying Jin:** Visualization, Investigation. **Feng Hang:** Resources. **Xuhua Mao:** Methodology. **Xing Jin:** Investigation. **Gang Wang:** Methodology, Funding acquisition. **Wei Chen:** Writing – review & editing, Supervision, Resources, Funding acquisition, 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.

Ethical statement

The cross-sectional and probiotic intervention trials received approvals from the Medical Ethics Committee of Yixing People's Hospital (Yixing, China) with the Chinese Clinical Trial Registry (ChiCTR2300067806 and ChiCTR2400080254). Animal experiments approved by Jiangnan University Animal Experiment Ethics Committee (20221230c0640630 [571]).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eng.2024.12.025.

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