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Beneficial effect of GABA-rich fermented milk on insomnia involving regulation of gut microbiota

Leilei Yu[a,b,d,1], Xiao Han[a,b,1], Shi Cen[a,b], Hui Duan[a,b], Saisai Feng[a,b], Yuzheng Xue[f,**], Fengwei Tian[a,b,d,*], Jianxin Zhao[a,b,c], Hao Zhang[a,b,c,e], Qixiao Zhai[a,b,d], Wei Chen[a,b,c,g]

[a] State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China
[b] School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu 214122, China
[c] National Engineering Research Center for Functional Food, Jiangnan University, Wuxi, Jiangsu 214122, China
[d] International Joint Research Laboratory for Probiotics at Jiangnan University, Wuxi, Jiangsu 214122, China
[e] (Yangzhou) Institute of Food Biotechnology, Jiangnan University, Yangzhou 225004, China
[f] Department of Gastroenterology, Affiliated Hospital of Jiangnan University, Wuxi, Jiangsu, China
[g] Beijing Innovation Centre of Food Nutrition and Human Health, Beijing Technology & Business University, Beijing 100048, China

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ABSTRACT

Insomnia is a common health problem in modern societies. GABA, an inhibitory neurotransmitter, can promote relaxation and reduce anxiety. In this study, milk was fermented with *Lactobacillus brevis* DL1-11, a strain with high GABA-producing capacity. The potential beneficial effects of this fermented milk on anxiety and sleep quality were evaluated in animal experiments. Sixty mice were divided into control, non-GABA fermented milk (NGFM), low-dose GABA fermented milk (LGFM, 8.83 mg/kg.bw), medium-dose GABA fermented milk (MGFM, 16.67 mg/kg.bw), high-dose GABA fermented milk (HGFM, 33.33 mg/kg.bw) and diazepam groups. The results of open field test and elevated plus-maze test indicated decreases in anxiety behavior after oral HGFM administration. Moreover, mice in the HGFM group exhibited a significantly prolonged sleep time after an intraperitoneal injection of sodium pentobarbital and a shortened sleep latency after an intraperitoneal injection of sodium barbital. These results indicate a beneficial effect of HGFM on sleep. Additionally, significant increases in the relative abundances of *Ruminococcus*, *Adlercreutzia* and *Allobaculum* and the levels of some short-chain fatty acids (SCFAs), such as butyric acid, were observed in the HGFM group. The results suggest that GABA-fermented milk may improve sleep and the protective pathways may involve in regulation of gut microbiota and increase of SCFAs level.

1. Introduction

Insomnia, which has negative impacts on the quality of life, mood, cognitive function and health of humans, has attracted increasing research attention. Approximately 10–15 % of people worldwide suffer from insomnia, and approximately 25–35 % have transient or occasional insomnia. These cases are attributed to increases in life pressures and the effects of various diseases (Souza Lopes et al., 2012). Insomnia can lead to decreased immunity (Irwin, 2002), cognitive impairment (Fortier-Brochu and Morin, 2014), depression (Cunnington et al., 2013) and increased risks of cardiovascular diseases (Khan and Aouad, 2017). Drugs including benzodiazepines and non-benzodiazepines are widely used to alleviate insomnia. However, these drugs have various side effects, and users can develop tolerance and dependence with long-term use (Cunnington et al., 2013). Therefore, a safer and more effective way to relieve insomnia is needed.

GABA is an inhibitory neurotransmitter that plays important physiological roles in humans, including reducing neuronal activity, regulating the heart rate, enhancing memory and regulating hormone secretion (Tong et al., 2020; Nakamura et al., 2009; Shang-Feng and Ai-Min, 2011). A recent study revealed that GABA extracted from fermented rice germ can normalize caffeine-induced sleep disorders in mice (Mabunga et al., 2015). In a randomized double-blind experiment, Byun et al. (2018) observed that a fermented rice germ extract containing GABA reduced the sleep latency and improved the sleep efficiency of insomnia patients. Another study demonstrated a significant

* Corresponding author at: State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China.
** Corresponding author.
E-mail addresses: xueyz001@163.com (Y. Xue), fwtian@jiangnan.edu.cn (F. Tian).
[1] These authors contributed equally to this study.

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increase in alpha wave activity and decrease in beta wave activity in the brains of volunteers for 60 min after the intake of 100 mg GABA, suggesting that this neurotransmitter can promote relaxation and reduce anxiety (Abdou et al., 2010). Orally administered GABA, which exhibited a blood peak after 30 min, was shown to shorten the sleep latency significantly and increase the non-REM sleep time in subjects (Yamatsu et al., 2016).

Many researchers have studied the role of the microbiota and its metabolites in health and disease. The gut microbiota affects many important host functions and is associated with a variety of neuropsychiatric disorders, including anxiety, depression (Lach et al., 2018), autism spectrum disorder and mood disorders (Mangiola et al., 2016). Interestingly, GABA is a growth factor for some bacteria and can thus modulate the gut microbiota. For example, KLE1738, a Gram-positive bacterium of the *Ruminococcaceae* family, uses GABA as a growth-supporting nutrient, and a genome analysis supported a GABA-dependent metabolism (Strandwitz et al., 2019). The effect of GABA on the gut microbiota might also contribute to improved sleep.

Lactic acid bacteria (LAB) are generally recognized as safe and are used in the fields of food, medicine and animal feed (Champagne et al., 2018; Ranadheera et al., 2018). LAB, including *Lactobacillus* spp., are considered the main producers of GABA (Wu et al., 2017). The most commonly reported *Lactobacillus* with GABA-producing ability include *Lactobacillus brevis* (Lim et al., 2017; Wang et al., 2018) and *Lactobacillus plantarum* (Shan et al., 2015; Ribeiro et al., 2018). Moreover, milk has long been consumed to promote sleep (dela Pena et al., 2015), and fermented milk, a nutritious and healthy food product (Barat and Ozcan, 2018; Temiz and Çakmak, 2018), is an ideal functional carrier of GABA. Therefore, in addition to GABA, GABA-rich fermented milk contains both *Lactobacillus* and milk with potential health effects, which is better than using extracted GABA alone. Previous studies mainly focused on optimizing the fermentation conditions required to produce GABA-rich fermented milk. In contrast, the association of GABA-rich fermented milk consumption with sleep improvements and gut microbiota regulation has not been studied. Therefore, this study aimed to screen high GABA-producing *Lactobacillus* strains from fermented milk and to investigate whether GABA-rich fermented milk produced by these strains would effectively improve sleep. The study provides new insights into the relationship between these sleep effects and the gut microbiota, as well as a theoretical basis for the application and development of GABA-producing probiotics.

2. Materials and methods

2.1. Preparation of GABA-rich fermented milk

Six *Lactobacillus brevis* strains WX7-1, WX7-3, WX17-3, HY2-1, DL1-11, RS6-12 and eight *Lactobacillus plantarum* strains DP1-12, HY6-2, HY8-2, HY9-1, DL2-1, DL3-1, DL5-1, 16-8-G-3 were derived from Culture Collection of Food Microorganisms of Jiangnan University (Wuxi, China), which isolated from traditional Chinese fermented food pao cai or the feces of healthy adults (Cherdyntseva et al., 2015). The strains were inoculated into 11 % skim milk containing 5 % sucrose and 1.5 % sodium glutamate with a 4:1:1 ratio of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. A total of 6×10^7 colony-forming units (CFU)/mL were inoculated. After fermentation at 37 °C for 7 h, the culture was stored at 4 °C for 12 h. The GABA content in the fermented milk was determined using high-performance liquid chromatography (HPLC; UltiMate™ 3000, Thermo, USA). A Hypersil GOLD column (100 mm × 2.1 mm) was used to separate the samples. The injection volume was 5 μL, and the flow rate was 0.2 mL/min. Sample absorbance was detected at a wavelength of 338 nm. Mobile phase A comprised 20 mmol/L sodium acetate solution, while mobile phase B comprised a mixture of 40 mmol/L sodium acetate solution and acetonitrile at 1:1 (v/v). The following gradient elution was set using mobile phases A and B: 0–6 min, increase in B from 30 % to 50 %; 6 − 11 min, increase in B from 50 % to 60 %; 11–12 min, increase in B from 60 % to 100 % and hold for 3 min; 15–16 min, decreased in B from 100 % to 30 % and 16–20 min, 30 % B for 4 min.

2.2. Animals and experimental design

Six-week-old male ICR mice with body weights (bw) of 25–30 g were obtained from the Shanghai Laboratory Animal Center (Shanghai, China). Sixty mice were divided randomly into six groups for treatment: control, non-GABA fermented milk (NGFM), low-dose GABA fermented milk (LGFM), medium-dose GABA fermented milk (MGFM), high-dose GABA fermented milk (HGFM) and diazepam. Mice were housed in cages in a temperature- (23 ± 3 °C) and humidity-controlled (55 ± 15 %) room equipped with a 12-h light/dark cycle. After a week of adaptation, mice in the control group were administered sterile saline daily. Mice in the NGFM group received fermented milk without GABA, which was produced by only *L. bulgaricus* and *S. thermophilus* and then adding with 10^8 CFU/mL *L. brevis* DL1-11. The number of *L. brevis* DL1-11 in the GABA-rich fermented milk is 10^8 CFU/mL, so this dose was added in the NGFM group to keep the same with other fermented milk groups containing GABA. Mice in the LGFM, MGFM and HGFM groups received once-daily oral doses of GABA-fermented milk (8.83, 16.67 and 33.33 mg/kg.bw, respectively). Mice in the diazepam group were administered diazepam solution (3 mg/kg.bw) as a positive control. All treatments were administered intragastrically via gavage once per day for 30 consecutive days. All experiments were performed between 8:00 and 16:00. Experimental animals were sacrificed using carbon dioxide at the end of the experiment. All of the study protocols were approved by the Ethics Committee of Jiangnan University, China (approval number: JN.No20180615c1201007).

2.3. Open-field test

The open-field experimental video analysis system comprised two parts: an open field box and a video capture system. The box had a height of 40 cm and bottom area of 40 cm × 40 cm. Thirty minutes after gavage, each mouse was placed in the center of the apparatus, and the activity was recorded for a 10-min period. The apparatus was cleaned with 75 % ethanol after each mouse test to eliminate any possible odor cues. The total movement distance, the time spent in the central area and the frequency of entry into the central area were recorded during the 10-min test period (Seibenhener and Wooten, 2015). The data were analyzed using EthoVision XT 11 (Nordas, Netherlands).

2.4. Elevated plus-maze test

The elevated plus-maze system comprised a plus maze and a video capture system. The plus maze hardware consisted of two open arms and two closed arms. All arms had dimensions of 30 cm × 6 cm × 15 cm. The entire maze was elevated to a height of 50 cm above the floor. Thirty minutes after gavage, each mouse was placed in the center of the maze facing one of the open arms. The number of entries into and the time spent in the open arms were recorded within a 5-min period, and the percentages of these events were calculated (Walf and Frye, 2007). The data were analyzed using EthoVision XT 11 (Nordas, Netherlands).

2.5. Sleep improvement test

The sleep improvement test was performed according to the method of Zhao et al. (2015), with some modifications. All tests were conducted in a quiet environment.

2.5.1. Sodium pentobarbital-induced sleep duration test

During the sleep tests, states of sleep and wakefulness were determined based on whether or not the righting reflex had disappeared,

respectively. The disappearance of the righting reflex for more than 30 s was considered to indicate sleep. Thirty minutes after gavage, sodium pentobarbital (49 mg/kg.bw) was administered intraperitoneally to each mouse. The sleep duration was recorded as the time interval from the loss of the righting reflex to its return.

2.5.2. Sub-threshold sodium pentobarbital dose-induced sleep experiment

At 30 min after gavage, each mouse received an intraperitoneal injection of sodium pentobarbital at a dose of 33 mg/kg.bw. This dose was predetermined to be the highest subthreshold dose at which 80–90 % of mice would retain the righting reflex. The mice were observed for 30 min to determine the number of sleeping mice and the sleep rate. The results were analyzed using the chi-square test.

2.5.3. Sodium barbital-induced sleep latency test

At 30 min after gavage, each mouse was injected intraperitoneally with sodium barbital at a dose of 320 mg/kg.bw. The sleep latency was recorded as the time interval from sodium pentobarbital injection to the loss of the righting reflex.

2.6. Fecal DNA extraction and 16S amplicon sequencing

Microbial genomic DNA was extracted from fecal samples using Fast DNA Spin Kit for Feces (MP Biomedicals, USA) according to the manufacturer's instructions. This DNA was then used as a template to amplify the V3-V4 region of 16S rRNA following previous study (Mao et al., 2015). Purified amplicons were subjected to paired-end sequencing on an Illumina MiSeq platform using standard protocols (Zhai et al., 2017). The original data files were analyzed using the QIIME platform (version 1.17) (Mao et al., 2015). The Ribosomal Database Project (RDP) Naive Bayes classifier was used to identify the OTU strain types.

2.7. Determination of short-chain fatty acids in mice feces

Fresh samples of mouse feces were collected in 1.5 mL centrifuge tubes, lyophilized and weighed accurately. The extraction and determination of short-chain fatty acids (acetic acid, propionic acid, butyric acid and total acid) in mice feces were performed according to previous study (Mao et al., 2016).

2.8. Statistical analysis

The experimental data were analyzed using SPSS 19.0 and plotted using GraphPad Prism 6 (GraphPad Inc., La Jolla, CA, USA). The results are expressed as mean ± standard deviation. Each experiment was repeated at least three times in parallel, and the analyses were performed using a one-way ANOVA (followed by Tukey's post-hoc test) and nonparametric tests. A p value < 0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. Screening of GABA-producing strains in fermented milk

Based on our previous research, HPLC was used to determine the GABA-producing capacities of 68 *Lactobacillus* strains isolated from fermented foods or the feces of healthy adults and cultured in MMRS medium. Consequently, 14 GABA-producing *Lactobacillus* strains were screened (Fig. 1A) (Han et al., 2019). These strains were then mixed with *L. bulgaricus* and *S. thermophiles* to produce GABA-rich fermented milk. Milk fermented with *L. plantarum* generally contained low levels of GABA ranging from 8.95 to 16.13 mg/100 g, compared to *L. brevis* which yielded GABA levels of 4.11–67.45 mg/100 g and the highest level produced by *L. brevis* DL1-11 (Fig. 1B). Supplementation with 0.10 % monosodium glutamate and fermentation at 37 °C for 9 h were identified as the optimal parameters. Fermentation with *L. brevis* DL1-11 under these conditions produced 84.10 ± 2.68 mg/100 g of GABA.

3.2. Open-field test

No significant difference in the total movement distance was observed between the groups. However, the administration of high-dose GABA led to significant increases in the time spent in the central area and the frequency of entry into the central area (Fig. 2A–C; $p < 0.01$). An analysis of the mouse trails yielded similar results (Fig. 2D-F).

3.3. Elevated plus-maze test

The results of the elevated plus-maze test are shown in Fig. 3. Compared with the control group, fermented milk without GABA had only a weak effect on the tendency of mice to remain in the open arm and the frequency of mice that entered the open arm. Both indexes increased gradually as the GABA content in fermented milk increased, while only the results of the HGFM group differed significantly from those of the control group ($p < 0.05$).

3.4. Effect of GABA-rich fermented milk on sleep improvement

GABA-rich fermented milk prolonged the pentobarbital-induced sleep duration and reduced the sodium barbital-induced sleep latency. These effects were only significant in mice treated with high-dose GABA fermented milk and diazepam (Fig. 4; $p < 0.05$).

After the intraperitoneal injection of 33 mg/kg.bw sodium pentobarbital, mice in the diazepam group had a sleep rate of 50 %, which differed significantly from that of the control group (Table 1; $p = 0.0325$). The sleep rate in the HGFM group was 30 %, compared to the rates of 10 % in the other groups. Although this rate represented an improvement relative to the control group, this difference was not significant.

3.5. Effect of GABA-rich fermented milk on the gut microbiota of mice

As shown in Fig. 5, no significant differences in the α diversity of the gut microbiota in mice were observed between the different groups. However, the administration of high-dose GABA fermented milk led to a significant change in β diversity ($p = 0.014$), indicating that this treatment changed the composition of the gut microbiota in mice. Moreover, the composition of the gut microbiota in mice from the HGFM group differed significantly from those in the MGFM and LGFM groups ($p = 0.004$ and $p = 0.017$, respectively). Therefore, different doses of GABA fermented milk appeared to have different effects on the composition and structure of mice gut microbiota.

At the phylum level, significant respective decreases and increases in the relative abundances of the *Firmicutes* and *Bacteroidetes* populations were observed (Fig. 5C). In contrast, no significant changes were observed in the populations of *Proteobacteria*, *Fusobacteria* and *Verrucomicrobia*. A cluster heat map of the group OTUs is shown in Fig. 6. It was founded that GABA-rich fermented milk promoted the growth of some bacteria species. Control and NGFM groups were clustered together, while HGFM were clustered together with diazepam group. At the genus level, the relative abundances of *Ruminococcus*, *Allobaculum* and *Adlercreutzia* were significantly increased and the relative abundances of *Bacteroides*, *Bilophila* and *Oscialospira* were significantly decreased in the guts of mice in the HGFM group, compared with the control group (Fig. 7).

3.6. Determination of short chain fatty acids in mice feces

As shown in Table 2, there were no significant differences in the concentrations of acetic acid and propionic acid between the groups. However, the high-dose GABA fermented milk group had significantly

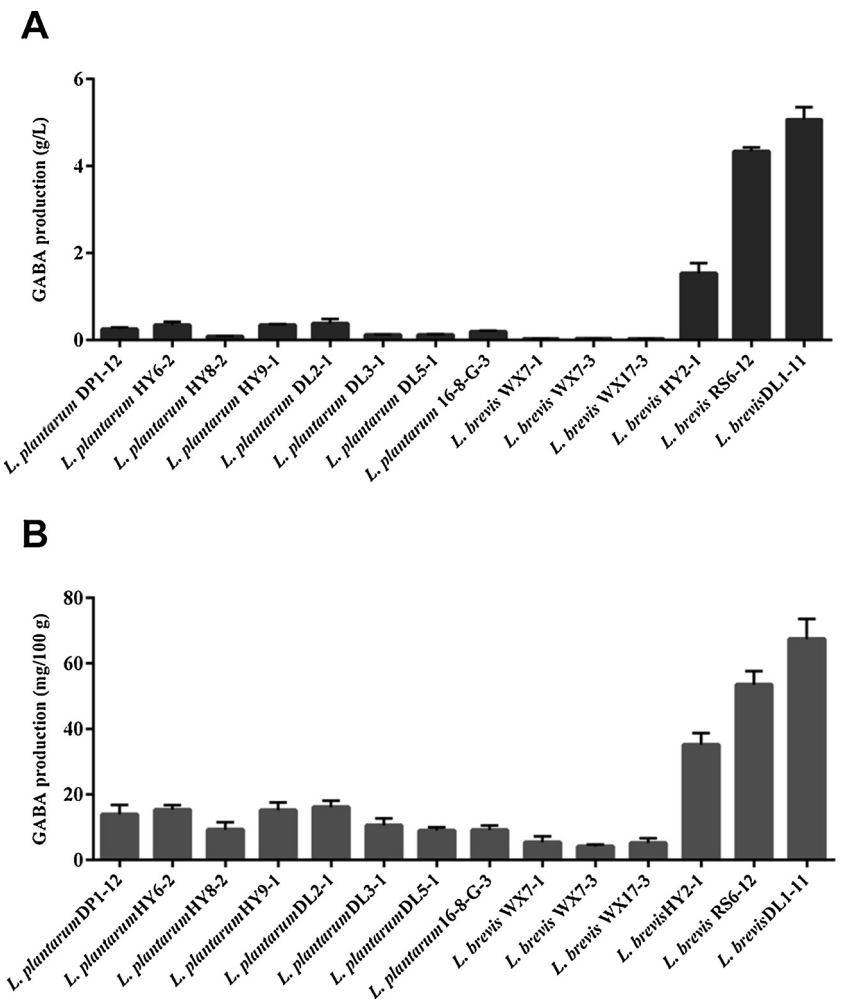

Fig. 1. Production of GABA by *Lactobacillus* strains in different systems: (A) MMRS medium. (B) Fermented milk. Each bar represents the mean ± standard deviation (SD).

higher concentrations of butyric acid and total acids than the control group ($p < 0.05$). Diazepam had no significant effect on these SCFAs.

4. Discussion

Numerous studies have shown that foods containing probiotics and prebiotics are beneficial to human health (Sperry et al., 2018; Shafi et al., 2019). Dairy produced by lactic acid bacteria (LAB) fermentation have been developed in a variety of consumer-recognized product forms (Torres et al., 2017; García-Gómez et al., 2019). LAB are considered the main producers of GABA (Diana et al., 2014). As shown in our previous work, *L. brevis* DL1-11, which was screened from among 68 *Lactobacillus* strains, produced the highest GABA yield of 5072 ± 280 mg/L in MMRS (Han et al., 2019). In this study, the top 14 GABA-producing strains were rescreened in fermented milk. Notably, the results showed that *L. brevis* yielded a higher level of GABA than *L. plantarum*, and *L. brevis* DL1-11 exhibited the highest ability to produce GABA in fermented milk. Similarly, Yunes et al. (2016) determined that *L. brevis* and *L. plantarum* were the main GABA-producing *Lactobacillus* spp. These bacteria synthesize GABA via the irreversible α-decarboxylation of L-glutamic acid by pyridoxal 5′-phosphate (PLP)-dependent glutamate decarboxylase (GAD). The entire process is accompanied by H^+ consumption. Accordingly, the GAD system is associated with the acid resistance exhibited in many *Lactobacillus* strains (Feehily and Karatzas, 2013). The *gad* operon comprises genes encoding a transcriptional regulator (*gadR*), glutamate decarboxylase (*gadA* or *gadB*) and Glu/GABA antiporter (*gadC*). In *L. brevis* DL1-11, *gadB* and *gadC* are expressed at high levels across different growth stages, resulting in a high yield of GABA (Han et al., 2019).

Insomnia is accompanied by diseases such as anxiety. An epidemiological study of French population aged 15 years or over revealed that 33.1 % of insomnia complainers were diagnosed with anxiety (Ohayon and Lemoine, 2002). Among a community-based sample of adolescents with comorbid insomnia and anxiety, anxiety disorder preceded insomnia in 73 % of the time (Johnson et al., 2006). Insomnia is both a common symptom and the main cause of anxiety. In turn, anxiety can also be a risk factor for chronic insomnia. Accordingly, anxiety relief is an important component in the treatment of insomnia. The open field test and elevated-plus maze test are the behavioral test methods used most commonly to assess anxiety behavior in mice (Carola et al., 2002). The results of open field test and plus-maze test experiments showed that mice treated with HGFM exhibited a significantly increased frequency of entry into the central region and open arms, as well as the durations spent in the central region/open arms. These results indicate that HGFM could effectively relieve anxiety. During the sleep improvement test, HGFM treatment led to a significantly prolonged sodium pentobarbital-induced sleep duration and decreased sodium barbital-induced sleep latency, both of which indicated improved sleep. Liu et al. (2015) studied the effects of GABA on di(2-ethylhexyl) phthalate-induced anxiety behavior in rats. In that study, GABA reduced the levels of nitric oxide and nitric oxide synthase in the prefrontal cortex, and relieved DEHP-induced anxiety behavior in

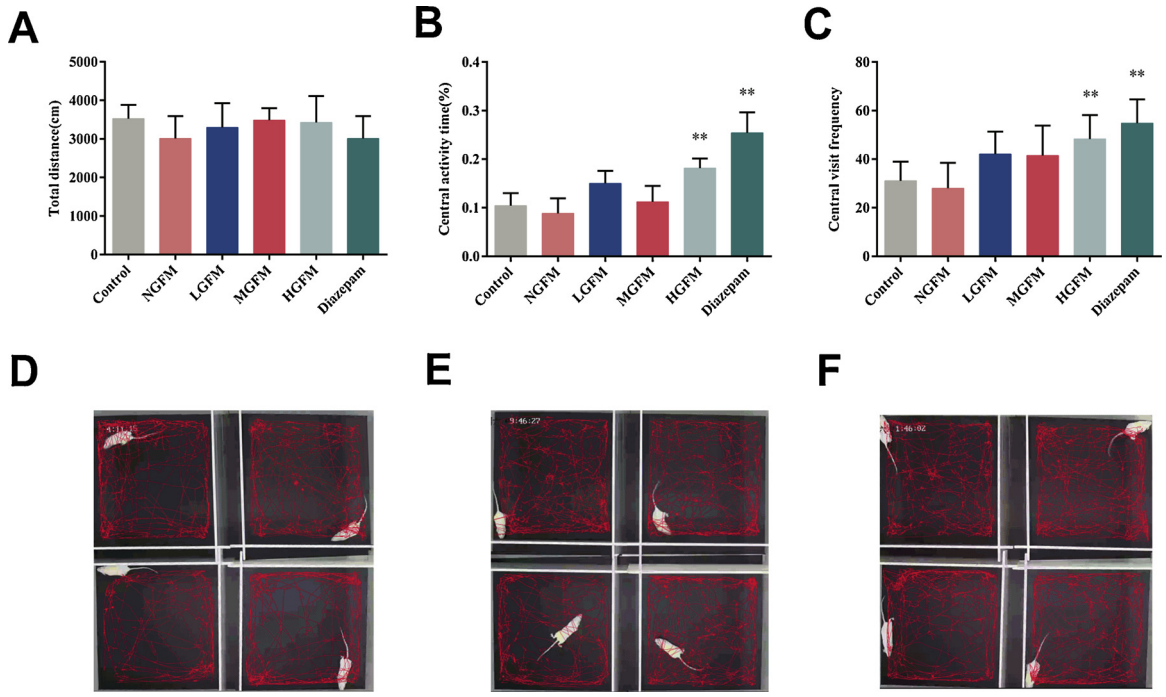

Fig. 2. Effects of treatment with GABA-rich fermented milk on open-field test outcomes. (A) Total movement distances. (B) Central activity times. (C) Frequency of central visits Trails of mice in the open field test in the (D) control group (E) HGFM group and (F) diazepam group. Each bar represents the mean ± standard deviation (SD). *p < 0.05 and **p < 0.01.

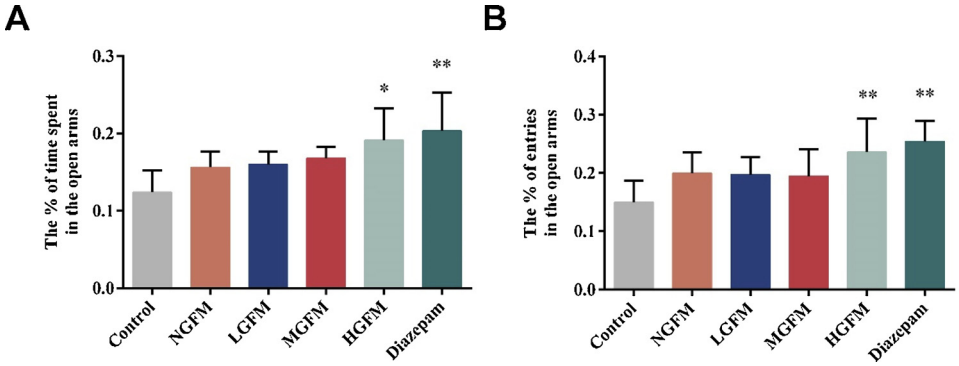

Fig. 3. Effects of treatment with GABA-rich fermented milk on elevated plus-maze test outcomes. (A) Percentages of time spent in the open arms. (B) Percentages of entries in the open arms Each bar represents the mean ± standard deviation (SD). *p < 0.05 and **p < 0.01.

rats during both the open field test and elevated plus maze test. Zhao et al. (2015) observed similar results with a high dose of GABA-containing black tea. Mabunga et al. (2015) used a mouse model of caffeine-induced sleep disorder to study the ability of a fermented rice germ extract containing GABA to improve sleep disorders. In the latter study, treatment with 100 mg/kg of the GABA-containing extract

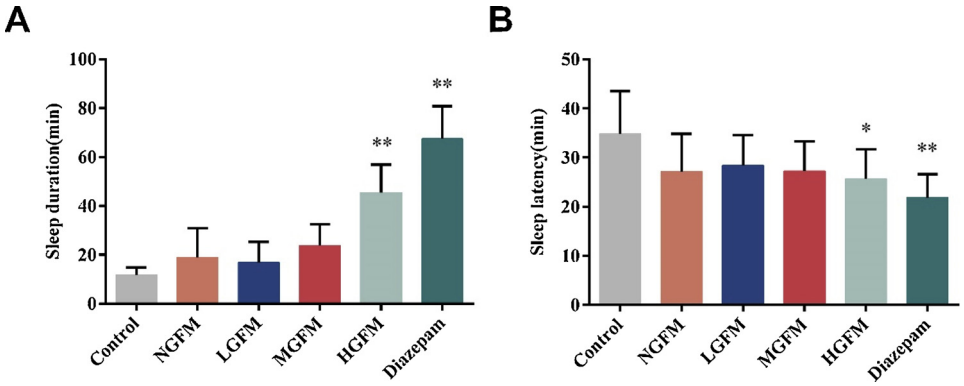

Fig. 4. Effects of GABA-rich fermented milk on sleep improvement. (A) Sodium pentobarbital-induced sleep durations (B) Sodium barbital-induced sleep latency periods. Each bar represents the mean ± standard deviation (SD). *p < 0.05 and **p < 0.01.

Table 1
Influence of GABA-rich fermented milk on sleeping rate in mice.

Group (n = 10)	Number of sleeping mice	Sleep rate (%)
Control	0	0
NGFM	1	10
LGFM	1	10
MGFM	1	10
HGFM	3	30
Diazepam	5	50*

*Significantly different from the control group, chi-square test, p = 0.0325.

normalized caffeine-induced sleep disorders.

Alpha diversity in the gut microbiota is a comprehensive indicator reflective of the richness and uniformity of the intestinal microflora, while the beta diversity is a comprehensive indicator reflective of differences between samples. After gavage with GABA-rich fermented milk, the mice exhibited significant changes in the composition of the gut microbiota. Moreover, treatment with different doses of GABA-rich fermented milk led to significant differences in the gut microbiota compositions in mice. The relative abundances of *Ruminococcus*, *Adlercreutzia* and *Allobaculum* increased significantly after the administration of HGFM. Previous study also demonstrated that GABA can modulate gut microbiota, including increasing relative abundance of *Ruminococcaceae* (Strandwitz et al., 2019).

The effects of exogenous GABA on the nervous system remain controversial. Generally, current research suggests that GABA cannot pass through the blood–brain barrier (BBB) (Knudsen et al., 1988; Van Gelder and Elliott, 1958), although some reports have described opposite findings (Kakee et al., 2001; Boonstra et al., 2015). Here, we suggest that GABA may have indirect effects on the nervous system that are mediated through its effects on the gut. First, as mentioned above, GABA alters the gut microbiota and may promote the production of certain sleep-promoting neurotransmitters. Second, tissues in the gastrointestinal tract express large numbers of $GABA_B$ receptors (Hyland and Cryan, 2010), and both $GABA_A$ and $GABA_B$ receptors regulate the release of 5-HT from intestinal cells (Schwörer et al., 1989). Moreover, the afferent neurons of the vagus nerve strongly express $GABA_B$ receptors. Upon GABA stimulation, the vagus nerve transmits signals to the ventrolateral preoptic nucleus, nucleus raphe, blue plaque and other structures, thus regulating the secretion of neurotransmitters in the brain and affects the related functions. In addition to alleviating inflammation and protecting the intestinal barrier, SCFAs are among the most important functional components of signal transmission between microorganisms and hosts. The total content of SCFAs in the intestine of mice were significantly increased after high-dose GABA fermented milk administration, it is may be associated with a significant increase in the relative abundance of potential SCFA-producing bacteria, such as *Ruminococcus* and *Allobaculum* (Ratajczak et al., 2019). Notably, the intestinal butyric acid level in HGFM group was significantly higher than that in the control group in this study. Particularly, butyrate has been shown to affect the brain indirectly by regulating immune system and vagus nerve activity, thereby exerting beneficial effect on host behavior. Moreover, butyrate can alter the brain functions of healthy animals or improve deleterious effects in models of cognitive and neurological dysfunction (Stilling et al., 2016). Thus, the increase in butyric acid may contribute to the improvement of sleep.

5. Conclusion

This study demonstrated that *L. brevis* DL1-11 had the highest GABA production capacity in fermented milk among the 14 screened

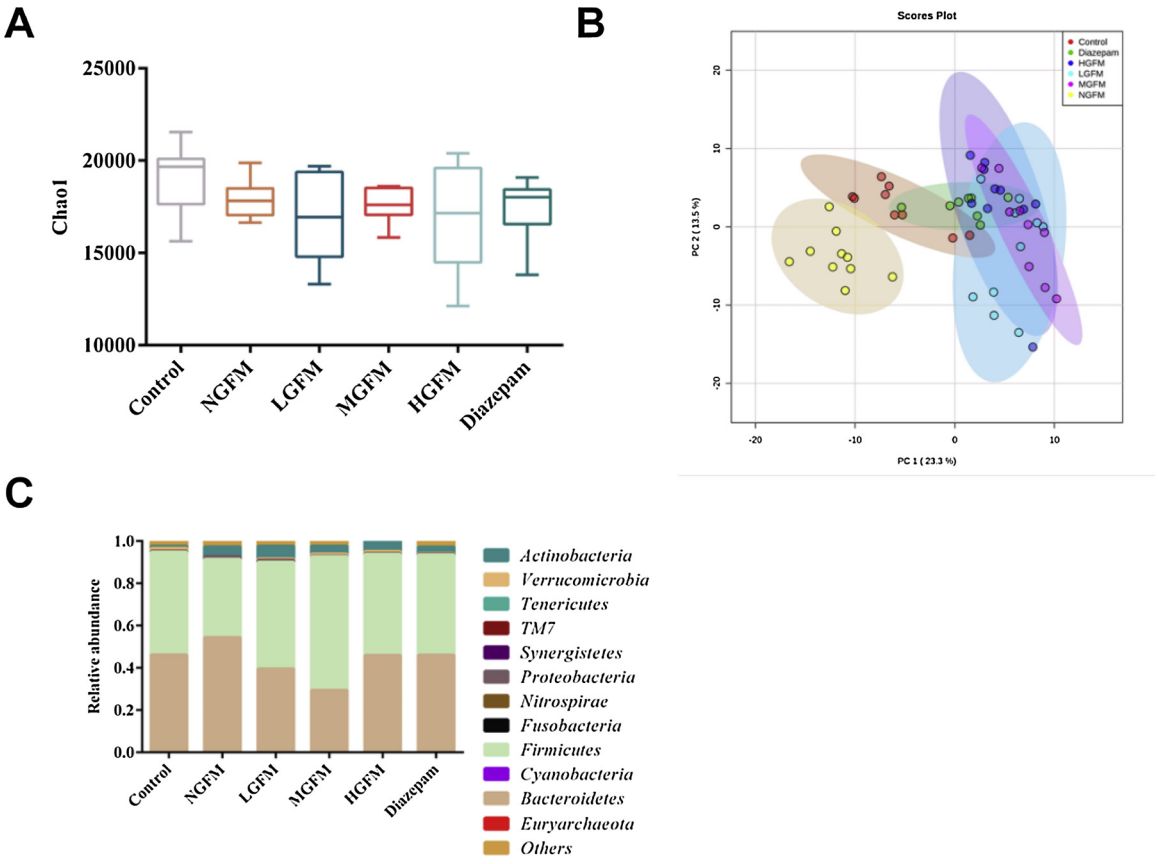

Fig. 5. Alpha diversity analysis of mouse fecal samples. (A) Chao1 index. Each bar represents the mean ± standard deviation (SD). (B) Beta diversity analysis of mouse fecal samples (C) Changes in the composition of the gut microbiota at the phylum level.

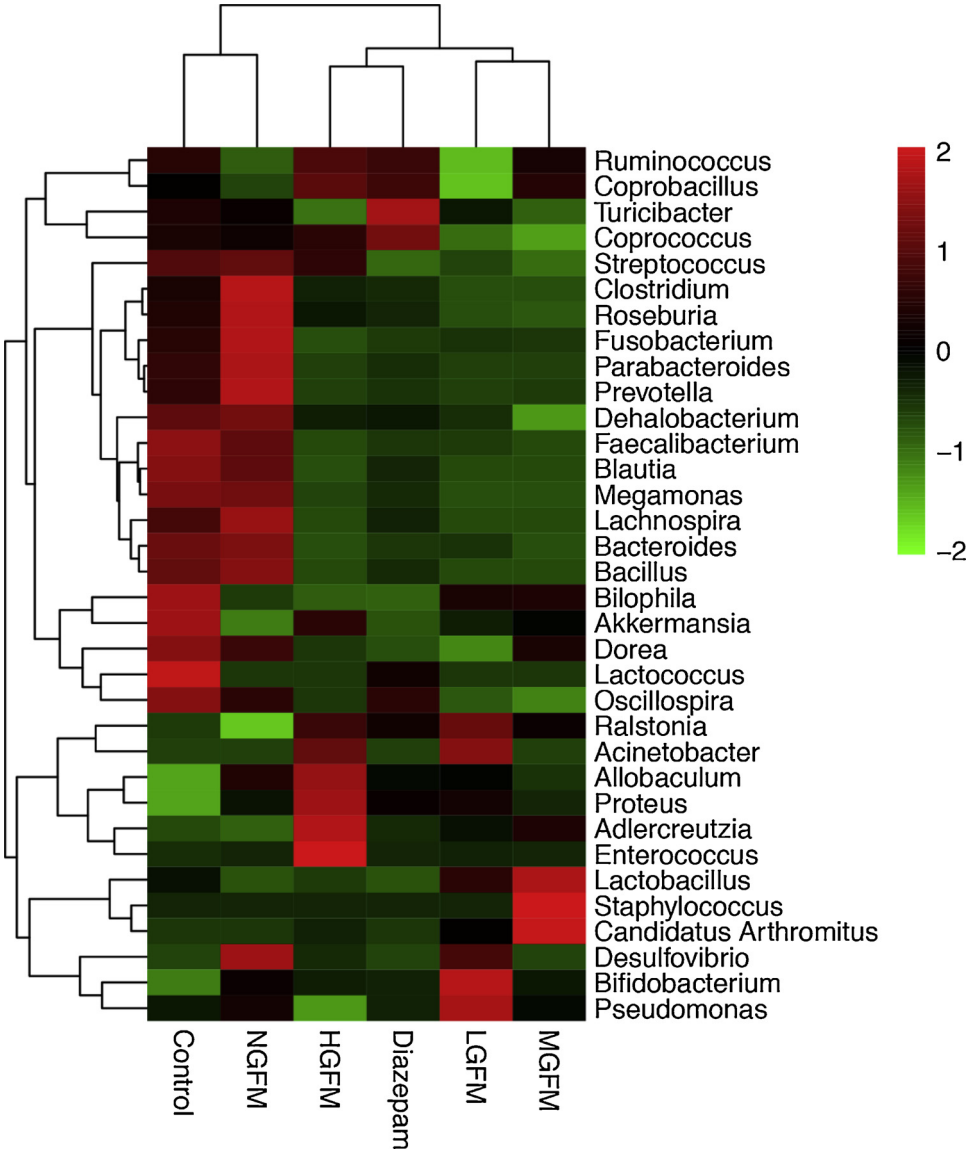

Fig. 6. Cluster heat map of the OTUs identified in mouse gut microbiota.

Lactobacillus strains. This enhanced capacity may be related to the strong expression of *gadB* and *gadC*. The results of open field, elevated plus-maze and sleep improvement tests demonstrated that HGFM effectively relieved anxiety and improved sleep. These effects may be related to changes in the structure and composition of the gut microbiota, such as significant increases in the relative abundances of *Ruminococcus*, *Adlercreutzia* and *Allobaculum*. Moreover, these changes may be associated with significant increases in the production of SCFAs in the intestine.

Conflicts of interest

There is no conflicts of interest regarding the publication of this paper.

CRediT authorship contribution statement

Leilei Yu: Conceptualization, Methodology, Writing - original draft. **Xiao Han:** Methodology, Investigation, Writing - original draft. **Shi Cen:** Software, Formal analysis. **Hui Duan:** Data curation, Writing - original draft. **Saisai Feng:** Data curation. **Yuzheng Xue:** Conceptualization, Supervision. **Fengwei Tian:** Conceptualization, Supervision, Writing - review & editing. **Jianxin Zhao:** Validation. **Hao Zhang:** Software, Validation. **Qixiao Zhai:** Visualization, Investigation. **Wei Chen:** Writing - review & editing.

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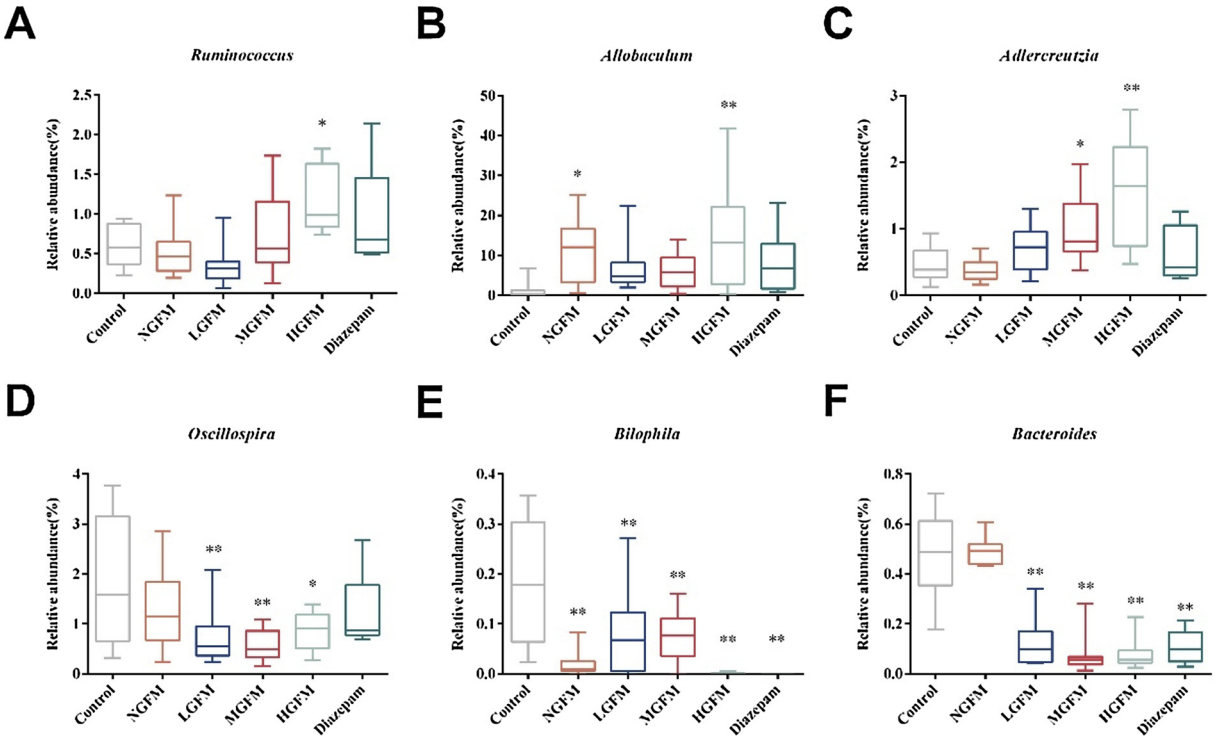

Fig. 7. Major differences in microbial populations at the genus level in the mouse gut microbiota. Each bar represents the mean ± standard deviation. Statistical analyses were conducted using a one-way ANOVA. *$p < 0.05$ and **$p < 0.01$.

Table 2
Concentration of SCFAs in the feces of mice from different groups.

Group	Acetic acid	Propionic acid	Butyric acid	Total acids
Control	19.04 ± 1.81^{a}	4.68 ± 0.99^{a}	1.77 ± 0.44^{a}	25.49 ± 2.18^{a}
NGFM	23.95 ± 3.88^{a}	5.19 ± 1.01^{a}	1.71 ± 0.75^{a}	30.85 ± 4.23^{ab}
LGFM	25.01 ± 4.71^{a}	5.15 ± 0.51^{a}	2.54 ± 0.57^{ab}	32.71 ± 4.66^{b}
MGFM	22.17 ± 1.41^{a}	5.59 ± 1.55^{a}	2.65 ± 0.71^{ab}	30.41 ± 3.47^{ab}
HGFM	24.23 ± 5.41^{a}	5.23 ± 1.79^{a}	3.00 ± 0.66^{b}	32.46 ± 6.29^{b}
Diazepam	22.25 ± 7.77^{a}	5.03 ± 1.81^{a}	1.91 ± 1.39^{ab}	29.18 ± 6.43^{ab}

SCFAs concentration are expressed as μmol/g samples. The results are expressed as means ± standard deviations (SD). Different lowercase letters mean significant differences among the groups ($P < 0.05$).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2020.126409.

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