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Sleep enhancement mechanism of GABA-Producing lactic acid bacteria XBMU-SN-23 fermentation broth in a p-chlorophenylalanine-induced insomnia mouse model

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ABSTRACT: γ -Aminobutyric acid (GABA) is an important neurotransmitter found abundantly in animals, plants, and microorganisms. *Lactobacilli* recognized as natural GABA producers, have garnered significant attention due to their safety and functionality. In our study, we aimed to isolate a high GABA-producing *Lactobacillus* strain from traditional fermented foods and investigate its growth characteristics, acid-producing capacity, and stress tolerance. Subsequently, a p-chlorophenylalanine (PCPA)-induced insomnia mouse model was established to evaluate the sleep-enhancing effects and underlying mechanisms of the strain's fermentation broth. We isolated six strains of GABA-producing lactic acid bacteria, among which strain XBMU-SN-23 exhibited the highest GABA production (2.150 g/L) as determined by high-performance liquid chromatography (HPLC) and was identified as *Lactiplantibacillus plantarum* via 16S rDNA sequencing. Treatment with XBMU-SN-23 fermentation broth significantly improved the quiet state of PCPA-induced insomniac mice. It mitigated PCPA-induced damage to hypothalamic neuronal cells, increased the levels of GABA, serotonin (5-HT), interleukin-1 (IL-1), and melatonin (MT), while simultaneously reducing glutamate (Glu) and dopamine (DA) in the serum and hypothalamus of the mice, thereby contributing to the restoration of neurotransmitter balance. Additionally, the treatment led to an increase in the expression of 5-HT_{1A}, GABA_AR _{α 1}, and GABA_AR _{γ 2} receptor proteins, which play a crucial role in the regulation of molecular pathways and collectively enhance sleep quality. These findings support the sedative-hypnotic benefits of XBMU-SN-23 fermentation broth and provide a theoretical foundation for the future development of functional GABA-producing probiotic products.

Keywords: GABA; PCPA-Induced insomnia mouse model; Lactic acid bacteria; Sleep-Enhancing mechanism

1. Introduction

The body uses sleep as a regular and recurring physiological process, which is crucial for sustaining daily physiological processes and survival. Numerous variables can interfere with the body's sleep-wake regulatory

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system, leading to sleep disorders^[1]. Inadequate or excessive sleep duration is one of the primary clinical indications of this disorder, a group of illnesses intimately associated with sleep and wakefulness. Approximately one-third of persons globally experience at least one sleep disturbance^[2]. Currently, Medications used to treat insomnia include benzodiazepines such as alprazolam and diazepam, benzodiazepine agonists (like eszopiclone), and ramelteon (a melatonin receptor agonist)^[3]. These medications exert their effects by enhancing the action of GABA neurotransmitters in the brain. However, significant side effects are associated with these drugs. For instance, they can cause daytime sleepiness, impair coordination and balance, and long-term use may lead to drug dependence and tolerance^[4]. Due to the numerous drawbacks of these synthetic medications, there is a growing demand for natural remedies for insomnia.

Lactic acid bacteria are a group of microorganisms capable of producing significant amounts of lactic acid by fermenting carbohydrates^[5]. These bacteria are widely present in nature and play a vital role in human health. The GABA generated through lactic acid bacteria fermentation is regarded as safe for consumption. For instance, Anggraini et al. indicates that lactate is a potential precursor for GABA production^[6]. GABA functions as an inhibitory neurotransmitter in the central nervous system. In addition to its potential positive effects on neuroprotection, sleep quality enhancement, alleviation of depression, and pain relief, there is growing interest in the various health benefits associated with GABA-rich foods, including anti-diabetic, anti-hypertensive, and anti-inflammatory properties^[7].

A naturally occurring non-protein amino acid, GABA is also known as piperidinic acid^[8]. Additionally, GABA plays a critical function in sleep and anesthesia arousal and is involved in the regulation of sleep, cognition, consciousness, and attention^[9]. Luo et al. demonstrated that GABA interacts with its receptor to promote sleep^[10]. The two types of GABA receptors, known as metabotropic and ionotropic^[11], bind to distinct receptors to perform different functions. Furthermore, GABA significantly influences human sleep during the early stages, lengthening the duration of non-rapid eye movement sleep and drastically reducing sleep latency^[12].

GABA can now be produced using three primary approaches: microbial fermentation, chemical synthesis, and plant enrichment. However, due to its straightforward process, inexpensive substrates, and high conversion rates, microbial fermentation is the most widely used method for GABA production^[13]. This process occurs when glutamic acid decarboxylase (GAD) catalyzes the breakdown of α -aminoglutaric acid. According to GAD, it is a crucial enzyme in the synthesis of GABA, facilitating the irreversible decarboxylation of L-glutamic acid into GABA. Besides, microbial fermentation naturally produces GABA during the food production process, making it not only more cost-effective but also safer. It can be directly utilized as a functional food ingredient for commercial applications^[14].

Probiotics are active microorganisms that provide health benefits to the host, primarily consisting of bacteria and yeast. When ingested in adequate amounts, they can promote various health advantages. By regulating the gut microbiota, probiotics may help alleviate stress and anxiety, which are often underlying

causes of insomnia^[15]. Compared to traditional sleep medications, probiotics are generally regarded as safe and tend to have fewer side effects. Consequently, probiotics hold promise as a natural treatment for insomnia.

In our study, we aimed to isolate, purify, and identify lactic acid bacteria capable of producing GABA from sources such as old yogurt and syrup in Lanzhou. Furthermore, we conducted a series of experiments to evaluate the growth curve, acid-producing capacity, and stress tolerance of the selected strain. Additionally, a PCPA-induced insomnia model was established to investigate the sleep-improving effects of the fermentation broth from this strain on mice, thereby laying the groundwork for the further application and development of GABA.

2. Materials and methods

2.1. Materials and reagents

Lanzhou's old yogurt and Jiangshui were purchased from a specialty supermarket in Lanzhou (Gansu, China). Agar powder, MRS broth medium, and sodium L-glutamate were obtained from Beijing Solarbio Technology Co., Ltd. (Beijing, China). Anhydrous ethanol was sourced from Tianjin Chemical Reagent Factory (Tianjin, China). Ninhydrin hydrate ($\geq 98\%$) was acquired from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). The GABA standard was purchased from Qingdao Jieshikang Biotechnology Co., Ltd. (Qingdao, China). Sodium chloride, ammonium sulfate, chromatographic-grade methanol, chromatographic-grade acetonitrile, and sodium acetate were obtained from Chemical Reagent Co., Ltd. (Shanghai, China). Dansyl chloride, pyridoxal phosphate, ammonium chloride, calcium chloride, and magnesium sulfate were sourced from Shanghai McLean Biochemical Technology Co., Ltd. (Shanghai, China). Peptone, beef extract, Tween 80, and yeast extract were purchased from Qingdao Haibo Biotechnology Co., Ltd. (Qingdao, China). XBMU-SN-23 fermentation broth was prepared by the laboratory at Northwest University for Nationalities, China. The PBS buffer, BCA protein quantitative detection kit, and RIPA lysis buffer were purchased from Wuhan Xavier Biotechnology Co., Ltd. (Wuhan, China). The PCPA, 5-HT, BDNF, IL-1, DA, and γ -amino acid receptor ELISA kits were obtained from Shanghai Enzymatic Link Biotechnology Co., Ltd. (Shanghai, China). Diazepam (2.5 mg) was acquired from Beijing Yimin Pharmaceutical Co., Ltd. (Beijing, China). Eosin staining solution was sourced from Zhuhai Beso Bioengineering Co., Ltd. (Zhuhai, China).

2.2. Screening of lactic acid bacteria

We added 0.5 mL of traditional fermented food to 4.5 mL of sterile physiological saline. Mix thoroughly using a constant-temperature shaking incubator (ZWYR-2401, Shanghai Zhicheng Analytical Instrument Manufacturing Co., Ltd., Shanghai, China). Then, perform a 10-fold serial dilution with sterile saline. We pipetted 200 μ L of the appropriately diluted bacterial suspension and spread it onto MRS agar plates. Incubate the plates at 37°C in a constant-temperature incubator for 48 hours. Select well-developed single colonies based on morphology and streak them repeatedly onto fresh MRS plates for 2-3 generations to ensure purification. We then performed Gram staining and examine the samples under a microscope (NE300,

Shenzhen Sinico Optical Instrument Co., Ltd., Shenzhen, China) to identify Gram-positive strains as candidates^[16]. Store these strains in 20% (v/v) glycerol tubes at -80°C for future use.

2.3. Determination of GABA in fermentation broth by HPLC

The pre-conserved strains were inoculated into MRS liquid medium for two consecutive generations. Subsequently, they were transferred to a fermentation medium containing 1.5% L-Glu with a 3% inoculum and incubated at 37°C for 30 hours. The fermentation broth was then centrifuged using a freezing centrifuge (2050R-H, Xiangyi Centrifuge Manufacturing Co., Ltd., Hunan, China) at 10,000 rpm for 10 minutes. Following this, it was subjected to high-speed centrifugation at 4°C. The supernatant obtained after centrifugation was utilized for quantitative analysis via high-performance liquid chromatography (1260, Agilent Technologies Co., Ltd., Beijing, China). For the analysis, 200 µL of the sample was combined with 200 µL of NaHCO₃-Na₂CO₃ buffer (pH 11) and 400 µL of a 10 g/L DNS-Cl solution. This mixture was reacted for 20 minutes at 30°C with sufficient shaking, and then filtered through a 0.45 µm membrane. The chromatographic column used was a ZORBAX SB C18 (4.6 mm × 250 mm, 5 µm). The mobile phase A consisted of 0.025 mol/L sodium acetate, while mobile phase B was acetonitrile. The flow rate was set at 1.0 mL/minute, the column temperature was maintained at 40°C, the injection volume was 10 µL, and the detection wavelength was 340 nm.

2.4. Identification of GABA-Producing strains by 16S rDNA sequences

The DNA from high GABA-producing *Lactobacillus* was selected as the template for PCR amplification, which was conducted following the protocol provided by the purchased bacterial DNA extraction kit. Universal primers were used: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3').

The 20 µL of PCR reaction was as follows: 2.0 µL of template DNA, 0.4 µL of Taq DNA polymerase, 1.6 µL of Mg²⁺, 2.0 µL of 10×PCR Buffer, 2.0 µL of dNTP (2.5 mmol/L), 2.0 µL of upstream primer and downstream primer (10 µmol/L), 8.0 µL of dd H₂O.

The PCR reaction conditions included pre-denaturation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 1 minute, followed by 30 cycles of amplification, a final extension at 72°C for 10 minutes, and storage at 4°C. The PCR products were analyzed using 0.8% agarose gel electrophoresis (150 V, 100 mA for 20 minutes).

After electrophoresis, the gel was cut, and the desired fragments were recovered and sequenced by Gansu Huake Biotechnology Co. The sequencing results were compared with the GenBank database of the National Center for Biotechnology Information (NCBI), and a phylogenetic tree was constructed.

2.5. Growth and physiological characteristics of strains

2.5.1. Determination of growth curve and capacity of acid production

The strain was inoculated into 10 mL of liquid medium with a 2% inoculum volume and incubated at a constant temperature of 37°C. Samples were taken every 2 hours, and the absorbance of the solution was

measured at 600 nm using a UV spectrophotometer (SPD15C, Mettler Bio-equipments Co., Ltd., Shanghai, China). A pH meter (PHS-3E, Shanghai Yidian Scientific Instrument Co., Ltd., Shanghai, China) was utilized to measure the pH values of the strains at various time intervals. Subsequently, the growth curves and acid-producing capacity curves of the strains were plotted^[17].

2.5.2. Acid resistance test *in vitro*

The MRS medium was adjusted to pH levels of 2.0 and 4.0 using 1 mol/L HCl. Optimal strains in the logarithmic growth phase were inoculated with a 2% inoculum, mixed thoroughly, and incubated in a constant temperature oven (LRH-150, Shanghai Yiheng Technology Co., Ltd., Shanghai, China) at 37°C for 24 hours. An UV spectrophotometer (LRH-150, Shanghai Yiheng Technology Co., Ltd., Shanghai, China) was employed to measure the absorbance of the solution at 600 nm, allowing for the calculation of the survival growth rate at different pH levels. The absorbance values of the solutions were recorded at 600 nm^[18]. The survival growth rate of a strain was calculated by the following formula:

$$K(\%) = 1 - \frac{A_x - A_0}{A_0} \times 100\% \quad (1)$$

K represents the survival growth rate of a strain (%); A_x denotes the absorbance value of the solution at 600 nm, measured at the x-th hour of strain incubation.; and A_0 represents the absorbance value of the solution at 600 nm, measured at the initial time of strain incubation.

2.5.3. *In vitro* bile salt tolerance test

The optimal strain in the logarithmic growth phase was inoculated into MRS medium containing 3% and 5% bile salt concentrations, using a 2% inoculum volume. The mixture was shaken thoroughly and incubated in a constant temperature oven at 37°C (LRH-150, Shanghai Yiheng Science and Technology Co., Ltd., Shanghai, China) for 24 hours. A UV spectrophotometer (LRH-150, Shanghai Yiheng Technology Co., Ltd., Shanghai, China) was utilized to measure the absorbance at 600 nm and to calculate the growth rate of the strains in media with varying bile salt concentrations. The calculation formula used is the same as that in section 2.5.2.

2.6. Effect of GABA on sleep improvement in insomnia mice induced by PCPA

2.6.1. Preparation of fermentation broth

Strain XBMU-SN-23 was selected and fermented at 37°C for 2 days, during which the incubation temperature and humidity were closely monitored. After the end of fermentation, the fermentation broth was centrifuged at a high speed at 8000 r for 10 minutes at 4°C, transferred to an autoclave (50G-YXQLS50G, Shanghai Boxun Industrial Co. Ltd. Medical Equipment Factory, Shanghai, China) for 15 minutes at 100°C, and then sealed and stored at 4°C.

2.6.2. Animal model preparation and grouping

A total of sixty healthy SPF KM male mice (20-23 g, 5 weeks old) were purchased from the Experimental Animal Center of the Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences

(Lanzhou, China). The license number is SCXK (Gan) 2020-0002. The experiment was conducted after a 1-week acclimatization period under standard environmental conditions, including a room temperature of 25 ± 2 °C, humidity of $65 \pm 5\%$, and a 12-hour alternation of day and night. All animal experiments were conducted in compliance with the National Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of Northwest Minzu University (xbmu-sm-2025103).

Sixty male mice were randomly divided into six groups in Table 1, with ten mice in each group^[19]: control group (Blank group, n=10 mice), positive control group (DZP group, n=10 mice), PCPA model group (Mod group, n=10 mice), XBMU-SN-23 low-dose group (Low-dose, n=10 mice), XBMU-SN-23 middle-dose group (Middle-dose, n=10 mice) and XBMU-SN-23 high-dose group (High-dose, n=10 mice).

Table 1. Establishment of experimental groups in animal

Groups	Gastric medicine	Modelling	GABA Content
Blank group	Saline (medicine)	-	0
DZP group	DZP Suspension	+	0
Model group	PCPA Suspension	+	0
Low-dose group	XBMU-SN-23 Fermentation broth	+	30 mg/kg
Middle-dose group	XBMU-SN-23 Fermentation broth	+	90 mg/kg
High-dose group	XBMU-SN-23 Fermentation broth	+	150 mg/kg
Gavage volume		10mL/kg/d	

From Day 1 to Day 3, a 2% suspension of 350 mg/kg PCPA was injected intraperitoneally into all groups, except for the Blank group, which received an equal volume of saline via intraperitoneal injection. The successful modeling in mice was demonstrated by behavioral changes, including increased activity levels, irritability, decreased appetite, and a disruption of circadian rhythm. These behavioral assessments are consistent with established methods for evaluating PCPA-induced insomnia and the efficacy of sedative-hypnotic interventions^[20, 21]. The DZP group was administered diazepam suspension at a dose of 2.5 mg/kg via gavage. The Low-dose, Middle-dose, and High-dose groups received XBMU-SN-23 fermentation solution at doses of 30, 90, and 150 mg/kg, respectively, also via gavage. Simultaneously, the Blank and Mod groups were gavaged with equal amounts of saline for 7 consecutive days. The body weights of the mice were measured daily. The detailed experimental procedure is summarized in **Figure 1**.

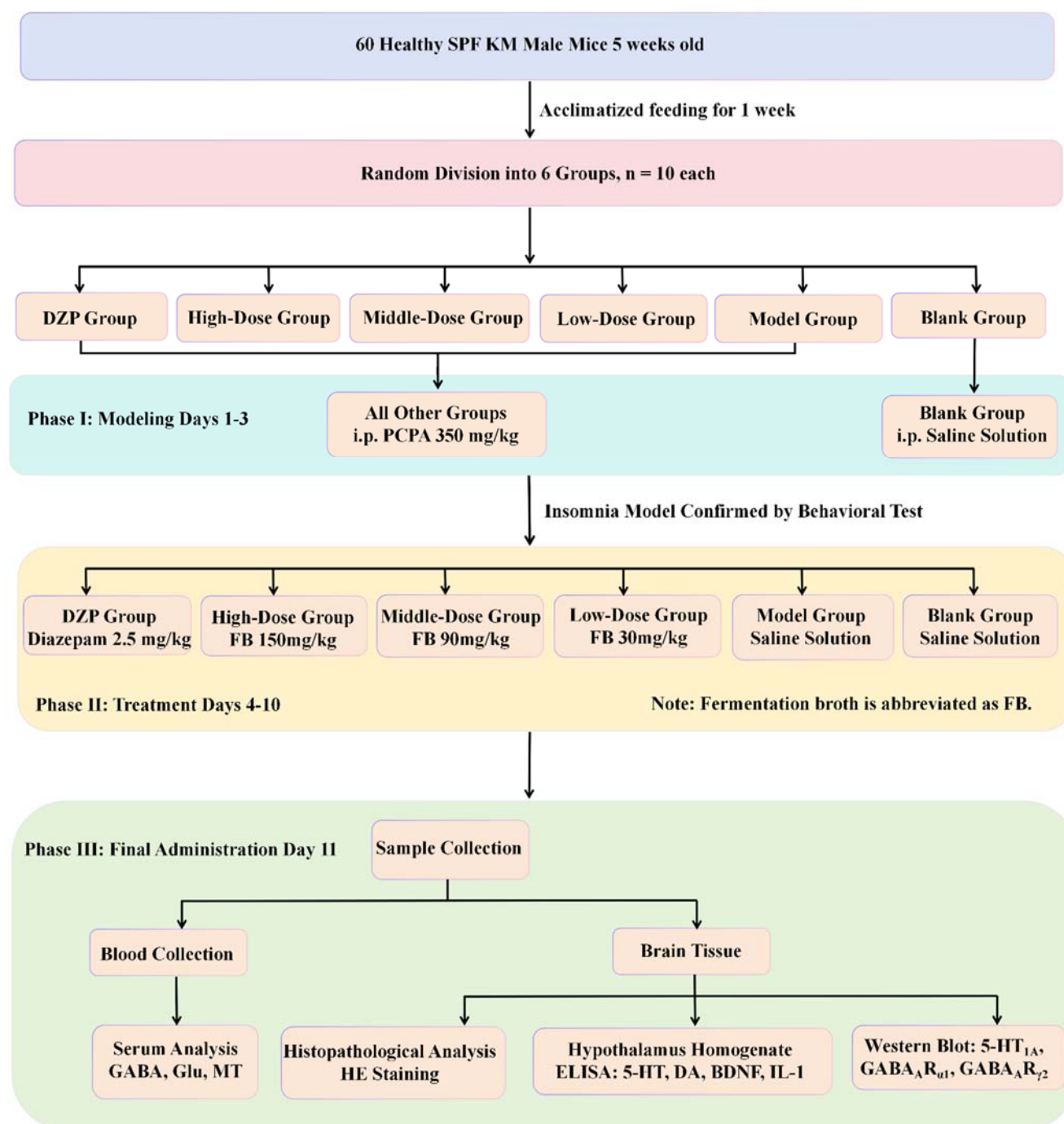


Figure 1 Schematic flowchart of the animal experimental procedure.

2.6.3. Observation of sleep latency and sleep duration in mice with PCPA-Induced insomnia

Mice were injected intraperitoneally with 2% pentobarbital sodium (35 mg/mL) one hour after the final administration. The sleep latency and sleep duration were then observed and recorded following the completion of the treatment. Sleep latency is the time from the injection of sodium pentobarbital until the disappearance of the flip-flop reflex^[22]. Sleep time is defined as the correct time from the disappearance of the flip-flop reflex until the mice roll over, as well as the inability to maintain a supine position for 30 seconds^[23].

2.6.4. Determination of brain index

After rinsing with physiological saline, the organ coefficients of each group of mice were measured, and the remaining samples were promptly placed in liquid nitrogen for preservation. The brain index (%) was calculated as follows:

$$X = \frac{Q}{W} \times 100\% \quad (2)$$

X represents the brain index (%), Q denotes the organ weight of mice (g), and W indicates the weight of the mice (g).

2.6.5. Determination of MT, GABA, and Glu in the serum of mice

Blood was taken from the mouse eye socket, and the plasma was centrifuged twice at 4°C (8000 rpm for 10 minutes). The supernatant was then collected and prepared for testing. Subsequently, the serum levels of melatonin (MT), GABA, and glutamate (Glu) in PCPA-induced insomnia mice were measured according to the instructions provided with the ELISA kit (Shanghai Enzyme Link Biotechnology Co., Ltd., Shanghai, China).

2.6.6. Brain tissue pathological analysis

Mouse brain tissues were excised and cut into 2-3 mm³ pieces following the method described in the reference^[24]. The samples were then embedded in paraffin and sectioned into 4 µm-thick coronal slices using a paraffin-embedding machine (LS-2065, Shenzhen DakoWei Medical Technology Co., Ltd., Shenzhen, China). After sectioning, the paraffin was sequentially removed using xylene, followed by 95%, 85%, and 70% ethanol. Using hematoxylin-eosin (HE) staining, the morphology and structure of the cells and tissues in the hippocampus of insomniac mice were meticulously observed and analyzed under a light microscope (NE300, Shenzhen Sinica Optical Instrument Co., Ltd., Shenzhen, China) at a magnification of 200×.

2.6.7. Determination of 5-HT, IL-1, BDNF, and DA levels in the hypothalamus of mice

Hypothalamic tissues were excised and immediately frozen in liquid nitrogen before being transferred to 4°C for thawing prior to analysis. A specific volume of phosphate-buffered saline (PBS, pH 7.4) was added, and the samples were thoroughly homogenized using a tissue grinder (CEBO-6, Shanghai Measurement and Bioscience Co., Ltd., Shanghai, China). The homogenates were then centrifuged twice at 8000 rpm for 10 minutes in a centrifuge (2050R-H, Xiangyi Centrifuge Manufacturing Co., Ltd., Hunan, China). The supernatant was collected and prepared for testing. The levels of serotonin (5-HT), interleukin-1 (IL-1), brain-derived neurotrophic factor (BDNF), and dopamine (DA) in mouse brain tissue were quantified using an ELISA kit (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China).

2.6.8. Determination of the expression levels of 5-HT1A, GABAARα1, and GABAARγ2 receptor proteins in brain tissue by Western Blot

A total protein extraction kit was utilized to extract proteins from mouse brain tissues^[25]. The tissues were placed in pre-cooled RIPA lysis buffer, ultrasonically homogenized in ice water, and incubated for 30 minutes. Subsequently, the samples were centrifuged (2050R-H, Xiangyi Centrifuge Manufacturing Co., Ltd.,

Hunan, China) at 12,000 rpm for 10 minutes at 4°C to obtain the supernatant containing the total protein samples of histones. The protein concentration was quantified using a BCA kit (Wuhan Xavier Biotechnology Co., Ltd., Wuhan, China). The 5-HT_{1A}, GABA_AR_{α1}, and GABA_AR_{γ2} proteins were separated by 10% SDS-PAGE gel electrophoresis. The target proteins were then semi-quantitatively analyzed following transfer, primary antibody incubation, washing, and secondary antibody re-incubation, with ACTIN used as an internal reference protein.

2.7. Data analysis and statistics

All experimental data are expressed as mean ± standard deviation ($\bar{x} \pm s$). To compare differences among multiple experimental groups, one-way analysis of variance (ANOVA) was performed. When significant main effects were detected ($P < 0.05$), pairwise comparisons were conducted using Tukey's significant difference (HSD) post hoc test, given equal sample sizes across groups. Statistical significance was defined as $P < 0.05$. All statistical analyses were performed using SPSS (version 19.0). The data were plotted by Origin software (2021b, OriginLab Inc.) and GraphPad Prism (version 19.0).

3. Results

3.1. Isolation and identification of GABA-Producing lactic acid bacteria

We successfully isolated six strains of GABA-producing lactic acid bacteria from traditional fermented foods. The initially screened *Lactobacillus* strain XBMU-SN-23 exhibited colony morphology, as shown in Figure 2A(a), after 48 hours of cultivation on MRS medium. The colonies appeared milky white with a raised center and emitted a distinct sour odor. Gram staining results (Figure 2A(b)) revealed purple-stained cells under microscopy, displaying the typical Gram-positive rod morphology, preliminarily indicating membership in the *Lactobacillus* genus. To further confirm its taxonomic status, 16S rDNA amplification was performed using DNA from strain XBMU-SN-23 as a template, yielding a single band of approximately 1500 bp (Figure 2B). Sequence alignment with the GenBank database and phylogenetic tree construction (Figure 2C) confirmed this strain as *Lactobacillus plantarum*. Regarding functional characteristics, GABA production was measured for six primary screening strains (Table 2). Among them, XBMU-SN-23 exhibited the highest yield at 2.15 g/L, surpassing most strains reported in the literature^[26], demonstrating excellent application potential.

Table 2. GABA content in fermentation supernatant of strains of lactic acid bacteria

Strain Number	XBMU-JE-15	XBMU-JE-16	XBMU-B-03	XBMU-M-12	XBMU-SN-23	XBMU-YD-22
GABA Content (g/L)	1.47	1.76	2.06	1.85	2.15	1.03

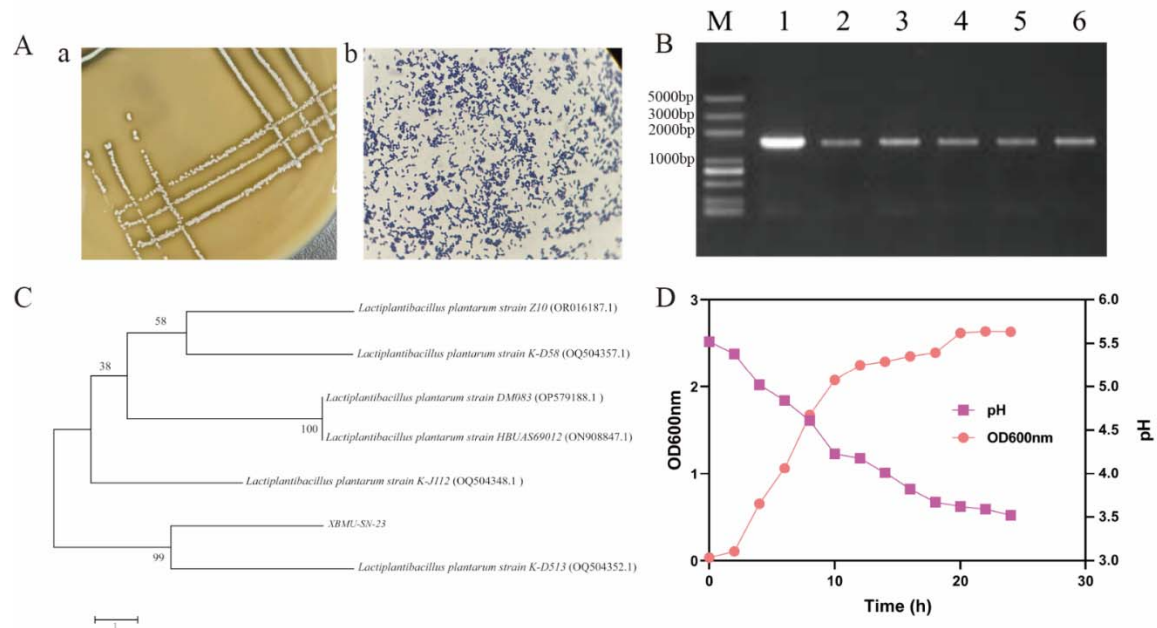


Figure 2 Identification and growth characteristics of Strain XBMU-SN-23. A shows the colony morphology and Gram staining microscopic observations of the strain (a is colony morphology and b is Gram staining), B shows the 16S rDNA amplification electrophoresis pattern, C presents the phylogenetic tree of strain XBMU-SN-23, D displays the growth curve and acid production capacity of strain XBMU-SN-23.

The growth and physiological characteristics of this strain are illustrated in Figure 2D. Between 0 and 10 hours, XBMU-SN-23 exhibited a high growth rate, followed by a plateau phase, reaching peak biomass at approximately 22 hours. Concurrently, the culture pH rapidly decreased during the initial 0-10 hours, indicating vigorous acid production during this period, with growth and acid production showing a positive correlation. After 18 hours, the culture entered a stable phase, with the pH reaching equilibrium. This growth profile helps determine optimal fermentation conditions, providing a foundation for enhancing product yield and efficiency.

3.2. Tolerance Analysis of GABA-Producing Lactic Acid Bacteria

To assess the probiotic potential, we evaluated the tolerance of XBMU-SN-23 to gastrointestinal stresses. As shown in Table 3, the strain demonstrated notable acid tolerance. The survival growth rate was 8.06% at pH 4.0 and increased to 34.58% at pH 2.0 after 24 hours, indicating a remarkable ability to survive in highly acidic conditions reminiscent of the stomach. Furthermore, XBMU-SN-23 exhibited bile salt tolerance, with survival growth rates of 26% and 20.2% at bile salt concentrations of 0.3% and 0.5%, respectively (Table 3). Although higher bile salt concentration slightly inhibited growth, the strain maintained considerable viability, suggesting its potential to survive and function in the intestinal environment.

Table 3. Acid resistance and bile salt tolerance of GABA-Producing lactic acid bacteria XBMU-SN-23

Time (h)	pH 2.0	pH 4.0	Bile salt concentration of 0.3%	Bile salt concentration of 0.5%
0 h	0.107	0.124	0.2	0.203
24 h	0.144	0.134	0.252	0.244
Growth rate(%)	34.58%	8.06%	26%	20.2%

3.3. Effect of XBMU-SN-23 fermentation broth on sleep duration in PCPA-Induced insomniac mice

Consistent with our behavioral observations, the PCPA-induced model group exhibited a pronounced hyperarousal state, characterized by increased spontaneous activity and irritability, which was visibly ameliorated following treatment with XBMU-SN-23 fermentation broth. This preliminary indication of a calming effect was quantitatively confirmed by the pentobarbital-induced sleep test. As shown in Table 4, the model group demonstrated significantly prolonged sleep latency and reduced sleep duration compared to the blank group. Notably, the sleep induction rate in the model group was also significantly lower than that in the blank control group, further validating the hyperarousal characteristic of the insomnia model^[27]. Treatment with XBMU-SN-23 fermentation broth dramatically reversed these deficits. All dosage groups showed a significant reduction in sleep latency and an increase in sleep duration compared to the model group. The middle-dose group (90 mg/kg) also exhibited a significantly higher sleep induction rate, restoring it to a level comparable to the blank group. These results, encompassing both qualitative behavioral improvements and quantitative sleep parameters, collectively demonstrate that XBMU-SN-23 fermentation broth effectively ameliorates the core behavioral deficits of insomnia, exhibiting potent sedative and hypnotic properties.

Table 4. Effects of fermentation broth on sleep latency and sleep duration in PCPA-Induced

Groups	Dosages (mg/kg)	Sleep latency (s)	Sleep duration (min)
Blank group	-	232.2±28.1	65.4±7.4
DZP group	2.5	208.5±58.6	71.4±4.5
Model group	-	485.3±32.2	24.5±5.6
Low-dose group	30	224.1±48.1	42.5±8.4
Middle-dose group	90	286.4±48.1	78.5±7.8
High-dose group	150	247.3±47.6	76.5±8.4

3.4. Effect of fermentation broth on body weight and cerebral index in PCPA-Induced insomniac mice

It can be observed from Table 5 that there was no significant difference in body weight among the groups, with the exception of the blank group ($P > 0.05$). At the conclusion of the experiment, the average weight of the blank group was the highest, reaching 35.20 ± 0.40 g, and its brain index was greater than that of the other groups. The final weight of the positive control group was the lowest at 31.47 ± 0.15 g, followed by the model group, which had a final weight of 32.20 ± 0.17 g and the lowest brain index. The organ weight of the mice may increase gradually due to the prolonged effects of PCPA. Different doses of XBMU-SN-23 fermentation broth can significantly enhance the weight of the mice and increase the brain index to varying degrees, indicating its potential effectiveness in treating the pathological changes in organs caused by PCPA and DZP, as well as alleviating the organ degeneration in insomnia mice induced by PCPA.

Table 5. Effects of fermentation solution on body weight and organ coefficient of mice

Groups	Initial weight (g)	Ending weight (g)	Brain index (%)
Blank group	23.07±0.32 ^a	35.20±0.40 ^a	0.54±0.24
DZP group	21.47±0.12 ^c	31.47±0.15 ^c	0.38±0.18
Model group	22.57±0.38 ^b	32.20±0.17 ^c	0.34±0.24
Low-dose group	21.63±0.15 ^c	33.60±0.17 ^b	0.45±0.27
Middle-dose group	22.83±0.06 ^{ab}	34.50±0.36 ^b	0.47±0.35
High-dose group	21.47±0.35 ^c	32.37±0.55 ^c	0.41±0.76

3.5. Effect of fermentation broth on serum levels of MT, GABA, and Glu in PCPA-Induced insomniac mice

GABA is the primary inhibitory neurotransmitter in the central nervous system, known for its sedative, anxiolytic, and sleep-promoting effects. As illustrated in **Figure 3A**, the serum GABA levels in the model group of mice were significantly lower than those in the control group ($P < 0.05$), indicating an impairment of central inhibitory function and an overexcitation of neurons in the state of insomnia. Conversely, the GABA levels significantly increased following the intervention with XBMU-SN-23 fermentation broth, suggesting that this treatment may restore central inhibitory signaling and alleviate the neuroexcitatory imbalance associated with insomnia by promoting GABA synthesis or inhibiting its degradation.

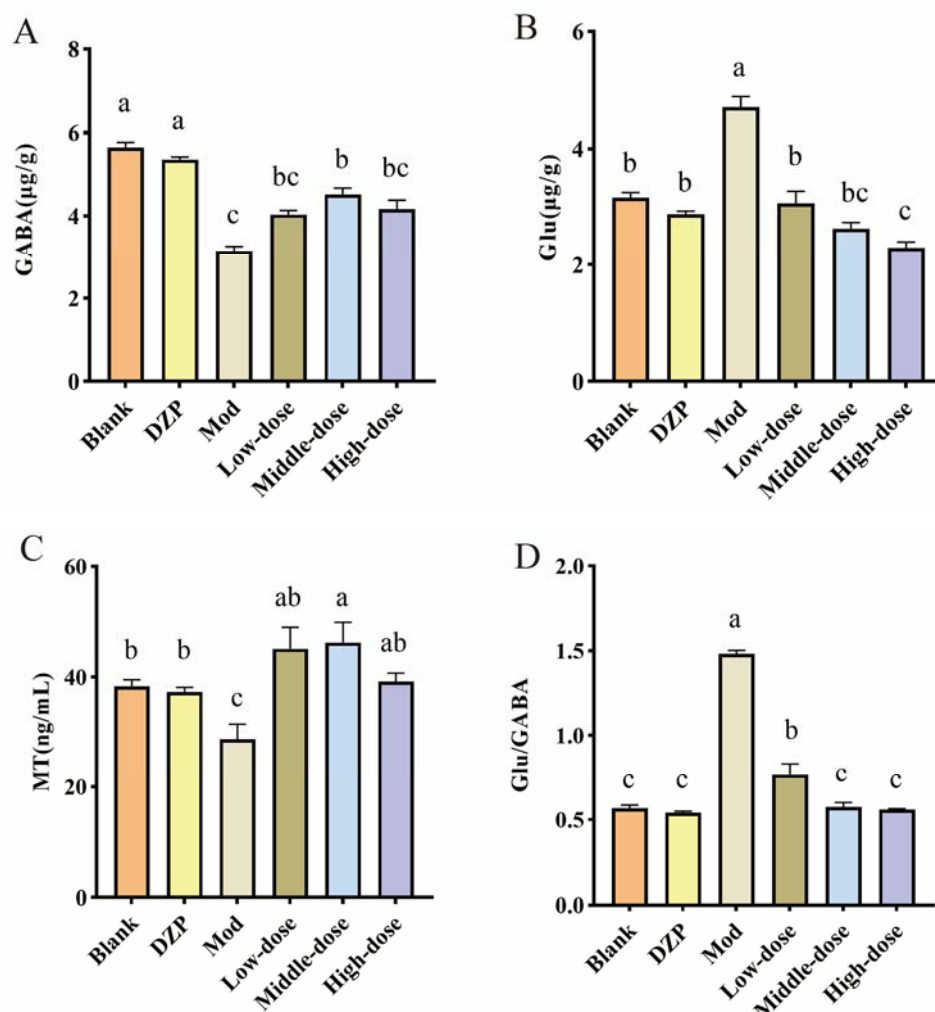


Figure 3 Effect of GABA on serum levels of MT, GABA, and Glu in PCPA-induced insomnia mice. A is the amount of GABA in the blood, B is Glu, C is MT, D is Glu/GABA ratio.

Glutamate (Glu) is the primary excitatory neurotransmitter in the central nervous system, and its excessive release can lead to neuronal over-activation. As shown in **Figure 3B**, Glu levels were significantly elevated in the model group. **Figure 3D** indicates that the Glu/GABA ratio was significantly increased compared to that of the control group ($P < 0.05$). This outcome aligns with previously reported findings regarding Glu in the literature^[27]. Following treatment with XBMU-SN-23 Fermentation Broth, the level of Glu was significantly reduced, and the Glu/GABA ratio approached the normal range. This supposes that XBMU-SN-23 Fermentation Broth mitigates central nervous system excitability by either inhibiting the

over-activation of glutamatergic neurons or promoting the metabolic clearance of Glu. Consequently, XBMU-SN-23 Fermentation Broth helps restore the dynamic balance of GABA and Glu, ultimately improving symptoms of insomnia.

Melatonin (MT) is a crucial hormone involved in the regulation of circadian rhythms and the sleep-wake cycle. As illustrated in **Figure 3C**, the serum MT levels in the model group of mice were significantly lower than those in the control group, indicating that the PCPA-induced insomnia model disrupted pineal gland secretion, leading to a reduction in endogenous sleep-promoting substances. Following treatment with the XBMU-SN-23 fermentation solution, MT levels showed a significant increase, suggesting that the fermentation solution may restore the regulation of circadian rhythms by activating pineal function or enhancing the MT synthesis pathway, thereby improving sleep quality.

In conclusion, these results suggest that XBMU-SN-23 Ferment can alleviate insomnia by modulating multiple targets within the neurotransmitter system.

3.6. Assessment of brain tissue damage

Histopathological findings of the mouse hippocampus and hypothalamus are presented in **Figures 4A and 4B**, respectively. In the blank control group, neuronal structures in both brain regions appeared normal and were neatly arranged. In contrast, the model group exhibited disorganized and loosely arranged hippocampal neurons, along with deformed and disordered hypothalamic neurons, indicating successful induction of brain tissue damage by the insomnia model. Following intervention with the GABA-rich XBMU-SN-23 fermentation extract, all treatment groups demonstrated varying degrees of reparative effects. The medium-dose group (90 mg/kg) showed the most significant improvement, with hippocampal neurons tending toward regular alignment and marked repair of hypothalamic cell atrophy and damage. The high-dose group did not exhibit enhanced protective effects. These consistent findings indicate that the fermentation extract effectively mitigates insomnia-induced damage to hippocampal and hypothalamic tissues, with the medium dose yielding optimal efficacy. This finding aligns with the literature indicating that the extract of ‘*Lilium pumilum*’ induces insomnia in mice by regulating PCPA through the hypothalamus^[28].

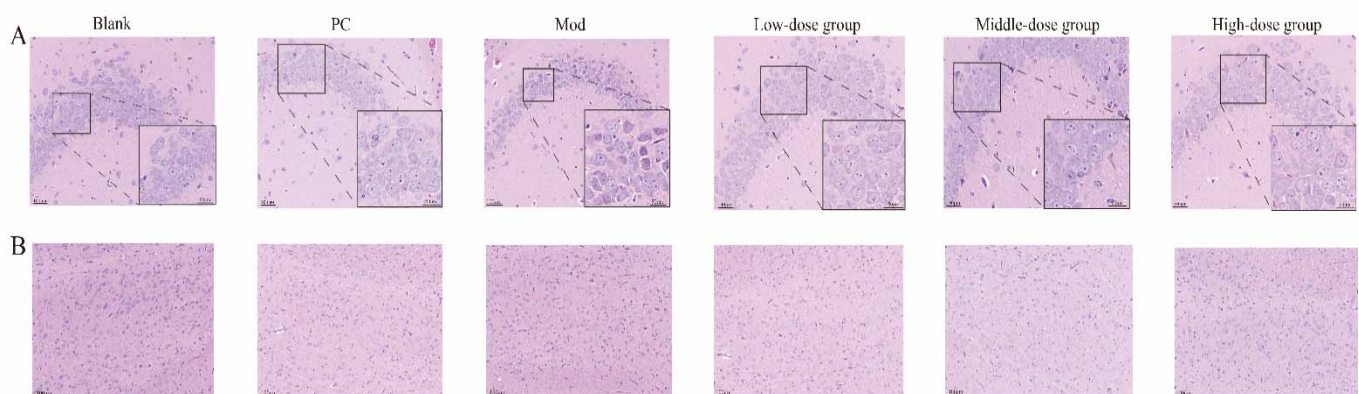


Figure 4 HE staining results of brain tissue structures in PCPA insomnia model mice. A shows HE staining of the hippocampus, and B shows HE staining of the hypothalamus.

3.7. Effect of fermentation broth on the levels of 5-HT, DA, BDNF, and IL-1 in PCPA-Induced insomniac mice

The hypothalamus, as the central integrator of sleep-wake regulation, coordinates complex neurochemical and neuroimmune interactions^[29]. To comprehensively elucidate the multi-target mechanisms of the fermentation broth of strain XBMU-SN-23, we systematically evaluated its effects on key hypothalamic neurotransmitters, including serotonin (5-HT), dopamine (DA), brain-derived neurotrophic factor (BDNF), and interleukin-1 (IL-1). We investigated how the fermentation broth alleviates insomnia symptoms by modulating these factors.

Our results indicate that PCPA significantly disrupts hypothalamic neurochemistry. As shown in **Figure 5A**, 5-HT levels in the model group decreased substantially compared to those in the control group. This finding aligns with the known mechanism of PCPA, which inhibits tryptophan hydroxylase and blocks 5-HT synthesis^[30], thereby affecting sleep. Treatment with XBMU-SN-23 fermentation broth effectively reversed this deficiency in a dose-dependent manner. Significant recovery of 5-HT levels was observed in the low, medium, and high dose groups, with the medium dose (90 mg/kg) producing the most pronounced effect, nearly restoring 5-HT to baseline levels. These results indicate that the fermented liquid can counteract PCPA-induced serotonergic dysfunction, potentially by supplying precursor molecules, modulating enteric 5-HT, or influencing the gut-brain axis to enhance central serotonergic signaling^[31, 32].

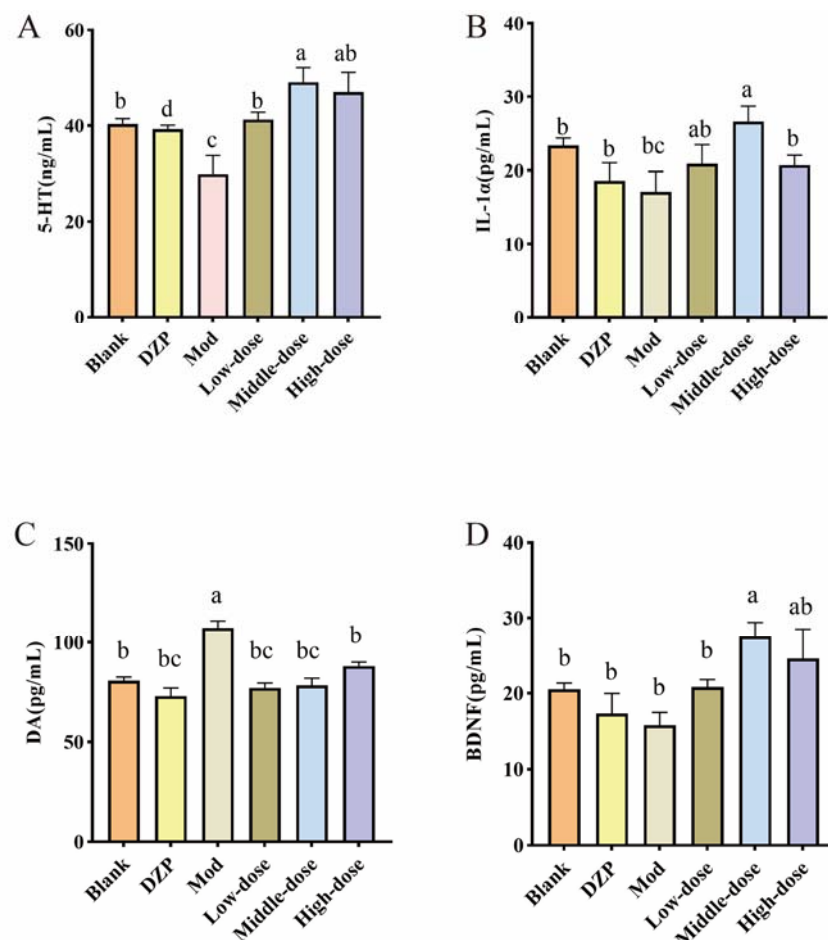


Figure 5 Effect of XBMU-SN-23 fermentation broth on factor levels in mouse hypothalamus tissue. A is the amount of 5-HT in the hypothalamus, B is IL-1, C is DA, D is BDNF.

Simultaneously, as shown in **Figure 5C**, hypothalamic dopamine (DA) levels were significantly elevated in the model group. This dopaminergic state corresponds with the hyperarousal phenotype of insomnia and may represent a compensatory response to 5-HT depletion, thereby disrupting the critical balance between sleep and wakefulness^[33]. Following intervention with XBMU-SN-23 fermentation broth, DA levels in both the diazepam group and the low-dose group decreased significantly, approaching normal ranges. This normalization of the 5-HT to DA balance indicates that the fermentation broth promotes sleep not only by enhancing 5-HT but also by suppressing DA, thereby restoring a neurochemical environment conducive to sleep.

In addition to neurotransmitters, the fermentation broth also significantly influenced neurotrophic and immune factors. **Figure 5D** shows that BDNF levels were significantly reduced in the model group, indicating that chronic sleep disruption impairs neural plasticity^[34]. The use of XBMU-SN-23 fermentate, particularly at moderate doses, significantly increases BDNF levels. This suggests that the sleep improvements conferred by the fermentate are associated with enhanced neurotrophic support, which may underlie the restoration of synaptic homeostasis and cognitive functions dependent on healthy sleep^[35].

Additionally, as shown in **Figure 5B**, IL-1 concentrations were significantly elevated in the medium-dose treatment group compared to the model group. IL-1 is a cytokine with immunomodulatory effects that participates in sleep regulation under both healthy and diseased conditions^[10]. This elevated IL-1 level may represent an additional mechanism by which XBMU-SN-23 fermentation broth promotes sleep, potentially by regulating the gut microbiota and its interactions with the host immune system, thereby leading to the release of sleep-regulating cytokines^[36].

In summary, these findings indicate that the XBMU-SN-23 fermentation broth plays a crucial role in rebalancing the neurochemical environment of the hypothalamus. It improves insomnia by promoting the increase of sleep-inducing substances (5-HT, IL-1), reducing a key arousal-promoting neurotransmitter (DA), and enhancing neurotrophic support (BDNF). These multi-target effects collectively contribute to its potent sleep-enhancing efficacy.

3.8. Effect of XBMU-SN-23 fermentation broth on GABA_AR and 5-HT_{1A} receptor protein in PCPA-Induced insomnia mice

The results of the Western blot analysis of GABA_AR and 5-HT_{1A} protein expression levels in the hypothalamus of mice are presented in **Figure 6**. Compared to the control group, the protein levels of 5-HT_{1A} and GABA_AR_{α1} receptors in the brain tissue of mice were significantly downregulated following PCPA induction. In contrast, the XBMU-SN-23 fermentation broth treatment group exhibited a significant upregulation of 5-HT_{1A} protein expression, while the middle dose of GABA also showed a significant increase ($P < 0.05$). This suggests that the 5-HT_{1A} receptor and the GABA_AR_{α1} subunit play key roles in the neurotransmitter system^[37]. This is consistent with previous ELISA results.

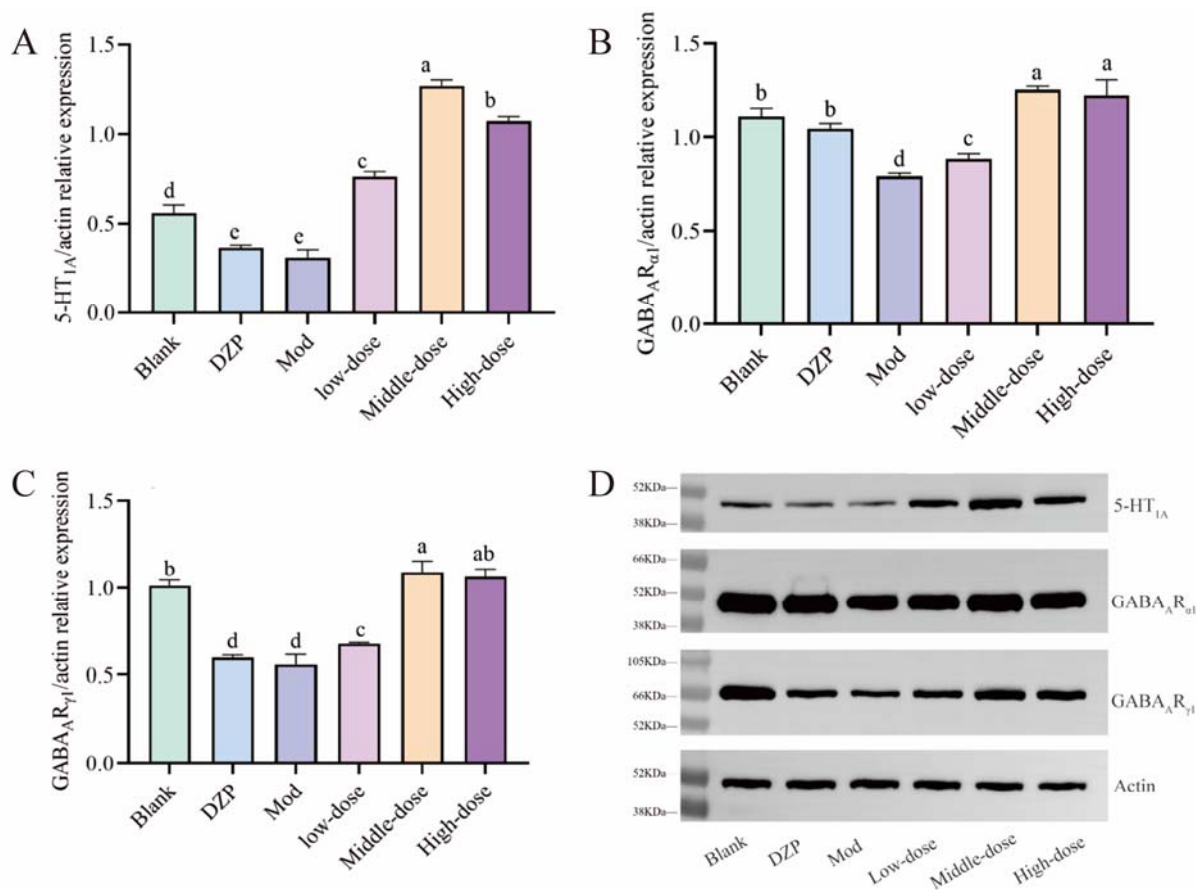


Figure 6 Effect of GABA on GABA_AR and 5-HT_{1A} receptor proteins in the hypothalamus of PCPA-induced insomnia mice. (A) 5-HT_{1A}/actin relative expression, (B) GABA_AR_{α1}/actin relative expression, (C) GABA_AR_{γ1}/actin relative expression, (D) Receptor protein expression.

4. Discussion

Insomnia is a significant global health issue, affecting approximately one-third of the population, with this proportion on the rise. Most existing medications for insomnia are the result of accidental discoveries and often come with addictive properties and other side effects. Therefore, there is an urgent need for safer and more effective therapeutic options. GABA is the primary inhibitory neurotransmitter in the central nervous system and has been shown to improve conditions such as insomnia, Alzheimer's disease, and Parkinson's disease^[38]. In our study, we isolated and identified a high-yielding GABA-producing strain, *Lactobacillus plantarum* XBMU-SN-23, from Jiangshui. Through systematic research, we confirmed that its fermentation broth significantly improves sleep quality in PCPA-induced insomnia mouse models through multitarget neural regulation.

Selecting microbial strains with high GABA production is essential for the effectiveness of fermented functional products. Our XBMU-SN-23 strain achieved a GABA yield of 2.150 g/L, which is significantly higher than that of several other reported GABA-producing lactic acid bacteria, such as *Lactobacillus plantarum* K154 (0.170 g/L)^[16] and *Lactococcus lactis* (1.153 mg/L)^[39]. This high-yielding characteristic not only underscores the industrial potential of XBMU-SN-23 but also ensures adequate GABA delivery in subsequent in vivo trials. Moreover, the strain exhibited strong tolerance to simulated gastrointestinal conditions, with a survival rate of 34.58% at pH 2.0 and 26% in 0.3% bile salts. These features suggest its

potential viability during passage through the human gastrointestinal tract—a critical attribute for probiotic strains intended for oral administration^[40].

LAB continue to experience growing consumer demand due to their significant benefits in antioxidant activity, gut regulation, and cardiovascular health maintenance. The strain XBMU-SN-23 examined in our study was identified as *Lactobacillus plantarum* through 16S rDNA analysis. This strain is not only a natural commensal microorganism in the human gastrointestinal tract and various fermented foods but is also recognized as a safe substance because of its long history of safe consumption^[41]. Furthermore, existing research indicates that *Lactobacillus plantarum* does not possess any known toxic or pathogenic factors^[42], providing a strong foundation for its use as a safe probiotic strain in food fermentation and health product development.

In our study, the fermentation broth of strain XBMU-SN-23 demonstrated multi-target restorative effects on imbalanced neurotransmitters and neuroendocrine systems associated with insomnia. In a PCPA-induced insomnia model, the fermentation broth significantly increased hypothalamic 5-HT and serum melatonin levels—both of which are critical for sleep initiation and circadian rhythm regulation^[43,44]. Concurrently, the significant increase in 5-HT_{1A} receptor expression suggests enhanced serotonergic signaling efficacy, consistent with reports that certain probiotics modulate the serotonergic system through the gut-brain axis^[45]. More importantly, the fermented liquid exhibits comprehensive regulatory effects on neurotransmitter balance. By increasing GABA levels and decreasing glutamate concentrations, it normalizes the Glu/GABA ratio, indicating a shift toward an inhibitory state within the central nervous system^[46]. Western blot results further demonstrated that the GABA_A receptor $\alpha 1$ and $\gamma 2$ subunits were synchronously upregulated alongside the 5-HT_{1A} receptor, confirming that the fermentation broth enhances the sensitivity and functionality of the inhibitory neurotransmitter system. These receptor-level changes are consistent with the mechanism by which ginsenosides regulate sleep^[47].

Notably, the fermentation extract demonstrated the most pronounced effect at a moderate dose (90 mg/kg), with no further enhancement observed at higher doses. This nonlinear dose-response suggests the presence of complex interactions among active components within the fermented liquid, which may act synergistically yet reach a saturation point. These findings also indicate that the sleep-promoting efficacy of the fermented liquid cannot be attributed solely to GABA's individual action; other active components, such as microbial metabolites like short-chain fatty acids^[48], likely contribute collectively to this effect. Additionally, histopathological analysis provided direct evidence of the neuroprotective effects of the fermented liquid. And the fermented liquid administered to the medium-dose group significantly alleviated PCPA-induced damage to neurons in the hippocampus and hypothalamus. These brain regions play crucial roles in sleep regulation, memory consolidation, and neuroendocrine control. The structural improvements observed in these areas correspond with the behavioral and biochemical enhancements, suggesting that the improved sleep quality may be partially attributable to the protection of these key sleep-regulating brain regions.

Beyond molecular discoveries, our research revealed that treated mice exhibited significantly restored calmness and reduced spontaneous movement in sedative-hypnotic-related behavioral phenotypes. This finding aligns closely with biochemical evidence of rebalanced Glu/GABA ratios and enhanced serotonergic activity (5-HT). Specifically, the reduction in the excitatory neurotransmitter glutamate and the increase in the inhibitory neurotransmitter GABA likely underlie the decreased excitability in the central nervous system, directly contributing to the observed sedative effects and improved sleep readiness^[49]. Furthermore, the shortened sleep latency, prolonged sleep duration, and increased sleep induction rate correspond with heightened sensitivity of GABAergic and serotonergic systems, as evidenced by the upregulation of GABA_A receptor subunits ($\alpha 1$, $\gamma 2$) and 5-HT_{1A} receptors. This multi-level consistency between behavioral normalization and neurochemical restoration strongly suggests that the sleep-promoting efficacy of XBMU-SN-23 results directly from its multi-targeted effects on neurotransmitter systems regulating the sleep-wake cycle.

5. Conclusion

In our study, we isolated and identified a GABA-high-yielding strain, *Lactobacillus plantarum* XBMU-SN-23, from traditional fermented foods. This strain exhibits optimal growth characteristics and tolerance to simulated gastrointestinal conditions. Administration of GABA-rich fermented broth significantly improved sleep disturbances in PCPA-induced insomnia mice, primarily by restoring the balance between excitatory and inhibitory neurotransmitters (Glu/GABA, DA/5-HT), increasing melatonin levels, and upregulating the expression of 5-HT_{1A} and GABA_A receptor subunits ($\alpha 1$, $\gamma 2$) in the hypothalamus. The intermediate dose (90 mg/kg) produced the most pronounced effect. These findings indicate that *Lactobacillus plantarum* XBMU-SN-23 fermented liquid enhances sleep quality through multi-target regulation of neurotransmitter systems and associated receptor pathways. In our study not only provides a theoretical foundation for developing natural sleep-promoting agents based on this strain but also highlights its significant potential as an alternative to traditional pharmaceuticals. In the future, we will focus on conducting safety assessments of GABA-producing bacterial strains, analyzing the interactions among multiple components in fermentation broths, and exploring the mechanisms of the gut-brain axis. This approach will clarify the safety margins of bacterial strains, reveal synergistic pathways among components, and elucidate the gut-to-brain signaling pathway, thereby advancing their safe and efficient application in the food and health supplement industries.

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