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Material basis and sleep-improving mechanisms of Lily-*Ziziphi Spinosae Semen* decoction: systematic evidence from LC-MS, network pharmacology and animal experiments

Fan Jia[a,1], Hui Zheng[a,1], Yao Xu[a], Jiani Jiang[a], Yuhang Wu[a], Jianxin Liu[b], Kai He[b,*], Yong Yang[a,*]

[a] *School of Pharmacy, Hunan University of Chinese Medicine, Changsha 410208, China*
[b] *School of Pharmaceutical Science, Hunan University of Medicine, Huaihua 418000, China*

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ABSTRACT

Lily-*Ziziphi Spinosae Semen* decoction (LZ) is known for its blood nourishing, mind calming, body sedation, and sleep promoting effects in traditional Chinese medicine. However, its material basis and underlying mechanisms have not yet been clearly defined. This study applies liquid chromatography-mass spectrometry, network pharmacology, and animal studies to reveal the material basis and sleep-improving mechanisms of LZ. The mixed decoction (LZ-ME) and single decoction (LZ-SE) were prepared to study their chemical components. Network pharmacology was used to predict the sleep-improving targets and signaling pathways of LZ. ICR mice were intragastrically administered saline (NC), melatonin (positive control group, 0.50 mg/kg), low (12.90 g/kg), medium (25.70 g/kg), and high (38.60 g/kg) dose of LZ-ME and LZ-SH for 30 days. The results showed that LZ-ME could prolong the sleep duration and shorten the sleep latency in sodium barbiturate induced mice model. The results of chemical composition showed that total polysaccharides, total flavonoids, total saponins, and total alkaloids in LZ-ME were significantly higher than those in LZ-SE (177.20%, 82.34%, 30.58%, and 11.66%, respectively). A total of 58 chemical components were identified by ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS), and 8 representative difference components of LZ-ME and LZ-SE were found. LZ-ME significantly increased tumor necrosis factor-α (TNF-α) in mice serum and neurotransmitter γ-aminobutyric acid (GABA) levels in mice hippocampus, decreased dopamine (DA) and glutamate (Glu) levels in mice hippocampus ($P < 0.05$). Furthermore, LZ-ME up-regulated the abundance of the beneficial bacteria *Lactobacillus* and *Eubacterium*_R, down-regulated the abundance of the harmful bacteria Lachnospiraceae etc. The polysaccharides, flavonoids (spinosin and 6″-feruloylspinosin) and saponins (jujuboside A and jujuboside B) were the main material bases for the sleep-promoting effects of LZ. These compounds may directly enhance levels of the GABA, reduced levels of Glu and DA and improve TNF-α levels. And they also may indirectly regulate GABA levels by influencing the relative abundance of *Lactobacillus* and *Eubacterium*.

1. Introduction

Insomnia refers to individuals often find themselves unsatisfied with their sleep duration or quality, even in a conducive sleep environment[1]. Insomnia is associated with many diseases, such as obesity, hypertension, diabetes, and psychological disorders[2].

[1] These authors contributed equally to this work.
[*] Corresponding authors.
E-mail address: yangyong@hnucm.edu.cn (Y. Yang); hekai69@email.swu.edu.cn (K. He)
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Prolonged insomnia leads to memory loss, immune function decreased, and aging accelerated, as well as increase the incidence of diseases such as Alzheimer's disease and Parkinson's disease[3]. Fierce social competition and the fast-paced lifestyle lead to a rapid increase in the incidence of insomnia, which seriously affects people's quality of life. The results of the World Health Organization (WHO) survey showed that approximately 45% of sub-healthy individuals suffer from sleep disorders over an extended period[4]. According to the "Annual Sleep Report of China" released in 2023, the incidence of insomnia in Chinese adults was as high as 38.2%, which means about 510 million Chinese people had sleep disorders[5]. Currently, benzodiazepine receptor agonists, melatonin receptor agonists, and antidepressants are the primary drugs for the treatment of insomnia. These agents have a rapid onset and clear mechanisms of action, while long-term using can lead to side effects such as residual effects, memory impairment, and withdrawal symptoms[6-7].

As evidence-based practice, traditional Chinese medicine (TCM) provides a lot of systematic theory and practical experience in insomnia intervention. The tranquilizing herbs and prescriptions, documented in ancient medical literatures, encapsulated valuable experiences in addressing sleep disorders. Lily-*Ziziphi Spinosae Semen* decoction (LZ) was recorded in 2 renowned ancient medical literatures "*The Essentials of the Golden Chamber*" (approximately 1 800 years ago) and "*Thousand Golden Formulas*" (around 1 300 years ago). LZ is considered to have blood-nourishing, mind-calming, body sedating, and hypnotizing effects[8]. LZ is a TCM formula composed of 6 herbs which are both medicinal and edible herbs, which contains fried *Ziziphi Spinosae Semen*, Lily Bulb, *Poria cocos*, *Longan Arillus*, *Lycii Fructus*, and *Triticum aestivum* L. Among these herbs, fried *Ziziphi Spinosae Semen* is considered as the first choice for treating insomnia in TCM[9]. The main active compounds in *Ziziphi Spinosae Semen* are saponins and flavonoids, these compounds have been found to influence the contents of 5-hydroxytryptamine (5-HT) and γ-aminobutyric acid (GABA) in the brain[10-11]. The steroid saponin composition of Lily Bulb was shown to improve dopamine (DA) and 5-HT deficiency associated disorders[12-13]. *P. cocos* is believed to fortify the spleen and nourish the heart to tranquilize in TCM, and polysaccharides from *P. cocos* can alleviate the metabolic disorder and restore the balance of gut microbiota in sleep-deprived rats by inhibiting the tumor necrosis factor-α/nuclear factor kappa-B (TNF-α/NF-κB) signaling pathway. Additionally, alcohol extract from *P. cocos* can interact with the $GABA_A$ receptor, enhancing the transmission of inhibitory neurotransmitters[14-15]. *Longan Arillus* is believed to nourish the blood and calm the mind in TCM, which is commonly used with *Ziziphi Spinosae Semen* in clinical practice[16]. Complexity is one of the characteristics of TCM prescriptions, the essential goal of herbs combination is to achieve synergistic health effects of multiple active ingredients, which is akin to the principles of cocktail therapy.

Although LZ had a long history of clinical application for sleep disorder therapy, the material basis and underlying mechanisms of LZ has not yet been clearly defined. In this study, the sleep-improving effects of LZ was evaluated according to the criteria and methods outlined in the "*Technical Standards for the Testing and Assessment of Health Food*" (2023, China). Spectrophotometry and ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) were used to analyze and compare the main chemical components differences of LZ decoction prepared by mixed and single extracts. The network pharmacological, which emphasized the relationships between drugs and organisms[17], were utilized to forecast potential interactions among multiple chemical components and various disease targets. Gut microbiota analysis was applied to investigate the gut-brain axis regulating role of LZ on insomnia. It is revealed that LZ might exhibit sleep-improving activity by enhancing the GABA levels, reducing glutamate (Glu) and DA levels and influencing the relative abundance of *Lactobacillus* and *Eubacterium* in mice gut. These results will provide theoretical and experimental basis for clinical and daily applications of LZ decoction.

2. Materials and methods

2.1 Preparation of the LZ standard decoction

All TCM materials in this experiment met the standards of "*Chinese Pharmacopoeia*" (2020) and were procured from Hunan Zhenxing Traditional Chinese Medicine Co., Ltd. The preparation of LZ standard decoction was carried out according to the study of Shang et al.[18] and with some modifications. All the raw materials were decocted together with 10 times amount of water for 2 h at the ratio of fried Lily-*Ziziphi Spinosae Semen*:Lily Bulb:*P. cocos*:*Longan Arillus*:*Lycii Fructus*:*T. aestivum* L. for 4:4:3:3:3:2. Then the residues was extracted twice. The filtrates were combined, concentrated under reduced pressure at 60 ℃ and constant volume to obtain LZ standard decoction by mixed extraction (LZ-ME) with a calculated content of 1.93 g/mL from the raw materials. Unlike LZ-ME, LZ-SE decoction was obtained by extracting the individual materials respectively, and then mixing each filtrate according to the same proportion of raw drug volume as LZ-ME. The decoction was concentrated under reduced pressure at 60 ℃ to achieve the final products which equivalent to 1.93 g/mL of the raw materials.

2.2 Evaluation the sleep-improving effect of LZ-ME

2.2.1 Animal experiment

Healthy, specific pathogen-free (SPF) grade ICR mice (male, 8 weeks old, weighing 18−20 g) were obtained from Hunan SJA Laboratory Animal Co., Ltd. (Hunan, China), with the experimental animal license number SYXK (Xiang) 2019-0004. The animal experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of Hunan University of Chinese Medicine, under approval number LL2021040704, adhering to the welfare and ethical principles of laboratory animals. All experimental animals were housed in SPF condition at the Animal Experiment Center of Hunan University of Chinese Medicine, with a 12 h light/dark cycle and free access to water and food. The ambient temperature was (23 ± 2) ℃ with a relative humidity of (60 ± 2)%.

2.2.2 Experiment on sleep-improving

The assessment for sleep quality improvement were performed based on the "*Technical Specification for Inspection and Evaluation of Health Food*" (2023, China), in which three dose groups is required for the animal experiment, including a group that 10 times of the human dose and a negative control group, a positive control group

if necessary. According to "*The Essentials of the Golden Chamber*", LZ consists of 20 g fried *Ziziphi Spinosae Semen*, 20 g Lily Bulb, 15 g *P. cocos*, 15 g *Longan Arillus*, 10 g *Lycii Fructus*, and 10 g *T. aestivum* L, which is a total of 90 g herbs. And these herbs are token as herbal decoction once a day. Therefore, the low, medium, and high dose of LZ for animal mice is 12.90, 25.70, and 38.60 g/kg, respectively. According to the "*Regulations on Declaration and Review of Amino Acid Chelates and Other Health Foods*" issued by the State Food and Drug Administration, the dosage of melatonin in human is 1–3 mg/day. Therefore, melatonin at 0.5 mg/(kg·day) was chosen for animal study.

After 7 days of habituation, ICR mice were randomly divided into 5 groups according to their weight (n = 10 in each group). The experimental groups were designed as follow: control group (NC, saline), positive control group (PT, 0.50 mg/kg bw melatonin), LZ-ME groups including low dose group (LZ-ML, 12.90 g/kg bw), medium dose group (LZ-MM, 25.70 g/kg bw) and high dose group (LZ-MH, 38.60 g/kg bw). Saline or drugs were intragastric administered to mice at 9:00 AM to 10:00 AM for 30 days. The gavage volume was 0.2 mL/20 g bw. The weights of each mouse were recorded at 3-day intervals.

2.2.2.1 Direct sleep experiment

The sleep and wake states of each group were observed immediately after the last dose. The disappearance of reversal reflex (when the mice were placed in the dorsal position and could not change to prone position for 30−60 s) was used as the criterion for determining sleep occurrence. The period between the disappearance of the reversal reflex and the awakening of the animals was the sleep duration. The number of sleeping animals and the duration of sleep in each experimental group were recorded.

2.2.2.2 Sub-threshold sodium pentobarbital dose-induced sleep experiment

The purpose of this experiment was to observe the synergistic effect between the tested agents and sodium pentobarbital. A pre-experiment was conducted to determine the highest sub-threshold dose (the highest dose of sodium pentobarbital at which 80%−90% of the mice would retain the reversal reflex) of sodium pentobarbital. The experimental mice were administered continuously for 30 days, and all mice were fasted for 24 h before the last LZ-ME administration. After receiving the last LZ-ME samples for 20 min, the mice were injected intraperitoneally with a sodium pentobarbital solution at a dose of 0.2 mL/20 g. The number of sleeping mice (the reversal reflex disappeared for more than 60 s) within 30 min was determined, and the incidence of falling asleep was calculated. The results were analyzed using the chi-square test.

2.2.2.3 The effects of LZ decoction on lengthen the hypnotic effect of sodium pentobarbital

To explore the potential synergistic effect between LZ standard decoction and sodium pentobarbital on sleep-promoting effects. The experimental mice were administered LZ standard decoction continuously for 30 days, and all mice were deprived of food and water for 24 h before the last administration of LZ decoction. After receiving the last LZ-ME samples for 20 min, the mice were injected intraperitoneally with a sodium pentobarbital solution at a dose of 0.2 mL/20 g (the dose of sodium pentobarbital was determined by our previous experiment). The sleep duration of each group of mice was recorded as the time interval when the reversal reflex disappeared over 60 s and the reversal reflex was recovered. The sleep duration of each group mice was determined.

2.2.2.4 Sodium barbital-induced sleep latency experiment

To observe whether the sleep latency of the experimental mice could be reduced after LZ standard decoction, the experimental mice were administered with LZ standard decoction continuously for 30 days, and all mice were deprived of food and water for 24 h before the last administration of LZ decoction. After receiving the last LZ-ME samples for 20 min, the mice were injected intraperitoneally with a sodium pentobarbital solution at a dose of 0.2 mL/20 g. The sleep latency of each group of mice was recorded as the time interval when injection of sodium barbiturate and the reversal reflex disappeared over 60 s. The sleep latency of each group mice was then determined.

2.2.2.5 Organ index determination

The heart, liver, spleen, lungs, and kidneys were dissected out and weighed, and the mouse organ index was calculated. The formula for calculating the mouse organ index is as follows:

$$\text{Organ index} = \frac{\text{Organ weight (mg)}}{\text{Mouse body weight (g)}}$$

2.3 Chemical component analysis of LZ standard decoction

The content of main active ingredients in LZ standard decoction, including total flavonoids (probably from fried *Ziziphi Spinosae Semen*, Lily Bulb, and *Lycii Fructus*), total polysaccharides (probably from Lily Bulb, *P. cocos*, *Longan Arillus*, and *Lycii Fructus*), and total saponins (probably from fried *Ziziphi Spinosae Semen*, Lily Bulb, and *P. cocos*) were investigated. These compounds were potential active substances against insomnia according previous studies. Additionally, UPLC-QTOF-MS analysis was employed to further elucidate the specific differences in components and their characteristics.

2.3.1 Determination of total polysaccharides content

A total of 5.0 mL distilled water was added to 5.0 mL of LZ-ME and LZ-SE sample respectively. After intense mixing, 1.5 mL of the prepared solution and add 7.5 mL of ethanol were mixed and incubated at 4 ℃ for 24 h. Centrifuge and discard the supernatant, evaporate the ethanol, and then dissolve it with distilled water and volume it up to 100 mL, then obtain the total polysaccharide test solution of LZ-ME and LZ-SE. Accurately absorb 2.0 mL of each test solution into a 10 mL tube, add 8.0 mL of 0.2% anthrone sulfuric acid solution and mix thoroughly, quickly cool he mixture in an ice-water bath for 5 min, heat in a boiling water bath for 10 min, remove in the ice water bath for another 5 min, then bring to room temperature for 10 min. Use the corresponding reagent as a blank, the absorbance value was measured at 585 nm and the total polysaccharide content was calculated[19].

2.3.2 Determination of total flavonoids content

According to the methods of "*Determination of Total Flavonoids in Health Food*" in the "*Technical Guidelines for the Inspection and Evaluation of Physicochemical and Hygienic Indicators of Health Foods*" (2020, China), the total flavonoid content in LZ standard decoction was determined. A total of 5.0 mL distilled water was added to 5.0 mL of LZ-ME and LZ-SE sample respectively. This yields the total flavonoid test solution for both LZ-ME and LZ-SE. Accurately absorb 3.0 mL of each test solution into a 10 mL tube, add 0.5 mL of 5% sodium nitrite solution and shake well, placed for 6 min. Then add 0.5 mL of 10% aluminum nitrate solution and shake well, placed for 6 min. Last add 5 mL of 4% sodium hydroxide solution and add distilled water to the mark and shake well, place it for 15 min, the absorbance value was measured at 510 nm and the total flavonoid content was calculated.

2.3.3 Determination of total saponins content

According to the method of "*Determination of Total Saponins in Health Food*" in the "*Technical Guidelines for the Inspection and Evaluation of Physicochemical and Hygienic Indicators of Health Foods*" (2020, China), the total saponins content in LZ standard decoction was determined. A total of 5.0 mL distilled water was added to 5.0 mL of LZ-ME and LZ-SE sample respectively. After intense mixing, 10.0 mL of the prepared solution and 10 mL of water-saturated *n*-butanol were mixed, separate the *n*-butanol layer, repeat the operation for 3 times, combine the 3 times of the *n*-butanol layer solution, washed with 10 mL of ammonia, repeat the operation for 2 times, discard the ammonia, evaporate the *n*-butanol solution in water, and dissolve and transfer to a 10 mL measuring flask, add methanol to the gradient, shake well, filter and take the filtrate to obtain the total saponin of LZ-ME and LZ-SE. Accurately absorb 3.0 mL of each test solution into a 10 mL tube, add methanol to the scale, shake well, filtered, and the filtrate was taken to obtain the total saponin test solution of LZ-ME and LZ-SE. Accurately absorb 1.0 mL of each test solution in 10 mL color tube, evaporate the solvent at low temperature, add 0.2 mL of 5% vanillin-chilled acetic acid solution, another add 0.8 mL of perchloric acid, shake well, and bath in 60 ℃ for 20 min, then cool quickly after removal. Last add 5 mL of glacial acetic acid solution, shaken well, and placed in the dark for 30 min. Subsequently, the absorbance value was measured at 560 nm and the total saponin content was calculated.

2.3.4 Determination of total alkaloids content

A total of 5.0 mL of LZ-ME and LZ-SE were transformed into a 10 mL volumetric flask. Add distilled water to the mark and shake well. Then take 5 mL evaporate the solvent at low temperature, add 5 mL acetic acid-sodium acetate solution, then obtain the total alkaloids test solution of LZ-ME and LZ-SE. Accurately absorb 3.0 mL of each test solution into a separating funnel, add 5 mL of 0.05% bromocresol green dye, another add 10 mL of chloroform, shaken for 3 min, and allowed to stand for 30 min, the chloroform layer was divided, and 0.5 g of anhydrous sodium sulfate was added for 0.5 h. Use the corresponding reagent as a blank, the absorbance value was measured at 415 nm and the total alkaloids content was calculated[20].

2.3.5 UPLC-QTOF-MS analysis

A total of 5.0 mL each of LZ-ME and LZ-SE were taken as described in Section 2.1, and transformed them into a 10 mL volumetric flask and shaked well. A total of 2.0 mL was taken and filtered through a 0.22 μm filter to obtain the test solution of LZ-ME and LZ-SE from the prepared solution. Then the filtered test solutions were analyzed using the UPLC-QTOF-MS instrument. Liquid chromatography conditions: Acquity UPLC BEH C_{18} column (2.1 mm × 100 mm, 1.7 μm), Mobile phase: 0.1% formic acid water (A)-acetonitrile (B); Gradient elution procedure: 0−6 min, 2%−13% B; 6 − 9 min, 13% − 24% B; 9 − 11 min, 24% − 25% B; 11 − 13 min, 25%−32% B; 13−17 min, 32%−42% B; 17−20 min, 42%−51% B; 20−24 min, 51%−87% B; 24−25 min, 100% B. Flow rate: 0.3 mL/min; injection volume: 1.0 μL; column temperature: 30 ℃ ; detection wavelength: 210 nm.

Mass spectrometry conditions: ion source: electrospray ionization (ESI); ionization mode: negative ion mode (full scan voltage: 2 500 V); capillary voltage: 2.5 kV; cone hole voltage: 40 V; ion source temperature: 120 ℃; atomization gas flow rate: 50 L/h; desolvent gas temperature: 300 ℃; desolvent nitrogen flow rate: 800 L/h; collision gas: argon; high energy channel collision energy: 20 − 50 V; primary mass spectrometry scanning range: *m/z* 50−1 500 Da.

Database establishment: with the help of literature databases such as CNKI, SciFinder and PubMed, the study searched the relevant literature on the study of *Ziziphi Spinosae Semen*, Lily Bulb, *P. cocos*, *Longan Arillus*, *Lycii Fructus*, and *T. aestivum* L. Then a comprehensive database was developed containing the following information on the major secondary metabolites from the specified herbs: names, relative molecular masses, molecular formulae, and molecular structures. Additionally, the . mol files for the identified compounds were downloaded using databases such as ChemicalBook, ChemSpider, and PubChem. The exact mass-to-charge ratios of the components were then calculated for the following ionization forms: $[M-H]^-$ and $[M+COOH]^-$.

2.4 Network pharmacological analysis of LZ standard decoction based on UPLC-QTOF-MS components analysis

Network pharmacological analysis was performed to predict the possible targets on the basis of chemical components of LZ standard decoction. On the one hand, the 58 chemical components were used to predict the overall possible targets. On the other hand, the flavonoids, saponins, phenylpropanoids, and alkaloids components were used to predict and attribute the possible targets of the four kinds of components.

2.4.1 Prediction of potential targets of LZ standard decoction

The SMILES files of 58 chemical components of LZ standard decoction were obtained by PubChem (https://pubchem.ncbi.nlm.nih.gov/). The potential targets of these components were predicted using the Swiss Target Prediction database (http://www.swisstargetprediction.ch/). All the protein names were standardized to gene names using UniProt (https://www. uniprot. org/). After deleting duplicates, the potential targets of all detected components in LZ standard decoction were complied. Besides, the potential targets of flavonoids, saponins,

phenylpropanoids, and alkaloids components also were sorted out, respectively.

2.4.2 Insomnia-related targets and intersected target acquisition

Using "insomnia" as the key word, human disease genes were searched for in the GeneCards (https://www. genecards. org/), Dis GeNET (http://www. disgenet. org) and OMIM (https://www. omim. org/) databases. After integrating the results and deleting duplicates, the insomnia-related targets were obtained. The online Venny 2.1 tool (https://bioinfogp. cnb. csic. es/tools/venny/index. html) was utilized to determine the intersected targets between insomnia-related targets and components targets that include 58 components and the 4 kinds of components. The intersected targets as the target genes of all detected components and four kinds of components in LZ standard decoction for the sleep-improving, respectively.

2.4.3 Protein-protein interaction (PPI) network construction and core targets screening

PPI networks are suitable for investigating interactions between proteins in biological systems. The target genes associated with sleep-improving from 58 components and four kinds of components in LZ standard decoction were uploaded to the STRING (https://cn.string-db.org/) database, respectively. And the biological species set to "*Homo sapiens*" for PPI network construction. The resulting PPI network was imported into Cystoscape 3.9.1 software, and the "Centiscape 2.2" plugin was utilized to identify core targets of all components and 4 kinds of components.

2.4.4 GO and KEGG enrichment analysis

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses are widely used methods in bioinformatics for functional annotation and pathway analysis, serving as the foundation for studying gene function and action pathways. Enrichment analyses for biological processes (BP), molecular functions (MF), cellular components (CC), and KEGG pathways were conducted using the DAVID database (https://david. ncifcrf.gov/), with screened for significance at $P < 0.05$.

2.5 Sleep-improving mechanisms of LZ-ME

2.5.1 Animal experiment

Healthy, SPF grade ICR mice (male, 8 weeks old, weighing 18−20 g) were obtained from Hunan SJA Laboratory Animal Co., Ltd. (Hunan, China), with the experimental animal license number SYXK (Xiang) 2019-0004. The animal experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of Hunan University of Chinese Medicine, under approval number HNUCM21-2312-20, adhering to the welfare and ethical principles of laboratory animals. The conditions for keeping all experimental animals were the same as in Section 2.2.1.

2.5.2 Experimental grouping and drug administration

After 7 days of habituation, ICR mice were randomly divided into 8 groups according to their weight (n = 6 in each group). The experimental groups were designed as follow: control group (NC, saline), positive control group (PT, 0.50 mg/kg bw, melatonin), LZ-ME groups including low dose group (LZ-ML, 12.90 g/kg bw), medium dose group (LZ-MM, 25.70 g/kg bw) and high dose group (LZ-MH, 38.60 g/kg bw), and LZ-SE groups including low dose group (LZ-SL, 12.90 g/kg bw), medium dose group (LZ-SM, 25.70 g/kg bw) and high dose group (LZ-SH, 38.60 g/kg bw). Saline or drugs were intragastric administered to mice at 9:00 AM to 10:00 AM for 30 days. The gavage volume of each drug was 0.2 mL/20 g bw. Mouse body weights were recorded at 3-day intervals.

2.5.3 Sodium pentobarbital-induced sleep duration experiment

Preliminary results indicated significant differences in the sleep-improving effects between LZ-ME and LZ-SE. To accurately analyze the relationship between behavioral experiment results and biomarkers, the sodium pentobarbital-induced sleep duration experiment was performed to evaluate the sleep-improving effect of LZ standard decoction according to Dong et al.[21]. The experimental procedures followed are consistent with those described in Section 2.2.2.3.

2.5.4 Determination of neurotransmitter and inflammatory factors

After conducting the sodium pentobarbital-induced sleep duration test, blood samples and hippocampal tissues were collected from experimental mice in the NC, PT, LZ-ML, LZ-MM, and LZ-MH groups for subsequent study. Blood samples were collected by removing the eyeball, then allowed to stand for 30 min before being centrifuged at 4 000 r/min for 10 min[22]. The resulting supernatants were transferred to labeled, enzyme-free EP tubes and stored at −80 ℃ for the analysis of interleukin-1β (IL-1β), interleukin-6 (IL-6), and TNF-α levels. All mice were immediately euthanized by cervical dislocation. The hippocampus of each mouse was swiftly excised on ice, placed in labeled frozen tubes, and promptly frozen in liquid nitrogen before being stored at − 80 ℃ for subsequent analysis of serotonin 5-HT, GABA, Glu, and DA levels[23].

Serum levels of IL-6, IL-1β, and TNF-α, as well as hippocampus levels of 5-HT, GABA, Glu, and DA were measured using ELISA kits (Shanghai Personal Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's instructions. Briefly, 50 μL of standards solution was added into each well, 10 μL testing sample and 40 μL sample diluent were added to the sample wells, blank group well dose not add anything. Then 100 μL horseradish peroxidase (HRP) labeled detection antibody were added to the standard and sample wells, and the reaction wells were sealed with a sealing film, incubated at 37 ℃ for 1 h, after washing 5 times, 50 μL of chromogenic agent A and B were added to each well and incubated at 37 ℃ for 15 min in the dark. After 15 μL stop solution was added to each well, the OD value of each well was measured at the wavelength of 450 nm microplate reader. The concentration of each sample was calculated according to the linear regression cutve of the standard sample[24].

2.5.5 Gene sequencing analysis of gut microbes from cecal contents

The cecal contents of each mouse were collected and placed in labeled frozen tubes. These samples were promptly frozen in liquid nitrogen and stored at −80 ℃ for gene sequencing analysis of gut microbes. Microbial full-length 16S rRNA gene sequencing was completed by the third-generation microbial sequencing technology commissioned by Shanghai Personal Biotechnology Co., Ltd. Specific method as following[25]: after samples were collected and ground into a centrifuge tube for extraction and lysis, total DNA was extracted with OMEGA Soil DNA Kit (M5635-02) (Omega Bio-Tek, Norcross, GA, USA). Molecular size determination was conducted *via* 0.8% agarose gel electrophoresis, and DNA concentration was quantified using a Nanodrop spectrophotometer. Then the full-length 16S rRNA gene in each sample was amplified using specific primers of the bacterial 16S rRNA V3-V4 region 338F (5′-barcode+ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′), the barcode in the preprimer is a 7−10 base oligonucleotide sequence used to distinguish between different samples in the same library.

The PCR reaction system consisted of 0.25 μL ABclonal DNApolymerase, 5 μL 5× Reaction Buffer, 5 μL 5× High GC Buffer, 2 μL dNTP (10 mmol/L), 2 μL template DNA, 1 μL forward primer (10 μmol/L), 1 μL reverse primer (10 μmol/L) and 8.75 μL ddH_2O. The PCR reaction procedure was as follows: pre-denaturation 98 ℃ 5 min, 25 amplification cycles:denaturation 98 ℃ 30 s, annealing 52 ℃ 30 s, extension 72 ℃ 45 s, 2 DNA fragment enrichment and synthesis , final extension 72 ℃ 5 min, storage at 12 ℃. After purification of PCR amplification products, TruSeq Nano DNA LT Library Prep Kit was used for library construction, and 2× 250 bp duplex end sequencing was performed on an Illumina NovaSeq machine using NovaSeq 6000 SP Reagent Kit (500 cycles). Microbiome biological information was analyzed using version QIIME2 2019.4.

2.6 Statistical analysis

Data analysis was performed using SPSS 26.0. Significant differences between multiple groups were assessed using one-way ANOVA, with results expressed as mean ± SD and a significance level of $P < 0.05$. Gene sequencing data were analyzed and visualized using the Gene Cloud Platform (https://www.genescloud.cn).

3. Results

3.1 The sleep-improving effects of LZ-ME

3.1.1 Results of direct sleep test

After the final administration of LZ-ME, the mice exhibited sedation and decreased activity. However, no loss of the righting reflex was observed. These results indicated that LZ-ME did not produce a direct hypnotic effect on the experimental mice.

3.1.2 Results of sub-threshold sodium pentobarbital dose-induced sleep experiment

The pre-experiment determined that the sub-threshold hypnotic dose of sodium pentobarbital in ICR mice was 30 mg/kg, which was utilized in subsequent formal experiments. As shown in Fig. 1A, compared to the NC group, the incidence of sleep in PT and LZ-MH groups was significantly higher ($P < 0.05$). Conversely, the LZ-ML and LZ-MM groups showed no significant difference compared to the

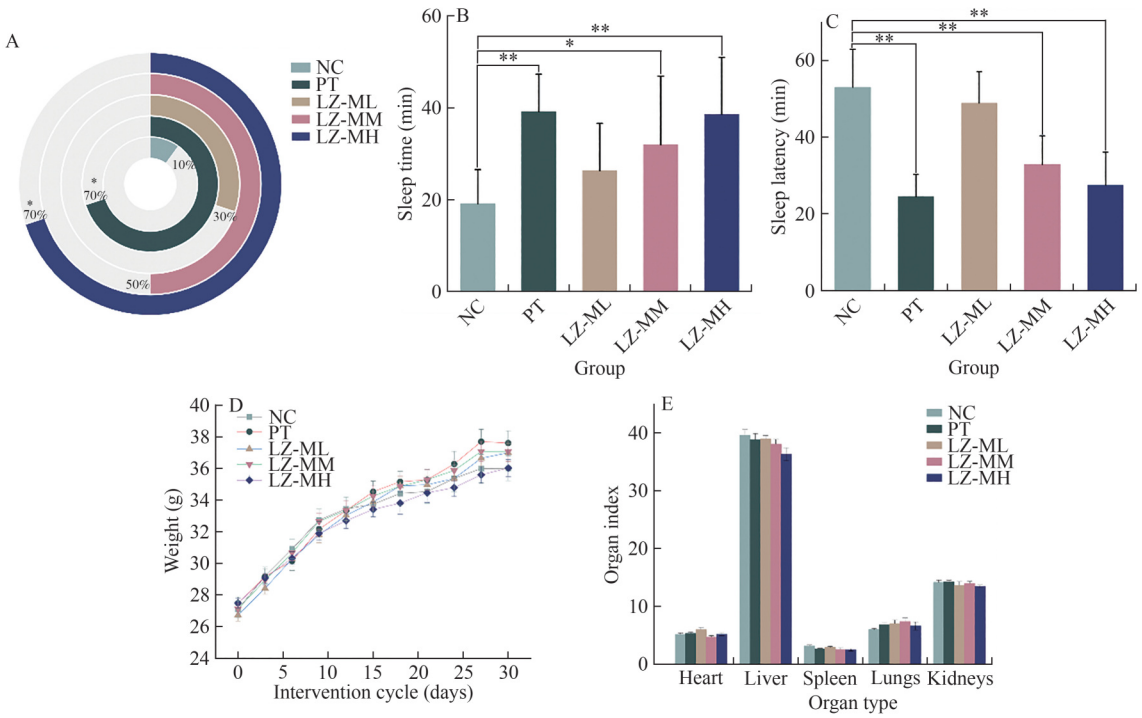

Fig. 1 Evaluation of LZ-ME sleep-improving effects ($n = 10$). (A) Incidence of sleep in each group of mice; (B) Sleep duration in each group of mice; (C) Sleep latency in each group of mice; (D) Weight changes in each group during the experimental period. (E) Organ index in each group of mice. $^*P < 0.05$, $^{**}P < 0.01$ *vs*. NC group.

NC group, although their incidence remained higher than that of the NC group. Notably, a clear dose-response relationship was observed among the three dose groups of LZ-ME.

3.1.3 Results of sodium pentobarbital-prolonged sleep duration experiment

Compared to the NC group ((19.18 ± 7.39) min), the sleep durations of LZ-MM group ((32.01 ± 14.89) min), and LZ-MH group ((38.63 ± 12.34) min) groups were significantly longer ($P < 0.01$). In contrast, the LZ-ML group ((26.35 ± 10.30) min) did not show significant difference ($P > 0.05$) (Fig. 1B). Specifically, LZ-ML, LZ-MM, and LZ-MH extended the sleep duration by 37.4%, 66.9%, and 101.4% compared to the NC group, respectively. The experimental results indicated that the medium and high doses of LZ-ME can significantly prolong the sleep duration induced by sodium pentobarbital in ICR mice.

3.1.4 Results of sodium barbital-induced sleep latency experiment

The pre-experiment determined that the sub-threshold hypnotic dose of sodium barbital in ICR mice was 260 mg/kg, which was used for subsequent formal experiments. The experimental results are shown in Fig. 1C. Compared with the NC group ((53.00 ± 9.86) min), all the sleep latency of PT ((24.58 ± 5.77) min), LZ-MM ((32.94 ± 7.59) min), and LZ-MH ((27.49 ± 8.59) min) were significantly shorter ($P < 0.01$). However, the LZ-ML ((48.88 ± 8.28) min) did not show significant difference. Shortened sleep latency was observed in all LZ-ME dose groups, with LZ-ML, LZ-MM, and LZ-MH shortening 7.77%, 37.84%, 48.13%, respectively, compared to the NC group. And a clear dose-response relationship was observed among the three dose groups of LZ-ME. The experimental results demonstrated that LZ-ME effectively shortens the sleep latency induced by sodium pentobarbital, and LZ-ME and sodium pentobarbital exhibited a synergistic sleep-improving activity.

3.1.5 Effect of LZ-ME on body weight and organ index in mice

During the experiment, the mice in each experimental group had shiny fur, a normal diet and activity level, moderate fecal moisture, and no diarrhea or loose stool. No significant lesions in the heart, liver, spleen, lungs, and kidneys were observed in any of the groups. The effects of LZ-ME on weight and organ indices in mice are shown in Fig. 1. As shown in Fig. 1D, there was no significant difference on body weight at 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 days, respectively. There was no significant difference in the organ index of the mice in each group (Fig. 1E).

3.2 Chemical components of LZ standard decoction

3.2.1 The total polysaccharide, total flavonoid and total saponin content in LZ standard decoction

LZ-ME and LZ-SE were LZ standard decoctions prepared with same materials by different methods. The total polysaccharides, total flavonoids, and total saponins in LZ-ME and LZ-SE were determined, and the results are presented in Fig. 2. Consistently, 4 kinds of components (total polysaccharides, total flavonoids, total saponins, and total alkaloid) in LZ-ME ((41.04 ± 0.51), (65.24 ± 1.60), (16.57 ± 0.05), and (4.32 ± 0.05) mg/g) were 77.20%, 82.34%, 30.58%, and 13.40% higher than those in LZ-SE ((23.16 ± 1.05), (35.78 ± 0.17), (12.69 ± 0.07), and (3.79 ± 0.07) mg/g), respectively. The findings were similar with Xu's study[26], which indicated that the total flavonoids and total saponins content of *P. cocos* and *Ziziphus spinosa* were significantly higher when extracted as a mixture compared to separate extractions.

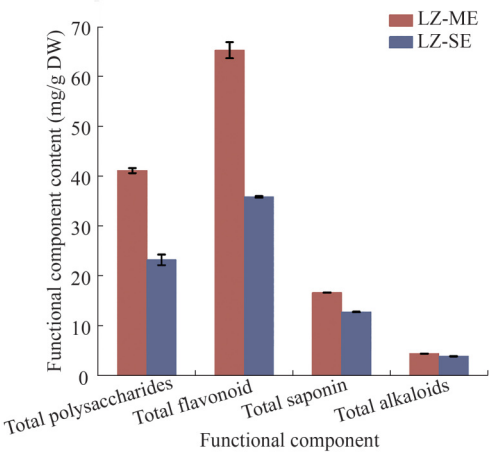

Fig. 2 Content of total polysaccharides, total flavonoids, total saponins and total alkaloid in LZ-ME and LZ-SE.

3.2.2 UPLC-QTOF-MS analysis results of LZ standard decoction

The total ion flow chromatograms of the two LZ standard decoction are shown in Fig. 3. UPLC-QTOF-MS data were analyzed using UNIFI software based on database, and 58 components were identified (Table S1). The detected components of LZ-ME and LZ-SE were classified. 13 flavonoids, 7 saponins, 10 phenylpropanoids, 7 alkaloids, 3 carbohydrates, 3 amino acids, and 7 fatty acids were identified in LZ-ME; 13 flavonoids, 6 saponins, 8 phenylpropanoids, 7 alkaloids, 3 carbohydrates, 3 amino acids, and 7 fatty acids were identified in LZ-SE. It was preliminarily found that alexandrin (saponins), chlorogenic acid (phenylpropanoid), and scopoletin (other classes) were the unique components of LZ-ME, which originated from *P. cocos*, Lily Bulb, and *Lycii Fructus*, respectively. Alexandrin is a triterpenoid saponin component of *P. cocos*, and it has been reported that *Ziziphi Spinosae Semen* can promote the dissolution of *P. cocos* triterpenoid components during mixed extraction[26].

The UPLC-QTOF-MS detection components were analyzed by principal component analysis (PCA), and the results are shown in Fig. 4A. From Fig. 4A, LZ-ME and LZ-SE were clearly clustered into 2 groups. A total of 41 differential components were yielded by screening the differential components of LZ-ME and LZ-SE utilizing extended statistical analysis. It included 10 flavonoids, 6 saponins, 7 phenylpropanoids, 5 alkaloids, and 13 other classes, and the specific differences are listed in Table S2. The total relative content of UPLC-QTOF-MS detection components was calculated according to the

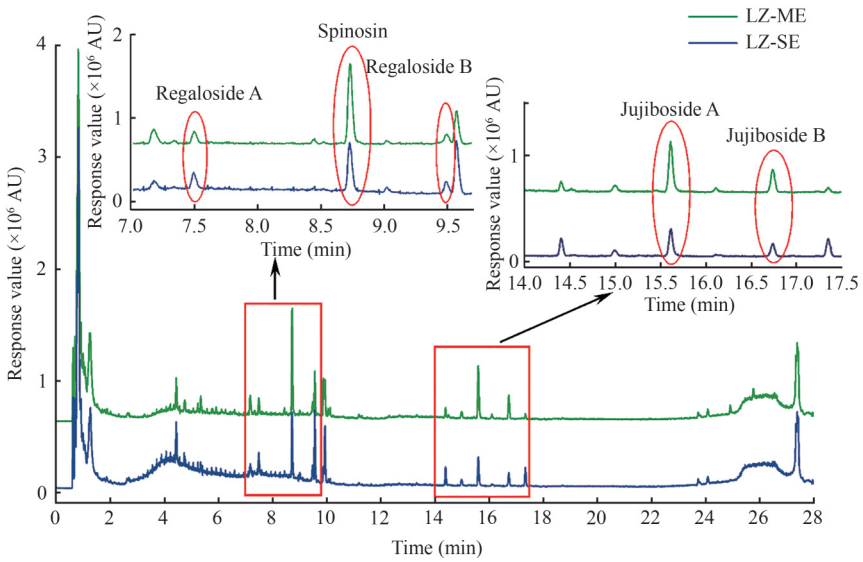

Fig. 3 Total ion chromatogram (TIC) plots in the negative ion mode of the 2 LZ standard decoction. (A) LZ-ME; (B) LZ-SE.

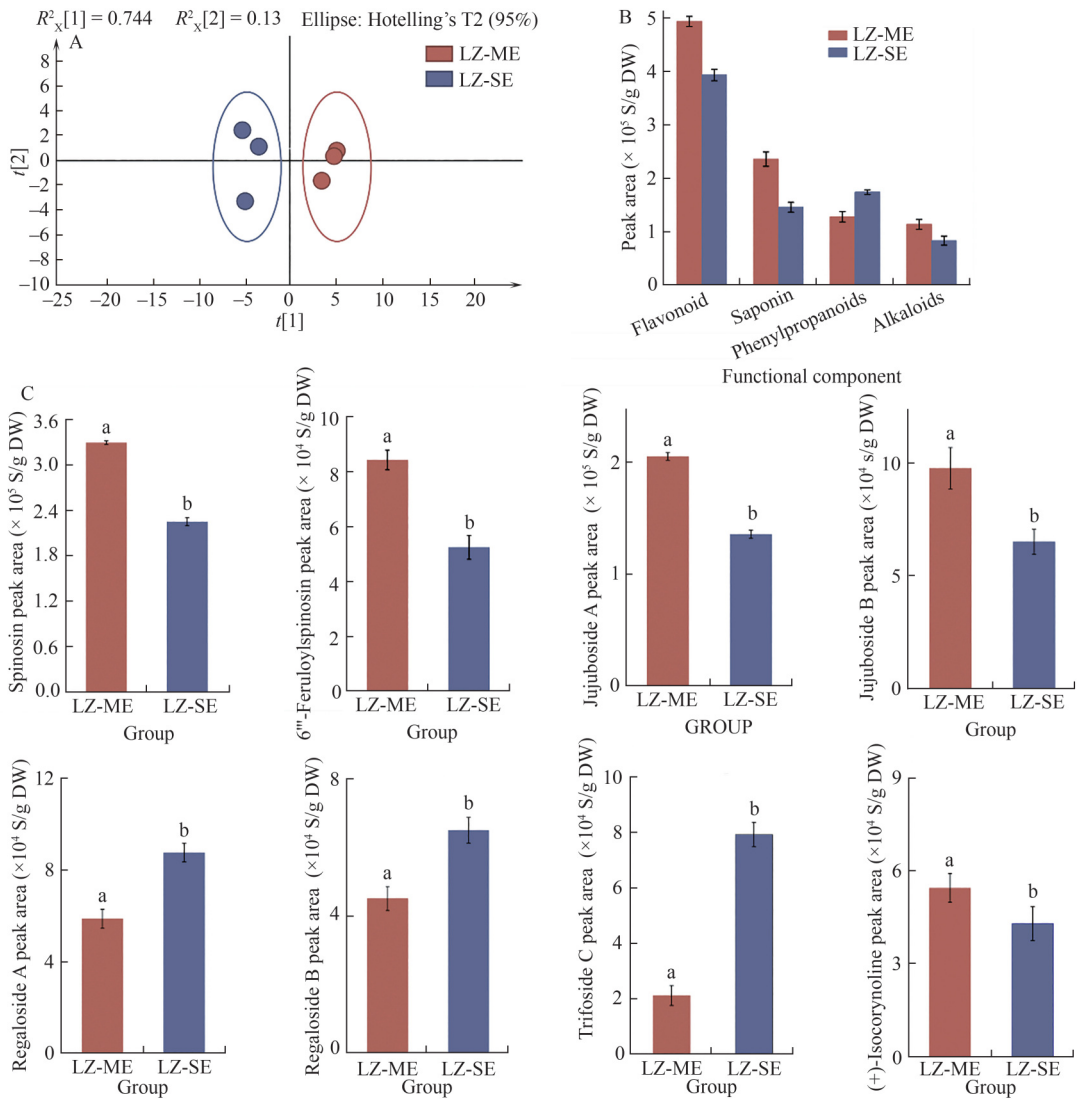

Fig. 4 Components difference of 2 LZ standard decoction. (A) The PCA plot; (B) The relative content of component groups identified by UPLC-QTOF-MS; (C) The relative content of 8 representative differential components. The mean of S/g is relative peak area per unit mass, S is relative peak area, g is unit of quality. Different letters are statistically different at $P < 0.05$.

response value of flavonoids, saponins, phenylpropanoids, and alkaloids, respectively (Fig. 4B). From Fig. 4B, the total response value of flavonoids, saponins, and alkaloids components in LZ-ME was significantly higher than that of LZ-SE. But the total response value of phenylpropanoids components in LZ-ME was significantly lower than that of LZ-SE. The UPLC-QTOF-MS results indicated that the total flavonoids, total saponins, and total alkaloids of LZ-ME were higher than that of LZ-SE. But the polysaccharides cannot be comprehensively characterized due to limited detection conditions. In conclusion, LZ-ME and LZ-SE had differences in component types and relative contents of common components, but the 3 unique components of LZ-ME had small relative contents and a low differential contribution rate for the difference. Therefore, the difference in common component contents was the main source of the component differences between LZ-ME and LZ-SE.

To further clarify the main differential components of the 2 LZ standard decoctions and reveal the primary components responsible for the sleep-improving effects of LZ standard decoction. The 8 representative differential components between LZ-ME and LZ-SE (Fig. 4C) were screened based on the response values (higher than 20 000), as well as the top 8 differential components. From the Fig. 4C, the contents of spinosin, 6‴-feruloylspinosin, jujuboside A, jujuboside B, and (+)-isocorynoline in LZ-ME were significantly higher than those of LZ-SE. However, the contents of trifoside C, regaloside A, and regaloside B in LZ-ME were significantly lower than LZ-SE. It is reported that *P. cocos* can promote the dissolution of jujuboside A and spinosin in *Ziziphi Spinosae Semen*, and *Ziziphi Spinosae Semen* can promote the dissolution of triterpenoids in *P. cocos*[26]. These results indicated that mixing extraction can promote the dissolution and solubilization of active components in LZ.

3.3 Network pharmacology analysis

3.3.1 Intersection targets acquisition of compound targets and insomnia-related targets

The target of the 58 components identified by UPLC-QTOF-MS were retrieved from the database. However, 7 components did not retrieve any target proteins. After integrating all retrieved targets and removing duplicates, 676 targets were identified for 51 components, 125 flavonoids targets, 66 saponins targets, 217 phenylpropanoids targets, and 283 alkaloids targets were identified for 12 flavonoids, 7 saponins, 8 phenylpropanoids, and 5 alkaloid components. Additionally, 1 821 insomnia-related targets were obtained by searching the GeneCards, DisGeNET, and OMIM databases. Ultimately, the 125 flavonoids targets, 66 saponins targets, 217 phenylpropanoids targets, and 283 alkaloids targets were cross-referenced with 1 821 insomnia-related targets, respectively. A total of 117 intersection targets of all components, 26 intersection targets of flavonoids components, 18 intersection targets of saponins components, 36 intersection targets of phenylpropanoids components, and 58 intersection targets of alkaloids components were identified (Fig. 5A).

3.3.2 The PPI network construction and the core target analysis

All components intersection target PPI network containing 117 nodes and 1 031 edges were created by uploading all components intersection targets to the STRING platform. Additionally, a formula-drug-component-intersection target network map was constructed (Fig. 6A). The core targets of all components and the 4 kinds of components were selected, and the core targets PPI network are shown in Fig. 5B. According to Fig. 5B, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), TNF, IL-6, and AKT serine/threonine

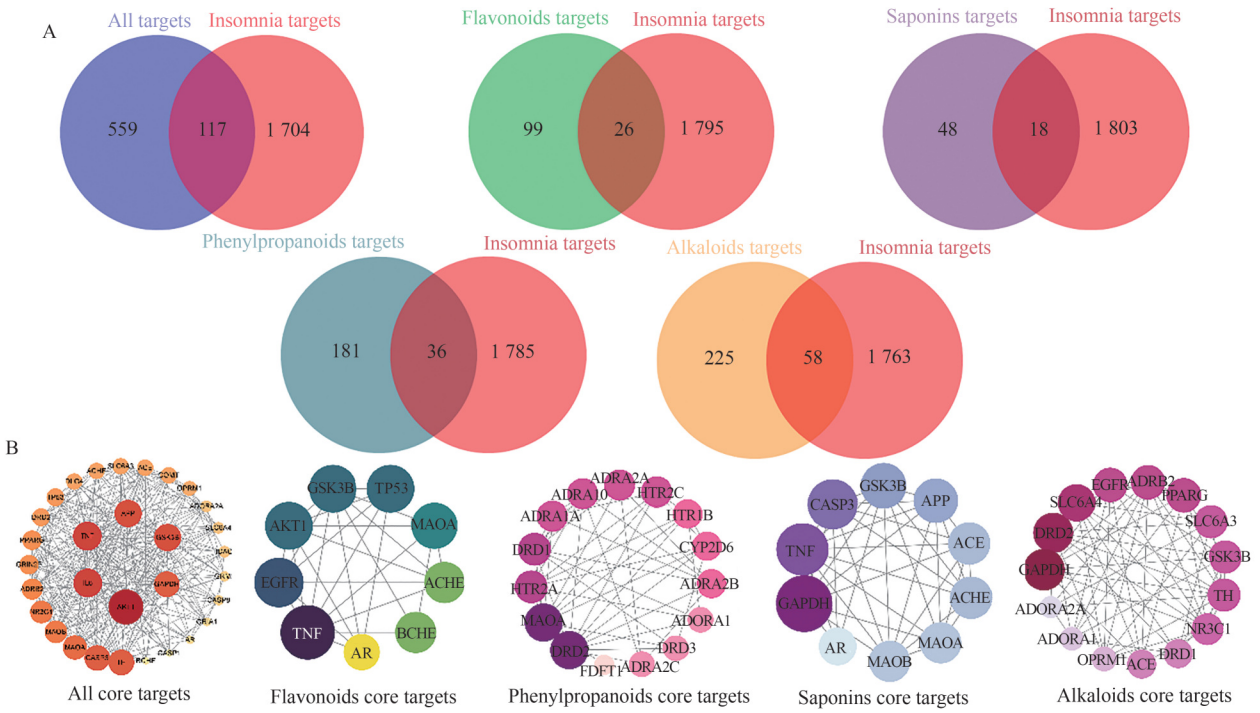

Fig. 5 Potential targets of LZ active compounds obtained by UPLC-QTOF-MS analysis. (A) The Venn diagram of the main active components in LZ and their potential anti-insomnia-related targets; (B) The PPI network and core targets of the main active components in LZ.

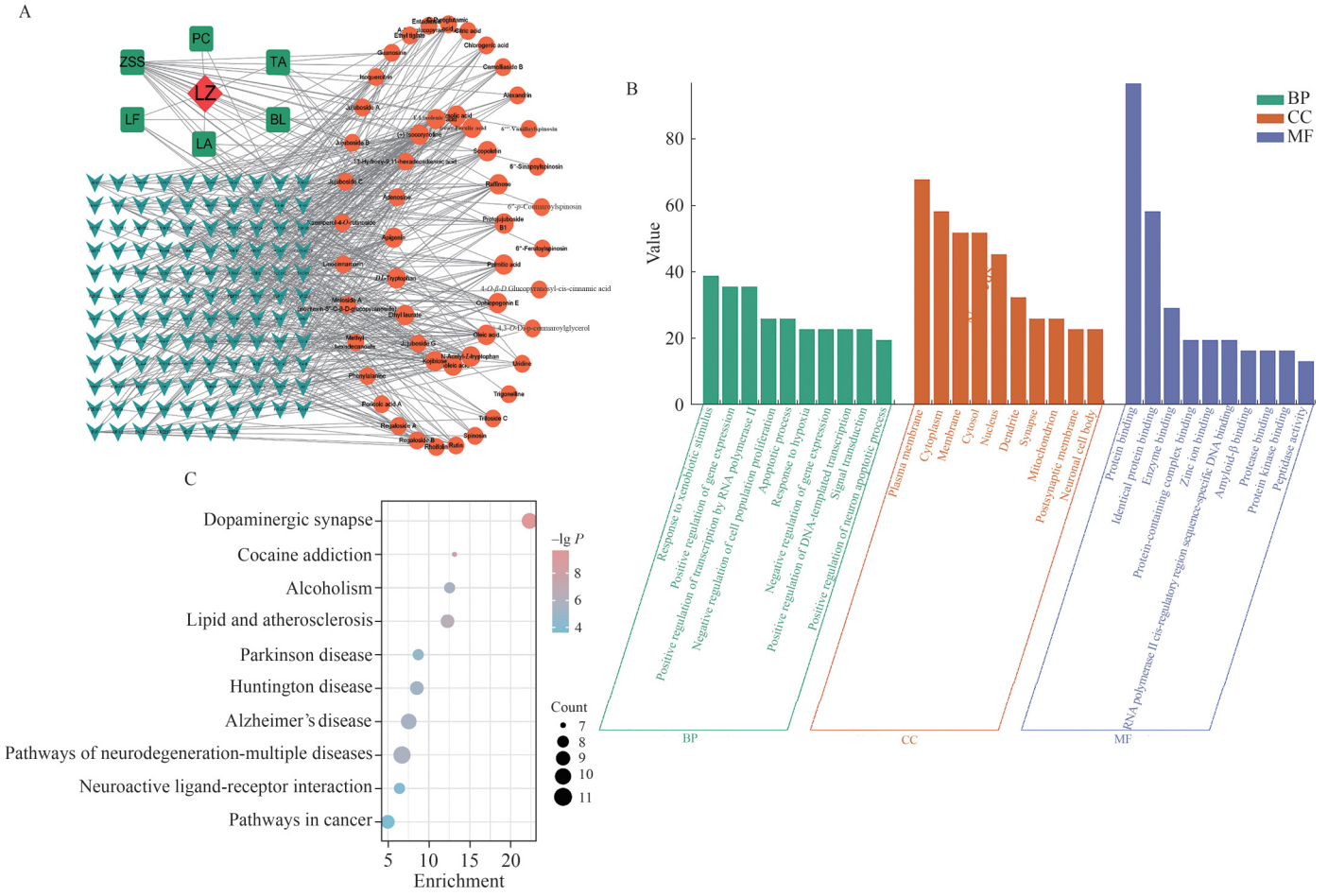

Fig. 6 Network pharmacology analysis result of LZ. (A) Formula-Drug-Component-Intersection target network map. Red represents the formula, green represents drugs of LZ, orange represents components involved in 6 drugs, blue represents the intersection target involved in components; (B) The GO enrichment result of core targets; (C) The KEGG enrichment result of core targets.

kinase 1 (AKT1) emerged as the highest-scoring targets of LZ standard decoction, suggesting that these proteins may play crucial roles in promoting sleep. Among them, GAPDH was the potential targets of saponins and alkaloid components. TNF was the potential targets of flavonoids and saponins components, and AKT1 was the potential targets of flavonoids, while phenylpropanoid components were not enriched on the four targets (Fig. 5B).

The core targets of all components and 4 kinds of components were introduced into the DAVID database for GO and KEGG analysis. The GO results showed that the targets of LZ standard decoction were mainly enriched in gene expression regulation, glutamatergic synapse, and protein kinase binding (Fig. 6B). KEGG enrichment results showed that the targets of LZ standard decoction were mainly enriched in dopaminergic signaling pathway, neuroactive ligand-receptor interaction, cAMP signaling pathway, TNF signaling pathway, and glutamatergic synapse, and the former 10 pathways are shown in Fig. 6C. And the results were similar with the study results of Bian et al.[27], TNF-α, IL-6, 5-HT, Glu, and DA may be potential targets for the sleep-improving effects of LZ standard decoction.

Notably, flavonoids, saponins, and alkaloid components were all enriched in glutamatergic synaptic and dopaminergic signaling pathways, while phenylpropanoid components were not enriched on either of these pathways. Combine with the former results of components content, flavonoids, saponins, and alkaloid components were speculated as the material basis of sleep-improving effect of LZ. Besides, AKT1 was a protein kinase, which overlapped with the protein kinase collection function in GO enrichment. In addition, the activation of PI3K/Akt signaling pathway could cause abnormal expression of inflammatory factors, such as TNF-α and IL-6. The targets mentioned above were all overlapping targets of the LZ active compounds that enriched in biological functions and pathways, which indicated that LZ might improve sleep by regulating these key proteins.

3.4 Mechanisms underlying the sleep-improving effects of LZ standard decoction

3.4.1 Effect of two LZ standard decoction and sodium pentobarbital on sleep duration

The pre-experiment determined that the hypnotic dose of sodium pentobarbital in ICR mice was 46 mg/kg, this dosage was used for subsequent formal experiments. As shown in Fig. 7, compared to the NC group ((20.10 ± 4.82) min), PT, LZ-ML, LZ-MM, LZ-MH, and LZ-SH groups treatment significantly extended the sleep duration

((45.45 ± 6.41), (36.26 ± 5.32), (37.50 ± 4.14), (68.25 ± 5.16), and (40.51 ± 2.75) min), respectively. No significant increase in sleep duration was observed in LZ-SE ((21.97 ± 4.47) min) and LZ-SM ((24.89 ± 3.12) min). It was also found that LZ-ME/LZ-SE treatment have a significant dose-effect relationship. At the same dose, the sleep prolonging effect of LZ-ME was significantly stronger than that of LZ-SE.

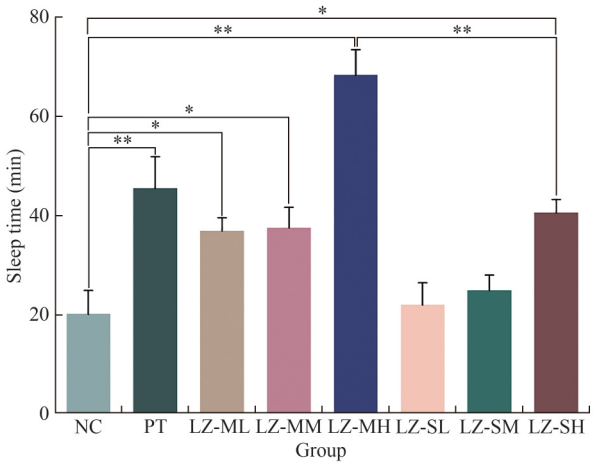

Fig. 7 Effect of 2 LZ standard decoction on sleep duration of sodium pentobarbital pretreated mice ($n = 6$). *$P < 0.05$, **$P < 0.01$.

The UPLC-QTOF-MS components analysis revealed that saponin and flavonoid might be the main sleep-improving components of LZ standard decoction. Besides, the high dose LZ-SE significantly prolonged the sleep duration of sodium pentobarbital in experimental mice, but the low dose group was not significant. Combine with the UPLC-QTOF-MS component analysis results, that alexandrin, chlorogenic acid, and scopoletin were the unique components of LZ-ME, if the 3 unique components were the main components for LZ to improving sleep, the three dose of LZ-SE had no effects on sleep-improving. It was not speculated that the 3 unique components were essential components for LZ to improve sleep. The experimental results not only verified the sleep-improving effects of LZ standard decoction, but also confirmed the that sleep-improving effects of LZ-ME is better than LZ-SE. Besides, mixed extraction is a practical method for LZ standard decoction preparation. Therefore, the subsequent mechanistic experiment would investigate the effects of LZ standard decoction (LZ-ME) on inflammatory factors, neurotransmitters and gut microbes.

3.4.2 *Effect of LZ-ME on serum inflammatory factors*

The results of LZ-ME on serum inflammatory factor contents in each group are shown in Fig. 8. According to Figs. 8A and B, compared to the NC group, only the IL-6 content of LZ-MM group was significantly increased, and there was no significant dose-effect relationship between LZ-ME and IL-1β or IL-6. But according to Fig. 8C, compared to the NC group, all the TNF-α content of LZ-ML, LZ-MM, and LZ-MH groups were dose-dependently increased ($P < 0.01$). Based on the above results, it was believed that elevated TNF-α levels may be one of the mechanisms by which LZ standard decoction exerts sleep-improving effects. Additionally, the experimental findings were consistent with the results obtained by network pharmacology. It was speculated that flavonoids components were mainly responsible for the TNF-α regulating effects of LZ standard decoction.

3.4.3 *Effect of LZ-ME on hippocampal neurotransmitters*

The hippocampal neurotransmitters content in each group are shown in Fig. 9. According to Fig. 9A, compared to the NC group, PT treatment significantly increased the 5-HT content ($P < 0.01$), this is because exogenous melatonin supplementation can increase the 5-HT concentration in the brain[28]. Compared to the NC group, only the content of 5-HT in LZ-MH group was significantly increased ($P < 0.01$). According to Fig. 9B, compared with the NC group, both the GABA content of LZ-MM and LZ-MH groups were significantly increased ($P < 0.01$), the GABA content of LZ-ML group was not significantly different, and the administered dose was positively correlated with GABA content. According to Figs. 9C and D, compared with the NC group, all the Glu and DA content in LZ-ML, LZ-MM, and LZ-MH groups were significantly decreased ($P < 0.01$), but there was no dose-effect relationship. The medium and high dose of LZ-ME dose-dependently increased GABA levels ($P < 0.01$). Furthermore, 3 doses of LZ-ME consistently reduced the levels of Glu and DA in the hippocampus. In conclusion, LZ-ME may exert sleep-improving activity *via* increasing GABA and decreasing Glu and DA levels. Based on the results of network pharmacology, flavonoids, saponins, and alkaloid components may contribute to the neurotransmitters-regulating role of LZ-ME decoction.

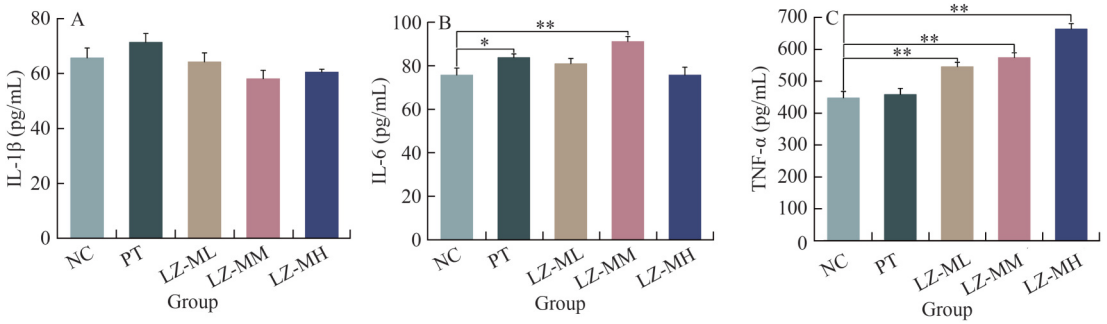

Fig. 8 Content of cell cytokines in mice of each group ($n = 6$). (A) Serum IL-1β content of mice in each group; (B) Serum IL-6 content of mice in each group; (C) Serum TNF-α content of mice in each group. *$P < 0.05$, **$P < 0.01$ *vs*. the NC group.

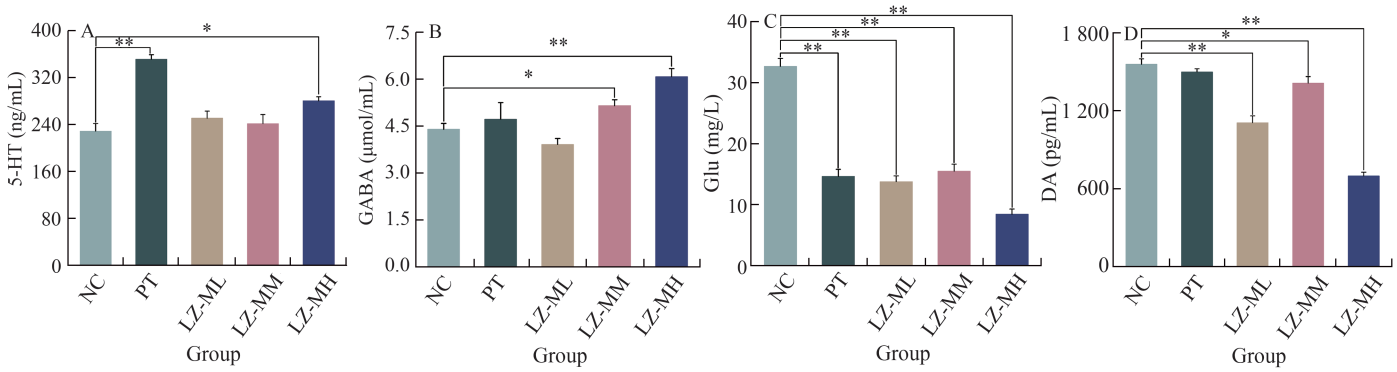

Fig. 9 Content of neurotransmitters in mice of each group ($n = 6$). (A) 5-HT content in the hippocampus of each group; (B) GABA content in the hippocampus of each group; (C) Glu content in the hippocampus of each group; (D) DA content in the hippocampus of each group. *$P < 0.05$, **$P < 0.01$ *vs*. the NC group.

3.4.4 Effect of LZ-ME on gut microbiota of the experimental mice

3.4.4.1 β-Diversity analysis results

β-Diversity refers to differences in species composition between groups. As shown in Fig. 10A, the proportions of PCo1 and PCo2 were 44.1% and 15.4%, respectively. Nonmetric multidimensional scale (NMDS) analysis showed that there was no significant differences in gut microbiota among the NC, PT, and LZ-MH groups (Fig. 10B). Both the median line of PT and LZ-MH groups were higher than NC group (Fig. 10C), although there was no significant differences.

3.4.4.2 Species composition analysis at the phylum and genus levels

Gut microbiota species composition analysis of experimental mice in the NC, PT, and LZ-MH groups are shown in Fig. 11. Compared to the NC group, PT and LZ-MH treatment altered the gut microbiota species in experimental mice. Fig. 11A shows the species composition Firmicutes and Bacteroidetes are the dominant bacteria at the phylum level. Compared to the NC group, both PT and LZ-MH treatment decreased the relative abundance of Firmicutes and increased the relative abundance of Bacteroidetes, although no statistically significant differences were observed among 3 groups. It has been demonstrated that continuous light caused the disturbance of human sleep-wake cycle, decreased the richness of Bacteroidetes and increased the richness of Firmicutes. The decreased of Bacteroidetes may be a biomarker for insomnia[29-30]. Therefore, LZ-MH may promote sleep by regulating the relative abundance of Firmicutes and Bacteroidetes in mice.

Fig. 11B shows the relative abundances of the top 10 dominant bacteria in mice at genus level. Compared to the NC group, LZ-MH treatment upregulated the relative abundance of *Muribaculum, Duncaniella*, *Desulfovibrio*_R, and *Lactobacillus*. Among them, *Muribaculum*, *Duncaniella*, and *Lactobacillus* are probiotics, which mainly involved in the metabolism of carbohydrates and vitamins[31], *Lactobacillus* has multiple physiological functions[32]. As an opportunistic pathogen, *Desulfovibrio*_R and *Faecalibacterium prausnitzii* often coexist, both of them contribute to the production of butyric acid and help to prevent diseases caused by butyric acid deficiency[33]. Compared to the NC group, the LZ-MH downregulated the relative abundance of Lachnospiraceae_UBA3283, *Alistipes*_A, and Lachnospiraceae_14-2. Lachnospiraceae is an opportunistic pathogen, it can damage glucose metabolism and cause inflammation, and promote the occurrence and development of diabetes, Crohn's disease and depression[34]. *Alistipes* is a harmful bacteria, it can aggravate livers fibrosis, colorectal cancer and cardiovascular disease, and promote the occurrence of diseases such as depression and obesity[35].

In conclusion, LZ-MH promoted the growth of probiotics and inhibited the growth of harmful bacterium or opportunistic pathogens.

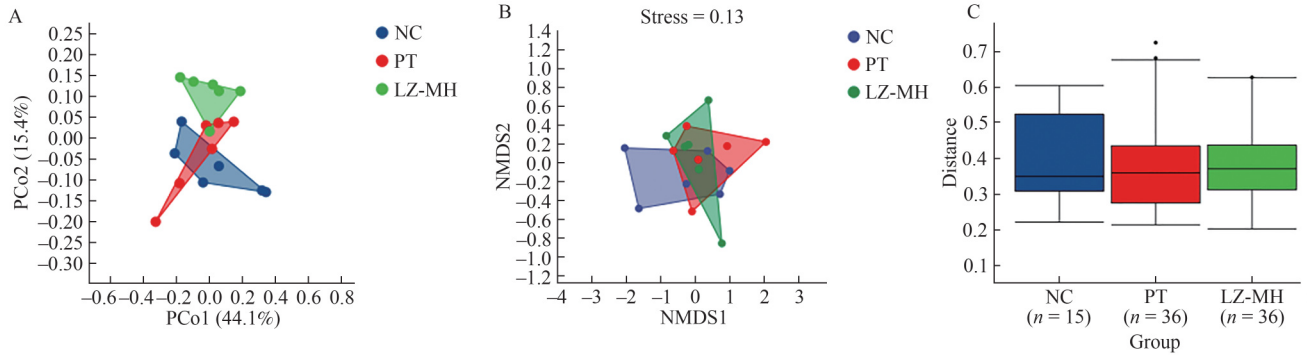

Fig. 10 Comparison of gut microbiota in NC, PT and LZ-MH groups ($n = 6$). (A) The primary coordinate analysis (PCoA) graph; (B) The NMDS graph; (C) The boxplot for difference analysis between groups, *n* refers to the times of sample comparisons.

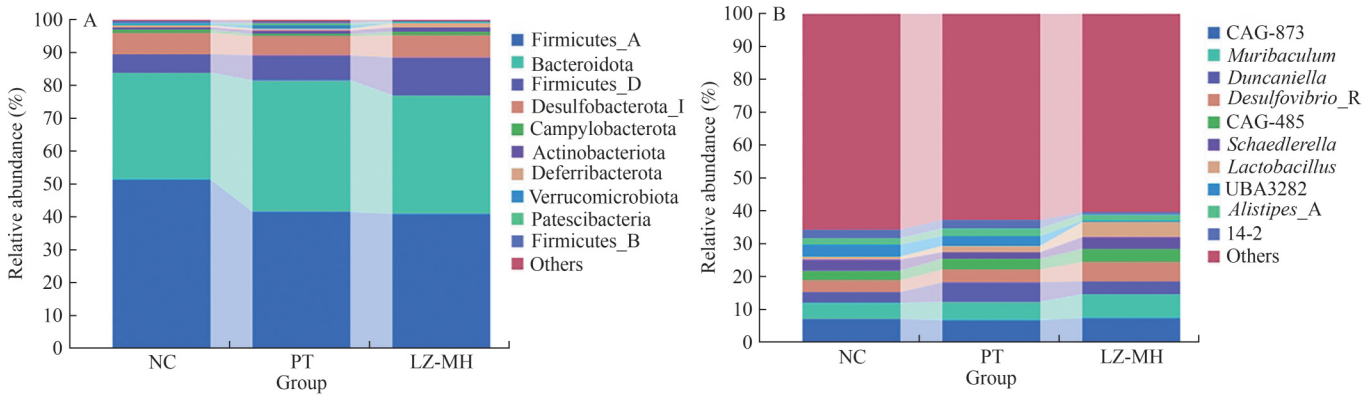

Fig. 11 Composition of gut microbiota species in NC, PT, and LZ-MH groups. (A) The histogram of gut microbiota composition at phylum level in different groups; (B) The histogram of genus level species composition.

The impact of LZ-MH on gut microbiota was not significantly different, this may because in normal mice, the gut flora is in a homeostasis state, LZ-MH may exhibit a strong microbiota-regulating role in pathological states in mice.

3.4.4.3 Analysis of the differential species of gut microbiota between LZ-ME and NC groups

Venn diagrams analysis, linear discriminant analysis (LDA) effect size (LEfSe) analysis and random forest analysis can be used to find differential species of gut microbiota in each group. Fig. 12A is the amplicon sequence variants (ASV) Venn figure of common or exclusive species between the NC and LZ-MH groups. Both sets of samples yielded 8 418 ASV, including 5 540 and 4 492 ASV counts for NC and LZ-MH groups, respectively. A total of 1 614 ASV counts were shared, 3 926 ASV were unique to NC, and 2 878 ASV unique to LZ-MH.

LEfSe analysis results (Fig. 12B) shows that there are 3 characterized microorganisms at gene and species levels among each group, respectively (LDA threshold greater than 4). At the gene level, *Mailhella* was the dominant genus in the NC group, and its abundance was significantly higher than LZ-MH group (Fig. 12C); *Lactobacillus* and *Eubacterium*_R were the dominant genus in the LZ-MH group, and their abundance were significantly higher than the NC group. At the species level, *Mailhella_massiliensis* was the dominant species of the NC group, its abundance was significantly higher than the LZ-MH group; *Eubacterium*_R_sp000436835 and *Lactobacillus_johnsonii* were the dominant species of the LZ-MH group, and their abundance were significantly higher than the NC group.

The results of the random forest analysis are shown in Fig. 13A (genus level) and Fig. 13B (species level). According to LEfSe analysis, two of the three landmark genus/species had certain influence values on the random forest analysis, and all of them were landmark genus in LZ-MH group. These findings indicated that the landmark genera, *Lactobacillus* and *Eubacterium*_R, as well as the landmark species *Eubacterium*_R_sp000436835 and *Lactobacillus_johnsonii*, exhibited significant differences between NC and LZ-MH groups. Both *Lactobacillus* and *Eubacterium*_R were recognized probiotics that play crucial roles in nutrient metabolism and maintaining intestinal balance. This suggested that LZ-ME may improve sleep by increasing the relative abundance of *Lactobacillus* and *Eubacterium*_R.

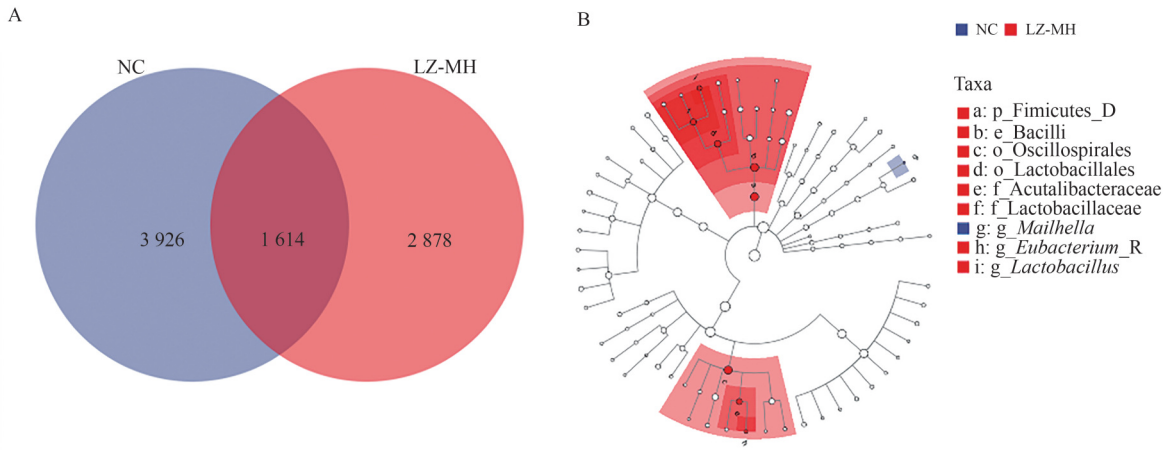

Fig. 12 Differentia gene or species between LZ-ME and NC group (n = 6). (A) ASV Venn figure of the 2 samples; (B) Results of LEfSe analysis, the current LDA threshold is 4; (C) Species difference of landmark gene/species level abundance.

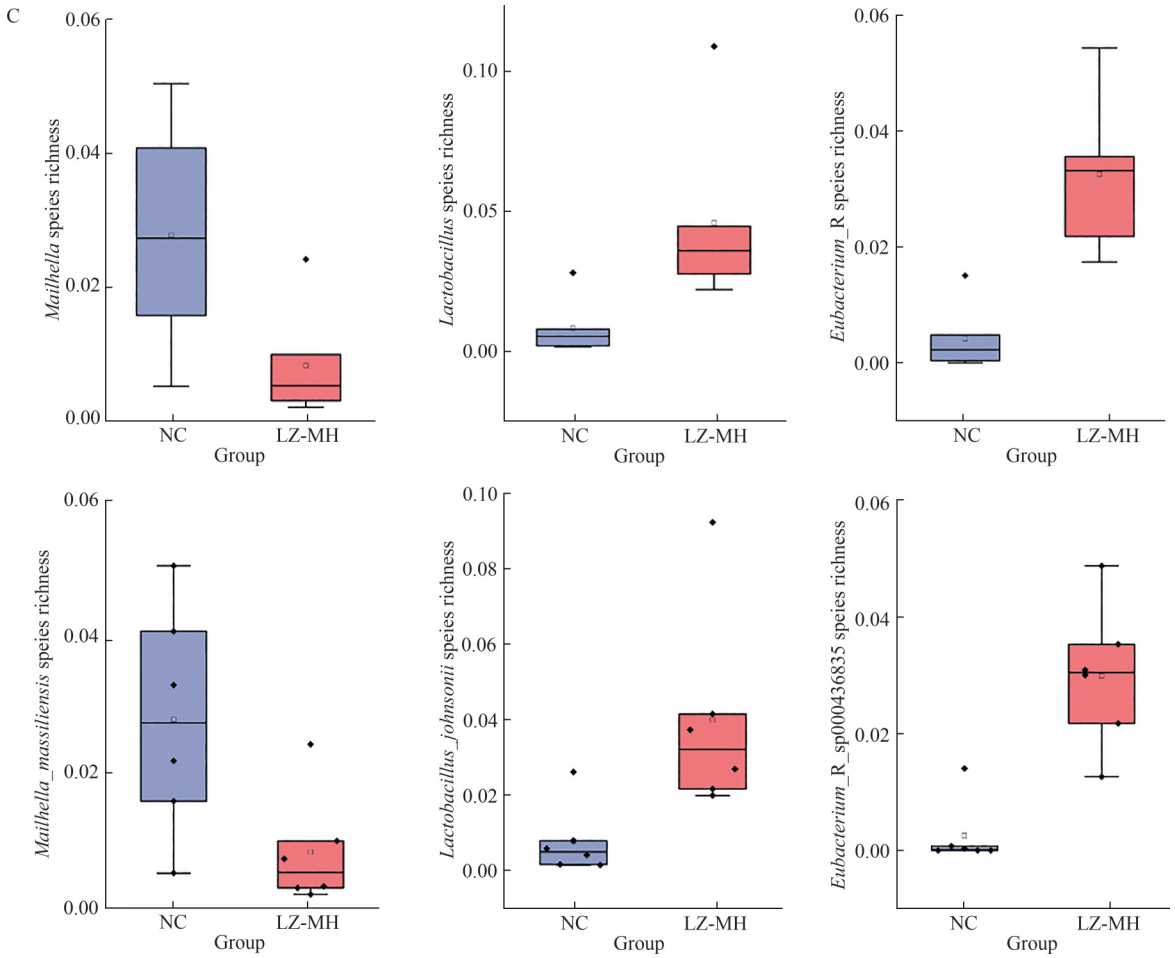

Fig. 12 (*Continued*)

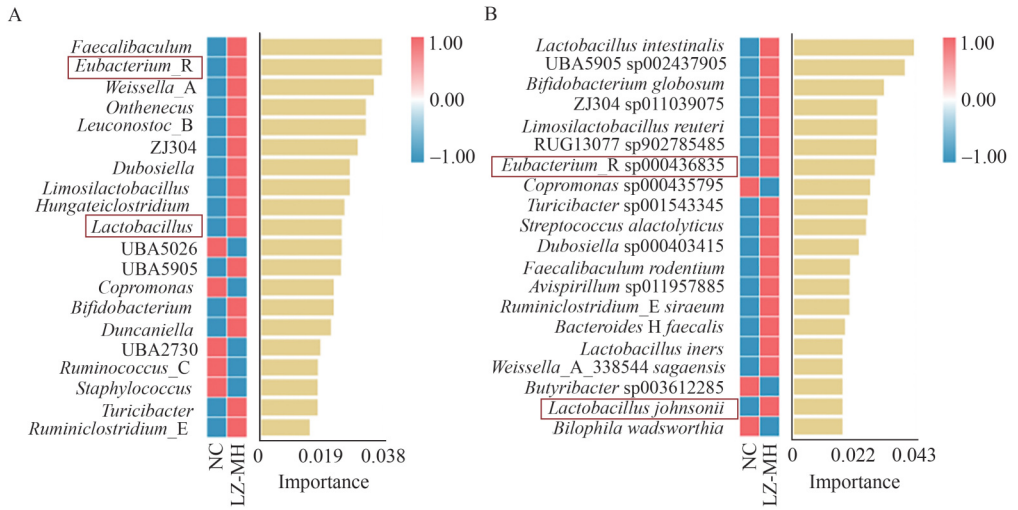

Fig. 13 Result of gene or species random forest analysis between LZ-ME and NC group (n = 6). (A) Results of gene level random forest analysis; (B) Species level random forest analysis.

4. Discusion

LZ is a traditional formula from "*The Essentials of the Golden Chamber*". Historically, LZ is well-known for its sleep-improving effects. However, its efficacy and underlying mechanisms have not been confirmed. This study for the first time evaluated the sleep-improving effects of LZ according to the criteria outlined in the "*Technical Specification for Inspection and Evaluation of Health Food*" (2023, China). Besides, the material basis and underlying mechanisms of the sleep-improving effects in LZ were also investigated.

Our results indicated that LZ-ME did not affect the growth and organs indexes of normal mice, LZ-ME had no direct effect on sleep,

high dose LZ-ME could significantly increase the hypnotic rate of sub-threshold sodium pentobarbital dose-induced. Medium and high dose LZ-ME could significantly prolong the sleep duration of sodium pentobarbital induced and reduce the sleep latency of sodium barbiturate induced. According to the criteria in the "*Technical Specification for Inspection and Evaluation of Health Food*" (2023, China), it was confirmed that LZ-ME had the sleep-improving function.

The preparation of TCM formula decoction is a complex process. The components of herbal decoction may undergo salt formation, molecular rearrangement, and hydrolysis under the boiling process. These transformations influence the dissolution efficiency of the components in herbs. Many scientific studies reported that different extraction methods result to the variation of the types and content of chemical components in herbal decoction. This study showed that mixed extraction herbs in LZ prescription leads to production of a higher active compounds content including total polysaccharides, total flavonoids, and total saponins than single extraction method. Previous study had found that the kind of chemical components in White Tiger decoction[36] obtained by mixed and single extraction were the same, but the content of saponin showed obvious differences, which was manifested by the higher content of new mangiferin was observed in the decoction of mixed extraction. It indicated that the dissolution of new mangiferin was promoted when Gypsum Fibrosum and Anemarrhenae Rhizoma were extracted together at a specific compatibility ratio. While the higher content of mangiferin and glycyriin was found in the decoction obtained by single extraction, indicating thatextracted methods should be chosen according to the clinical application purpose. Besides, Shang el al.[18] evaluated the chemical consistency between traditional and dispensing granule decoctions of Tao-Hong-Si-Wu decoction, the results showed that there was obvious difference in components between the 2 preparing methods following the same dosage ratio. The pharmacological function of TCM formulas is closely related to their chemical components, and different extraction methods affect the type and content of the chemical composition in TCM formula. Deng et al.[37] studied different extraction methods on the chemical composition and anti-cataract effects of Buqing Decoction. The results showed that compared to the single extraction, the mixed extraction group significantly reduced the degree of lens turbidity, slowed down the turbidity rate, and exhibited higher efficacy. In this study, the 2 LZ standard decoctions prepared using mixed and single extraction methods showed significant differences in components and sleep improvement effects, which provided new evidence for the scientific basis of mixed decoction.

The UPLC-QTOF-MS analysis results proved that the total response contents of flavonoid and saponin components in LZ-ME were significantly higher than that of LZ-SE, and the total response content of phenylpropanoid components was significantly lower than that of LZ-SE. Previous study showed that the saponin components in *P. cocos* and *Ziziphi Spinosae Semens* dissolved each other due to similar dissolution, and polysaccharides could combined with least water-soluble flavonoids to form spongy compounds, which in turn increased the solubility and stability of flavonoids in the solvent, such as quercetin and baicalein[38]. Besides, it is possible that the acidity of some polysaccharide solutions can reduce the pH of the degradation buffer to a certain extent, thus promoting the stability of the flavonoids[39]. By comparing the components and the sleep-promoting effects of the 2 LZ standard decoction, spinosin, 6‴-feruloylspinosin, jujuboside A, and jujuboside B were found to be the main active compounds responsible for the sleep-promoting effects of LZ. It has been shown that spinosin and 6‴-feruloylspinosin affected sleep improvement effects by regulating GABA levels[40-41]. Sapogenin generated by the hydrolysis of jujuboside A can cross the blood-brain barrier to promote the combination of GABA with its receptors involved in sedation and hypnosis. Sapogenin exerts its sedative and hypnotic effects by promoting the mRNA expression of GABA receptor subunits (α1, α5 and β2) in the hippocampal and downregulated the secretion of related inflammatory factors in the intestinal mucosal system[42-44]. Jujuboside B can increase the expression of GABA receptors and Cl^- influx and exert the sedative

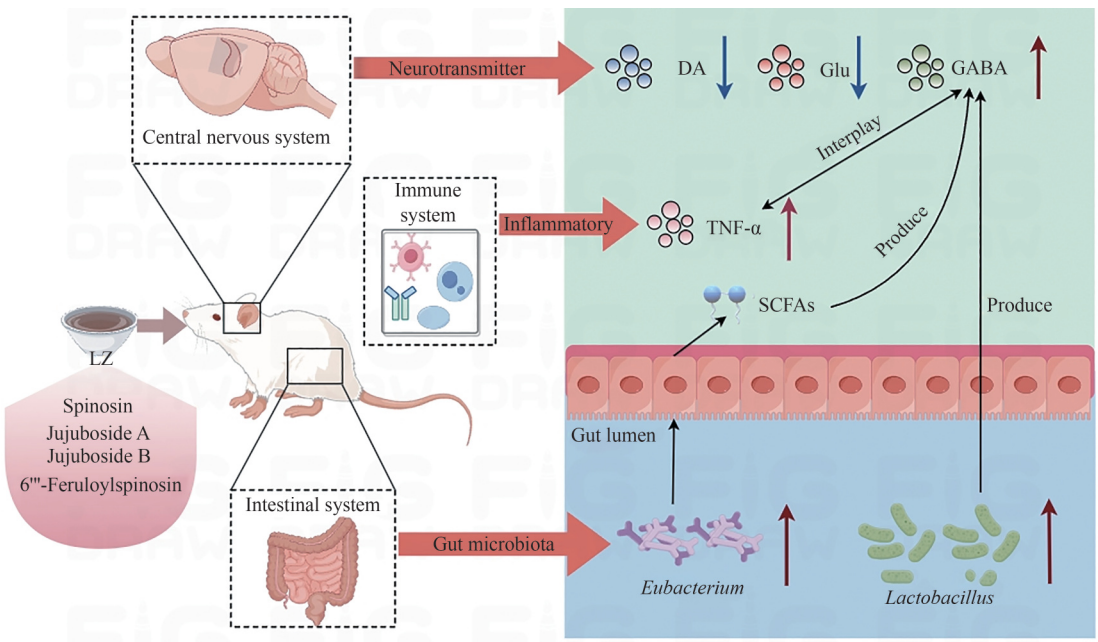

Fig. 14 Mechanism diagram of the sleep-improving effects of LZ.

and hypnotic effect[45]. Currently, there was little information on the sleep-improving activity of trigonelline and (+)-isocorynoline. It is demonstrated that sanjoinine A in *Ziziphi Spinosae Semen* has sedative-hypnotic activity, but it was not identified in LZ decoction because of its low content. It was speculated that alkaloid might be the components for sleep-improving function of LZ decoction, but that need more deeply study.

Network pharmacology is a modern analytical method for revealing the mechanisms of drug-disease interactions from the perspective of the overall biological network[46]. This study used network pharmacology technology to analyze the potential targets of 58 components in LZ standard decoction identified by UPLC-QTOF-MS. Four key targets including GAPDH, TNF, IL-6, and AKT1, glutamatergic synapse and dopaminergic signaling pathways were found to be targets of active compounds in LZ decoction. These findings provided theoretical supports and reliable directions for subsequent mechanistic research. Further animal study showed that LZ-ME improved the TNF-α and GABA levels, reduced Glu and DA levels in mice to improve sleep. It was consistent with the results reported by Liu et al.[47], which also reported that anti-insomnia effects of dodder extract achieved by regulating GABA, Glu, and DA levels in central nervous system. GABA is the main inhibitory neurotransmitter in the central nervous system, while the DA and Glu are excitatory neurotransmitters. It is well known that increasing GABA content and decreasing DA and Glu levels are helpful for sleeping[48]. The secretion levels of TNF-α exhibits diurnal variations. By modulating the neuroendocrine system and promoting nerve cell growth in the brain, TNF-α plays an important role in regulating sleep and the human bioclock[49-50]. Additionally, due to neural-immune interactions, the secretion of inflammatory factors is regulated by neurotransmitters such as GABA. High-dose GABA intake promote the synthesis of TNF-α in the immune organs of tilapia, leading to an increase in serum concentrations of TNF-α[51]. In this study, both TNF-α and GABA levels were significantly increased in a dose-response relationship after LZ-ME treatment, suggesting that LZ may increasing GABA levels by promoting TNF-α production, subsequently contributing to improved sleep quality.

Sleep and gut microbiota exhibit complex interactions. Gut microbiota and their metabolites reaching the central nervous system through the "gut-brain axis," and influencing the production of neurotransmitters and inflammatory factors. They also pass through the intestinal epithelial barrier into the circulatory system, affecting the sleep-wake cycle[52-53]. This study revealed that LZ may affects neurotransmitter levels by increasing the abundance of specific beneficial bacteria, including *Lactobacillus* and *Eubacterium*. *Lactobacillus* is GABA-producing microorganisms, which played a role in modulating neural activity. While *Eubacterium* is one of the important butyrate-producing members, it can generate butyric acid. And butyric acid as one of short chain fatty acids (SCFAs), it may stimulate enteroendocrine cells to produce neurotransmitters such as GABA[54-55]. These neurotransmitters influenced sleep behavior through neural pathways, blood circulation, and immune responses. Gut microbial metabolites such as short-chain fatty acids, secondary bile acids and indoles have an important role in modulating somatic functions. In this study, LZ-ME administration up-regulated the abundance of the *Lactobacillus* and *Eubacterium*_R, and down-regulated the abundance of the Lachnospiraceae, etc. It is rational for us to speculate that these significantly changed microbiota may change the SCFAs, bile acids and indoles in mice intestine to regulating mice sleep. Therefore, it is great importance to study the role of LZ-ME significantly changed gut microbial metabolites on mice sleeping using nude mouse. Besides, there are some limitations of this paper. Firstly, the sleep-improving effect of LZ was studied in normal mice, some important indicates like gut microbiota showed little significant difference between the normal group and treatment groups. Moreover, although we have proved the sleep-improving effect of LZ is contributed to its regulating role on levels of neurotransmitter and inflammatory factors, such as GABA, TNF, and IL-6, further studies still needed to uncovering the related signaling pathways and the key targets.

5. Conclusion

This study proved that LZ had sleep-improving effect. It also proved the ancient wisdom in TCM that co-extracting herbsin LZ prescription results to production of a higher active compounds content, including total polysaccharides, total flavonoids, saponins, and total alkaloids. The active compounds of LZ such as spinosin, 6‴-feruloylspinosin, jujuboside A, and jujuboside B improve the sleep of mice by targeting GAPDH, TNF, IL-6, AKT1, and glutamatergic synapse, dopaminergic signaling pathways. LZ-ME administration increased TNF-α in mice serum, and neurotransmitter GABA levels in mice hippocampus, meantime significantly decreased DA and Glu levels in mice hippocampus. Additionally, LZ-ME also up-regulated the abundance of the beneficial bacteria *Lactobacillus* and *Eubacterium*_R, down-regulated the abundance of the harmful bacteria Lachnospiraceae in mice intestine. Both of these microbes play vital role in regulating neurotransmitters in brain and thus affecting sleep quality.

Conflict of interest

The authors declare there is no conflict of interest.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2025.9250493.

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