Effect of adzuki bean sprout fermented milk enriched in γ -aminobutyric acid on mild depression in a mouse model

Zhen Wu,^{1*} Pengyu Wang,² Daodong Pan,^{1,2,3*} Xiaoqun Zeng,¹ Yuxing Guo,² and Guangsheng Zhao⁴

¹State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-products, Key Laboratory of Animal Protein Deep Processing Technology of Zhejiang, School of Food and Pharmaceutical Sciences, Ningbo University, Ningbo, Zhejiang 315211, P. R. China

²School of Food Science & Pharmaceutical Engineering, Nanjing Normal University, Nanjing 210046, P. R. China

³National R&D Center for Freshwater Fish Processing, Jiangxi Normal University, Nanchang, Jiangxi 330022, P. R. China

⁴Hangzhou New Hope Shuangfeng Dairy Co., Hangzhou, Zhejiang 310000, P. R. China

ABSTRACT

This study focused on the ability of adzuki bean (*Vigna angularis*) sprout fermented milk, which is rich in γ -aminobutyric acid (GABA), to relieve anxiety and mild depression. A high-yield GABA-producing strain, *Lactobacillus brevis* J1, from a healthy cow was screened, and its physiological and probiotic properties were evaluated. The effect of adzuki bean sprout fermented milk was investigated in vivo in a chronic depression mouse model. The results showed that *Lb. brevis* J1 had excellent probiotic properties, grew well at low pH and 3% NaCl, and adhered to the surface of HT-29 cells. The GABA-enriched ($241.30 \pm 1.62 \mu\text{g/mL}$) adzuki bean sprout fermented milk prepared with *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, and *Lactobacillus plantarum*, and *Lb. brevis* J1 can reduce and possibly prevent mild depression-like symptoms in mice (C57/B6) by increasing social interaction and enhancing the pleasure derived from movement. The research revealed that the GABA_B-cyclic AMP-protein kinase A-cAMP-response element binding protein (GABA_B-cAMP-PKA-CREB) signaling pathway was related to the depression-like symptoms and that levels of 5-hydroxytryptamine, norepinephrine, and dopamine in the hippocampus of mice increased after treatment with the adzuki bean sprout fermented milk. Our results suggest that GABA-enriched dairy products have the potential to prevent or treat mild depression-like symptoms in mice, which suggests a new approach for a dietary therapy to treat chronic social stress.

Key words: γ -aminobutyric acid, *Vigna angularis*, *Lactobacillus brevis*, γ -aminobutyric acid (GABA)-enriched fermented milk, mild depression

INTRODUCTION

With rapid urbanization in developing countries over the past several decades, mild depression in city dwellers caused by psychological, social, and economic pressures has become a serious and long-term public health problem. Worse, mild depression can lead to persistent depressive disorders, especially in young people (Anakwenze and Zuberi, 2013). Although antidepressant drugs are effective, they are not universally so and can cause strong dependency (Chuang et al., 2011). Therefore, dietary therapy has increasingly been seen by physicians and patients as an alternative approach to the treatment of chronic mild depression (Felton and Cervenka, 2015).

γ -Aminobutyric acid (GABA) is a common non-protein amino acid that can act as an inhibitory neurotransmitter to relieve depression (Barrett et al., 2012) while fulfilling several other important physiological functions, such as lowering blood pressure (Alves et al., 2016) and managing diabetes by converting glucagon-producing cells to large numbers of β -like cells (Weir and Bonner-Weir, 2017). It also has been shown to produce an anti-stress effect in humans (Vaiva et al., 2004) and to lessen the symptoms of depressive disorders (Okada et al., 2000). Recent advances in food and nutrition science have highlighted the possibility of modulating certain specific physiological functions through food intake (Jiménez-Colmenero, 2013), including using functional foods containing a high concentration of GABA to treat chronic mild depression. In general, food-grade GABA is derived from germinated cereals and microorganisms (Diana et al., 2014), such as germinated brown rice and barley (Lin et al., 2015). *Lactobacillus* strains are also a good source of GABA; lactic acid bacteria such as *Lactobacillus brevis* and *Lactobacillus plantarum* can produce high amounts of GABA (Wu et al., 2018). However, these types of basic food sources are not plentiful enough to meet the potential adult demand for food products made with GABA.

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*Corresponding authors: woodsen163.com and daodongpan@163.com

Lactic acid bacteria (LAB) are commonly used as commercial starters to make many fermented foods and beverages; they have many beneficial effects, such as reducing the risk of gastrointestinal diseases (Minervini et al., 2009; Sharifi Yazdi et al., 2017). Different kinds of *Lactobacillus* found in fermented food have been shown to display GABA-accumulating properties, such as *Lb. paracasei* in the fermented fish product funa sushi (Komatsuzaki et al., 2005), *Lb. brevis* in salt-fermented Jot-gal (cod gut; Lee et al., 2010), *Lb. rhamnosus* in Nostrano cheese, and *Lb. otakiensis* in Pico cheese (de los Reyes-Gavilan et al., 2015; Ribeiro et al., 2018). Research has also revealed that *Lactobacillus* taken orally can increase GABA content in the brain, which helps regulate many physiological and psychological processes (Janik et al., 2016). Oral treatment with *Lb. rhamnosus* was found to attenuate stress-related disorders in male C57BL/6 mice (Bharwani et al., 2017). *Lactobacillus* strains possess the ability to produce high levels of GABA, and they can be used as functional starters to manufacture novel fermented dairy foods with beneficial health effects (Sharma and Kanwar, 2017).

Plant-derived foods provide many potential health benefits. The adzuki bean (*Vigna angularis*) was domesticated in China about 12,000 years ago. Since then, it has been consumed as a common type of bean that is rich in protein and dietary fiber (Oliveira et al., 2017). After germination, some antinutritional factors such as levels of phytic acid and protease inhibitors decrease, and many functional secondary metabolites, such as GABA and soy isoflavones, are formed that provide health benefits to humans (Shi et al., 2010; Fayed, 2015). Okada et al. (2000) first showed that defatted rice germ enriched with GABA can reduce symptoms such as sleeplessness, somniphathy, and depression in patients. Patterson et al. (2019) also demonstrated the positive effect of GABA on metabolism and depressive-like behavior.

Dietary therapy is a good approach to the treatment of chronic illness, and natural compounds and natural approach to food processing are currently popular among consumers (Gahruie et al., 2015). Because both fermented dairy products and germinated adzuki beans produce GABA, we screened LAB strains to identify a high-yield GABA-producing *Lactobacillus* and prepared adzuki bean sprouts for a fermented milk product with high levels of GABA. A mild depression-like mouse model with C57/B6 male mice was used to explore the possible mechanism by which the GABA-enriched fermented milk affects depression. The novel fermented milk product in this study represents a new approach for using plant-derived components in the development of dairy products for the adjuvant treatment of chronic diseases.

MATERIALS AND METHODS

Screening of GABA-Producing LAB

Eighty LAB strains were isolated from 20 types of fermented kimchi, 10 samples of gastrointestinal tract contents from healthy fish (*Ctenopharyngodon idellus*), and 10 samples of fresh milk. The LAB strains were incubated in de Man, Rogosa, and Sharpe (MRS) broth for 24 h at 37°C. To identify LAB with high GABA-producing capability, strains were grown in MRS broth containing 1.0% (wt/vol) monosodium glutamate (MSG) and then the supernatants were centrifuged for detection. The GABA contents were preliminarily identified by thin layer chromatography using butyl alcohol, glacial acetic acid, and water (5:3:2 vol/vol/vol) plus 0.1% (wt/vol) ninhydrin (Li et al., 2009). After chromatography, the paper was dried at 90°C for 10 min and the specific shift value (R_f) was calculated. For the quantification of GABA production in a selected strain, an amino acid analyzer (L-8900, Hitachi, Tokyo, Japan) was used to detect the GABA concentration in the fermented culture media of the screened strains.

The GABA-producing LAB were further identified by fermentation potential tests and 16S rDNA analysis (Komatsuzaki et al., 2005). Their fermentation potential on different sugars was determined using an API rapid CH fermentation strip kit (bioMérieux, Marcy l'Etoile, France). The 16S rDNA sequence analysis procedure was as follows: total DNA of a strain in 1.0 mL of MRS medium was extracted using a bacterial genomic DNA extraction kit (Takara, Shiga, Japan). The general primers 1492R and 27F were used for amplification. The PCR fragments (1,500 bp) were purified using a quick PCR purification kit (Takara) and sequenced by Sangon Biotech (Shanghai, China). Then, a phylogenetic tree was constructed by generating complete alignment of the 16S rDNA gene of the selected members in GenBank by using MEGA software (<https://www.megasoftware.net/>) bootstrap values. Finally, the identified strain was deposited in the China General Microorganisms Collection (CGMCC, Beijing, China) under ID number 15954.

Physiological and Probiotic Properties of Isolated *Lb. brevis* J1

The LAB strains were collected and inoculated at 1.0% in MRS culture medium containing 0.05, 0.10, 0.20, or 0.30% (wt/vol) bovine bile salts at 37°C for 18 h. The survival rate was calculated based on the value of the viable cells. Gastric and intestinal fluid tolerance tests were carried out as previously described (He et al., 2019).

The adhesion ability of the collected *Lb. brevis* J1 strain was measured according to the method of Mazzearella et al. (2017) with some modifications. The HT-29 cells were cultured at 37°C in 5% CO₂ and placed on coverslips (5 × 10⁵ cells per well) in Dulbecco's modified Eagle medium and cocultured with carboxyfluorescein succinimidyl ester (CFSE)-labeled *Lb. brevis* J1. Cells were then fixed with 4% (wt/wt) paraformaldehyde for 8 min at 4°C and washed 3 times with PBS. Then cells were incubated with 0.1% (wt/wt) Triton X-100 for 10 min, 4',6-diamidino-2-phenylindole (DAPI) for 15 min and mounted using 50% glycerol. The signals were visualized using laser scanning confocal microscopy (FV 3000, Olympus, Tokyo, Japan).

Fermented Milk with Sprouted Adzuki Beans

During the milk fermentation, adzuki bean sprout refiner (20% wt/vol) and MSG (1.0% wt/vol) were added to fresh milk and sterilized at 121°C for 5 min. Then, *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, and *Lactobacillus plantarum* 15953 (strain CGMCC15953, which can transfer soybean isoflavones to soybean isoflavone aglycone) and strain *Lb. brevis* J1 were combined in a ratio of 1:1:3:1 to make the GABA-enriched fermented milk at 40°C. Milk fermented without adzuki bean sprouts or *Lb. brevis* J1 was used as the negative control. The pH of each fermented milk sample was measured using a pH meter (Ins mark IS128C, Shanghai, China). The adzuki bean sprouts were prepared as follows: adzuki beans were soaked in water at 30°C for 6 h until the moisture content was about 30% (wt/wt). Then, bean samples (~200 g each) were placed in germination trays in a controlled-growth chamber. Germination proceeded at 25°C and pure water was sprayed on the sprouts by an ultrasonic humidifier every 4 h. The sprouted adzuki beans were removed from the germination chamber after 84 h and collected for milk fermentation.

Water-Holding Capacity and GABA Content of the Adzuki Bean Sprout Fermented Milk

The water-holding capacity (WHC) was measured as described by Sodini et al. (2005). A homogenized sample of 10 g of fermented milk (Y) was centrifuged at 4,000 × g for 10 min at 4°C, and the whey supernatant from the sample was removed and weighed (WE). The WHC was calculated as follows: WHC (%) = [(Y – WE)/Y] × 100.

The GABA content was determined using an amino acid analyzer (Hitachi L-8900) as previously described. 3,5-Disulfosalicylic acid was added to the fermented

milk (1:1, vol/vol) to remove protein and centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was filtered through a 0.22-μm syringe filter and detected using the analyzer.

Animals, Grouping, and Mouse Model Design

Five-week-old C57/B6 male mice (around 20 g) were purchased from the Shanghai Xipuer-Bikai Laboratory Animal Co. [SCXK(Hu) 2018-0006; Shanghai, China], and kept in the animal room at 22 ± 1°C under standard housing conditions of a 12:12 h light/dark cycle for 7 d before the experiment. All animal experiments carried out in accordance with the UK Animals (Scientific Procedures) Act of 1986 and associated guidelines. For the mild depression-like mouse model design, the GABA dosage (3.43 mg/kg) was determined according to a previous study (Okada et al., 2000). Fluoxetine, a common antidepressant, was used as a positive control at a dose of 20 mg/kg of BW for each mouse. The GABA concentration in the adzuki bean sprout fermented milk was 241.3 ± 1.62 μg/mL, and that in normal fermented milk was 3.67 ± 0.42 μg/mL in the mice experiment.

All mice were weighed and randomly assigned to 7 groups of 12 mice each: (1) healthy mice administered 0.4 mL of saline per day (0.9 % NaCl, control); (2) mild depression-like mouse model, with mice administered 0.4 mL of saline per day (model); (3) mice administered common fermented milk (normal, 0.4 mL of fermented milk without GABA per day); (4) mice administered full dose of adzuki bean sprout fermented milk (high, 0.4 mL of GABA-enriched fermented milk sample per day); (5) mice administered half dose of adzuki bean sprout fermented milk (medium, 0.2 mL of GABA-enriched fermented milk with 0.2 mL of saline water sample per day); (6) mice administered a low dose of adzuki bean sprout fermented milk (low, 0.1 mL of GABA-enriched fermented milk with 0.3 mL of saline water sample per day); and (7) fluoxetine-treated mice (20 mg/kg of BW per day).

After grouping, mice were exposed to social frustration stress for 10 d to induce mild depressive symptoms. The procedure was as follows: CD1 mice were screened for strong attack ability and then C57/B6 mice were placed as invaders in a large white cage housing the CD1 mice. Each cage contained one CD1 mouse and one C57/B6 mouse. The mice were allowed physical contact for 10 min/d and then the C57/B6 and CD1 mice were separated by a small transparent plate for the rest of the day. The plate allowed the C57/B6 mouse to see the CD1 mouse; even though it could not be attacked, the sight would still cause psychological

stress. The C57/B6 mice were exposed to different CD1 mice every day, and the social interaction experiments were conducted over 10 d.

Depressive-Like Behavior Analysis

Sucrose Preference Test. The sucrose preference test is a classic method to evaluate the pleasure index of mice (Su et al., 2017). The test was carried out according to Liu et al. (2015) with slight modifications. Before the test, 1.0% (wt/vol) sucrose in water was given to the test mice for 24 h. After acclimation, the mice were weighed, placed singly in cages, and made to fast for 24 h. Then, each mouse was given a bottle of 1.0% (wt/vol) sucrose water and a bottle of tap water. After 12 h, the positions of the sucrose and tap water were exchanged and the mice were fed for another 12 h. Finally, the remaining sucrose and tap water were collected and weighed for the sucrose preference test analysis.

Social Interaction. The social ability of mice was evaluated using a social interaction experiment according to the methods of Kaidanovich-Beilin et al. (2011), with some modifications; specifically, the empty chamber was replaced by a mineral water bottle. The mice were placed in a 3-cabin metal cage for 30 min to adapt to the environment, and the contact area was set at 3 to 5 cm² around the cage. Then, 1 mineral water bottle was placed on one side of the whole cabin and the door connecting the other 2 compartments was opened. The time it took for the mouse to reach the mineral water bottle within 2.5 min was recorded. The mineral water bottle was replaced with the same kind of CD1 mouse, and communication times between the same kinds of mice were recorded. The ratio of the 2 recorded times was calculated as the social interaction rate.

Forced Swimming Test. The forced swimming test is also known as the despair experiment. It was performed according to the method described by Smith et al. (2019) and others. Each mouse was individually forced into an open cylindrical container (10 × 25 cm). The container contained 15 cm of water at a constant temperature of 25 ± 1°C. The experiment lasted 6 min and the first 2 min was considered the acclimation period. The period when the mouse floated motionless in the water and only kept their nose above the water was considered immobilization, and the cumulative time of mouse immobility during the 4 min after the acclimation period was measured.

Tail Suspension Test. The tail suspension test was determined according to the method of Xu et al. (2016), with slight modifications. The mice were inverted and

attached to a hanging hook with tape at a distance of about 1 cm from the tip of the tail. Mice were then placed in a universal sound-proof behavior box and hung upside down with their heads 20 cm from the bottom of the box. The mice struggled to overcome the abnormal posture, but the activity appeared intermittently with signs of despair. A mouse-tail suspension video analysis system recorded each mouse's activity over 6 min. The first 2 min was considered the adaptation period, and the accumulated time of the mouse's immobility during the subsequent 4 min was then measured.

Quantification of Monoamines

Before euthanasia, mice were weighed and injected with 5 mg/kg carprofen (Rimadyl, Pfizer, New York, NY) as an analgesic. Then, mice were anesthetized (isoflurane 2–3% mixed with 30% O₂ and 70% N₂O) and immediately killed by decapitation; tissues were stored at –80°C. Concentrations of 5-hydroxytryptamine (5-HT), norepinephrine (NE), dopamine (DA), and cyclic AMP (cAMP) were measured by ELISA kits (ZCI BIO, Shanghai, China) according to the manufacturer's instructions.

Western Blotting

The samples were centrifuged at 12,000 × *g* for 30 min at 4°C. The total protein concentration of the supernatant was determined using a bicinchoninic acid (BCA) protein assay kit (Nanjing Jiancheng Biological Co., Nanjing, China) according to the manufacturer's instructions. After heating at 100°C for 5 min, the proteins were separated on SDS-PAGE gels and transferred to polyvinylidene difluoride membranes for immunoblotting. The membrane was then blocked with blocking solution (5% skim milk) at room temperature for 2 h followed by an overnight incubation at 4°C with antibodies against GABA B receptor (GABA_B), protein kinase A (PKA), cAMP-response element binding protein (CREB), and phosphorylated (p)-CREB. Horseradish peroxidase-conjugated goat anti-rabbit IgG (H⁺L) was used as the secondary antibody. After washing in PBS, the membranes were incubated with secondary antibodies (1:5,000 dilution, Cell Signaling Technology, Danvers, MA), followed by washings 3 times with Tris-buffered saline-Tween. Immunoreactivity was detected with an enhanced chemiluminescence detection system and visualized in an imaging system. β-Tubulin (1:1,000; Cell Signaling Technology) was used as control. The images were quantified using Image-Pro Plus software (Media Cybernetics, Rockville, MD).

Figure 1. γ -Aminobutyric acid (GABA)-producing ability (A) and growth characteristics (optical density, OD, at 600 nm) of *Lactobacillus brevis* J1 grown in medium with monosodium glutamate (MSG) at different pH values (B; 3.0, 4.0, 5.0, and 5.8) and NaCl concentrations (C; 1, 3, 5, and 7%). (A) Seven screened lactic acid bacteria strains identified by thin layer chromatography; R_f = specific shift value. Lane 5 shows high GABA production of *Lb. brevis* J1; the micrographs show morphology of screened strain number 5 (*Lb. brevis* J1). (B, C) Effect of different initial de Man, Rogosa, and Sharpe (MRS) pH (B) and concentrations of NaCl (C) on *Lb. brevis* J1 growth.

Statistical Analysis

Experimental data were analyzed using GraphPad Prism 7.0 (GraphPad Inc., San Diego, CA), and analysis of significant differences was performed by one-way ANOVA ($P < 0.05$). Data are expressed as means $\pm$ standard deviations. All experiments were repeated 3 times.

RESULTS

Isolation, Identification, and Preservation of GABA-Producing LAB

Lactic acid bacteria strains that could convert MSG to GABA were screened and identified using thin layer chromatography and 16S rDNA analysis. Among them, strain 5 (*Lb. brevis* J1) derived from the fresh tract contents of a cow displayed high GABA production of 9.87 ± 0.18 g/L (Figure 1A). The *Lb. brevis* J1 strain had 99% similarity to *Lactobacillus brevis* ATCC14869.

The 16S rDNA sequence was submitted to the National Center of Biotechnology Information with accession number MH266120. The strains with the best physiological properties and carbon source fermentation ability (*Lb. brevis* J1; Table 1) were used for further study.

Physiological Characteristics and Probiotic Properties of Isolated Strains

The target strain was cultured in MRS medium with different initial pH values and concentrations of NaCl for 24 h at 37°C. As shown in Figure 1B, at pH 3.0, the growth of *Lb. brevis* J1 was significantly inhibited. Optimal growth was achieved at pH 5.0. At pH 5.0, *Lb. brevis* J1 showed high glutamic acid decarboxylase activity. As shown in Figure 1C, *Lb. brevis* J1 grew well when the NaCl concentration was $<5\%$ but grew very little when it was $>7\%$. The tolerance of *Lactobacillus* to bile salts is an important factor that determines whether they can reach and colonize the intestinal tract.

The results in Figure 2A show that the survival rate of *Lb. brevis* J1 (>55%) was higher under conditions of <0.2% (wt/vol) bovine bile salt, decreasing significantly to 37% at 0.3% bovine bile salt ($P < 0.05$). For the stimulated gastric and intestinal juice experiment, the survival rate of *Lb. brevis* J1 was significantly decreased in gastric and intestinal juice when the culture time was extended from 2 to 4 h ($P < 0.05$), although the survival rate was still >50% after 4 h (Figure 2B). *Lactobacillus brevis* J1 displayed good adhesion abil-

ity in the HT-29 cell model as shown by fluorescence microscopy (Figure 2C). With different ratios of *Lb. brevis* J1 in fermented milk, the GABA content differed: the ratio of *Lb. bulgaricus*: *Strep. thermophilus*: *Lb. plantarum* 15953: *Lb. brevis* J1=1:1:3:1 (group C in Supplemental Figure S1; <https://doi.org/10.3168/jds.2020-19154>) had the highest GABA content of $241.3 \pm 1.62 \mu\text{g/mL}$, whereas the GABA content in the *Lb. brevis* J1-free group (group D in Supplemental Figure S1) was much lower ($P < 0.05$), with a concentration of $215.2 \pm 1.31 \mu\text{g/mL}$ (Supplemental Figure S1).

Table 1. Carbon source assimilation (positive or negative) of strain *Lactobacillus brevis* J1

Carbohydrate ¹	Result
Glycerol	—
Inositol	—
Inulin	+
D-Arabinose	—
Sorbitol	—
Raffinose	—
D-Ribose	+
α-Methyl-D-glucoside	—
Glycogen	—
L-xylose	—
Amygdalin	—
Gentiobiose	+
β-Methyl-D-xyloside	—
Esculin	+
D-Lyxose	—
D-Glucose	+
Cellobiose	—
D-Fucose	—
D-Mannose	—
Lactose	—
D-Arabitol	—
L-rhamnose	—
Sucrose	+
Gluconate	+
Erythritol	—
Mannitol	—
Melezitose	+
L-Arabinose	+
α-Methyl-D-mannoside	—
Starch	—
D-Xylose	+
N-Acetyl-glucosamine	+
Xylitol	—
Adonitol	—
Arbutin	—
D-Turanose	—
D-Galactose	—
Salicin	+
D-Tagatose	—
D-Fructose	+
Maltose	+
L-Fucose	—
L-Sorbose	—
Melibiose	—
L-Arabitol	—
Dolcitol	—
Trehalose	+
2-Keto-gluconate	—

¹Carbohydrate adoption relays on the API 50CH system (bioMérieux, Marcy l'Étoile, France).

Properties of Adzuki Bean Sprout Fermented Milk

The pH and viable cell count of the control fermented milk and adzuki bean sprout fermented milk were measured during fermentation. As shown in Figure 3A, at 8 h, the pH of adzuki bean sprout fermented milk and control fermented milk both decreased below 4.6. The rate of viable bacteria in the GABA-enriched adzuki bean sprout fermented milk was higher than that in the normal fermented milk, which could help shorten the fermentation time (Figure 3B).

Effect of GABA-Enriched Adzuki Bean Sprout Fermented Milk on the Depression-Like Mouse Model

As shown in Figure 4A–C, the sucrose water preference of the control group was significantly higher ($P < 0.05$) than that of the model group, indicating that the depression-like mice model was successfully induced. Compared with the model group, the sucrose water preference of the mice in the adzuki bean sprout fermented milk group was increased in all of the treated groups ($P < 0.05$). We also observed a significant improvement in the social interaction of the mice ($P < 0.05$), especially in the medium- and high-dose groups ($P < 0.05$). Compared with the positive control group, the high-dose group displayed a better response group in facing fear during the forced-swimming and tail-suspension experiments ($P < 0.05$; Figure 4D, E).

Monoamine Transmitter Content in Mouse Hippocampus

To date, most antidepressants have been shown to work by increasing levels of the monoamine transmitters 5-HT (serotonin), NE, and DA (Yi et al., 2008). To investigate whether GABA-enriched adzuki bean sprout fermented milk similarly affected these neurotransmitters to alleviate symptoms of depression, we analyzed their concentrations in hippocampus tissue of 7 groups

Figure 2. Probiotic properties of isolated *Lactobacillus brevis* J1. Effects of different concentrations of bile salts (A) and simulated gastrointestinal fluid environment (B) in vitro on *Lb. brevis* J1 growth. (C) Images of *Lb. brevis* J1 adhesion in HT-29 cells with confocal laser scanning microscopy. *Lactobacillus brevis* J1 labeled with carboxyfluorescein succinimidyl ester (CFSE, Figure 2C-b), and HT-29 cells labeled with 4',6-diamidino-2-phenylindole (DAPI, Figure 2C-a). Figure 2C-c is the differential interference contrast image, and Figure 2C-d is the merged images of Figure 2C-a and Figure 2C-b. The different letters (a-e) in panels A and B indicate significant differences between groups ($P < 0.05$); error bars indicate the standard deviation.

of mice. As shown in Figure 5A-C, compared with the blank control group, the concentrations of 5-HT, NE, and DA in the model group of depressed mice were significantly reduced ($P < 0.05$). Mice in the medium-

and high-dose groups showed significantly increased levels of 5-HT and NE ($P < 0.05$), whereas mice in the medium-dose group had significantly increased levels of DA ($P < 0.05$).

Protein Expression of GABA_B-cAMP-PKA-CREB Pathway in Mouse Hippocampus

The cascade response process of GABA_B receptor activation, second messenger cAMP, PKA activation, CREB phosphorylation, gene transcription regulation, and the subsequent biological effects play an important role in mood regulation. As shown in Figure 6, compared with the blank control group, GABA_B expression in the model group was significantly decreased ($P < 0.05$), indicating that the depression-like symptoms correlated with the inhibition of GABA_B expression in the hippocampus. The GABA-fortified fermented milk group showed an increase in GABA_B expression in a dose-response manner. However, only the high-dose GABA-enriched adzuki bean sprout fermented milk group showed significantly increased cAMP content in the hippocampus (Figure 5D). For PKA and p-CREB, the medium- and high-dose GABA-enriched fermented milk groups showed significantly increased levels ($P < 0.05$).

DISCUSSION

Increasing evidence suggests that GABA dysfunction plays a major role in depressive disorders and that enhanced levels of GABA neurotransmitters in nerve cells can dramatically improve the response to chronic social-frustration stress (Diana et al., 2014). In this research, we explored the therapeutic potential of GABA-producing germinated adzuki beans and high-yield GABA-producing *Lb. brevis* J1. We developed a GABA-enriched adzuki bean sprout fermented milk

product, which was used to make a novel fermented milk with traditional Chinese food elements and key functional properties.

The digestive time of food in the stomach is usually between 2 and 4 h. Human gastric juice is a strong, food-dissolving, complex acidic solution, which can hydrolyze microorganisms in the gastric environment (Nardone and Compare, 2015). Bile salt stress in the gastric tract can damage cell viability by disrupting the cell membrane and cell wall components. Therefore, measuring the tolerance of *Lactobacillus* to gastric and intestinal juice helps confirm the probiotic effect of *Lactobacillus* in the gastrointestinal tract. In this study, we showed that *Lb. brevis* J1 has good physiological and probiotic characteristics and can tolerate high concentrations of bile salt (0.3%) with a survival rate >50% and has good adhesion ability to HT-29. Furthermore, during the milk fermentation process, the adzuki bean sprout fermented milk displayed a larger number of viable bacteria and a higher acidity rate compared with the control fermented milk.

Yogurt has a 3-dimensional network gel structure formed by the accumulation of denatured casein particles. Fat globules and *Lactobacillus* strains are embedded in this gel fiber network (Berrazaga et al., 2019). This complex structure enhances the WHC of fermented milk. The adzuki bean sprout fermented milk had a more uniform and denser milk gel structure and its WHC was slightly higher fermented milk without adzuki bean sprout. Because of the dietary fiber components in the bean sprouts, a more solid network structure was formed, which can preserve and retain water, and thereby improve the WHC of fermented

Figure 3. Properties of milk fermented with sprouted adzuki bean. (A) Changes in pH during milk fermentation between the control and adzuki bean sprout fermented milk; (B) changes in growth (log cfu) during milk fermentation. The error bars indicate the standard deviation.

milk. In addition, a variety of nutritional factors such as fatty acids, free amino acids, and GABA in the adzuki bean sprouts encourage the growth of *Lb. brevis* J1 (Li

et al., 2011), which is why the colony counts of strains were much higher in the adzuki bean sprout fermented milk than in conventional fermented milk.

Figure 4. Effect of γ -aminobutyric acid (GABA)-enriched adzuki bean sprout fermented milk on the depression-like mouse model. (A) Sucrose preference test of mice after different doses of adzuki bean sprout fermented milk; (B) social interaction analysis of different adzuki bean sprout fermented milk-treated mice groups; (C) trajectory maps of mice (dots) in different adzuki bean sprout fermented milk groups; (D) immobility (time spent immobile, s) of mice in the forced-swim test (FST); and (E) immobility of mice in the tail-suspension test (TST). Groups: (1) healthy mice administered 0.4 mL of saline (0.9% NaCl, “control”); (2) mild depression-like mouse model, with mice administered 0.4 mL of saline (0.9% NaCl, “model”); (3) mice administered common fermented milk (“normal,” 0.4 mL of fermented milk without GABA); (4) mice administered a low dose of adzuki bean sprout fermented milk (“low,” 0.1 mL of GABA-enriched fermented milk with 0.3 mL of saline water sample per day); (5) mice administered half dose of adzuki bean sprout fermented milk (“medium,” 0.2 mL of GABA-enriched fermented milk with 0.2 mL of saline per day); (6) mice administered full dose of adzuki bean sprout fermented milk (“high,” 0.4 mL of GABA-enriched fermented milk sample per day); and (7) mice treated with fluoxetine (20 mg/kg of BW per day; “fluoxetine”). The dots in the rectangle represent the test mice; the 3 sections indicate the cabins for communication of the test mice. Different letters (a–d) indicate significant differences between groups ($P < 0.05$), and error bars indicate SD.

We studied the effects of the adzuki bean sprout fermented milk in a depression-like mouse model. In general, mice will actively make tentative contact with strange mice out of curiosity, but if mice are exposed to chronic social frustration stress, they will evade and not make contact with the strange mice (Nikoui et al., 2016). When mice were treated with the GABA-enriched adzuki bean sprout fermented milk, they showed a significantly higher index of sugar water preference and higher rates of social interaction and swimming immobility than mice in the control group. The trajectory maps of all of the fermented milk groups were more uniform and regular than those for the model group. These data indicate that the GABA-enriched adzuki bean sprout fermented milk can effectively relieve mild depression-like symptoms in a mouse model. Chronic mild stress impairs GABA release and uptake in the

medial prefrontal cortex, leading to the lowered GABA levels found in major depression (Ma et al., 2016). Through the facilitated GABA transporter system, GABA can enter and exit the brain. Researchers have found that participants receiving a GABA-fortified beverage had less psychological fatigue after completion of a task than participants who did not receive the GABA supplement (Kanehira et al., 2011).

Neurotransmitters have predominated in the study of the pathogenesis of depression for more than 50 yr. It is generally accepted that one or more deficiencies in monoamine transmitters explain the physiopathology of depression (Bunney and Davis, 1965). Studies have shown that GABA administration can affect the levels of monoamine transmitters in the hippocampus (Petty, 1995). Serotonin (5-HT) is a critical monoamine neurotransmitter in the brain. Many studies have shown

Figure 5. Quantification of monoamines in mice hippocampus measured by ELISA. The concentrations of 5-hydroxytryptamine (5-HT; A), norepinephrine (NE; B), dopamine (DA; C) and cyclic AMP (cAMP; D) were measured in differently treated groups. Groups: (1) healthy mice administered 0.4 mL of saline (0.9% NaCl, “control”); (2) mild depression-like mouse model, with mice administered 0.4 mL of saline (0.9% NaCl, “model”); (3) mice administered common fermented milk [“normal,” 0.4 mL of fermented milk without γ -aminobutyric acid (GABA)]; (4) mice administered a low dose of adzuki bean sprout fermented milk [“low,” 0.1 mL of GABA-enriched fermented milk with 0.3 mL of saline sample per day]; (5) mice administered half dose of adzuki bean sprout fermented milk [“medium,” 0.2 mL of GABA-enriched fermented milk with 0.2 mL of saline per day]; (6) mice administered full dose of adzuki bean sprout fermented milk [“high,” 0.4 mL of GABA-enriched fermented milk sample per day]; and (7) mice treated with fluoxetine (20 mg/kg of BW per day; “fluoxetine”). Different letters (a–d) indicate significant differences between groups ($P < 0.05$), and error bars indicate the standard deviation (SD).

that it plays a regulatory role in neurobehavior and emotional regulation, and that altered 5-HT activity in the brain may lead to depression, anxiety, or mania (Chen et al., 2019). The deficiency can be due to abnormal endogenous release and secondary exhaustion caused by chronic systemic stimulation. In this study, the high-dose fermented milk group may have experienced decreased symptoms of depression due to an increase in 5-HT content. Similar trends were found in NE levels. Dopamine is another neurotransmitter, and it is most relevant to the reward mechanism. Studies have found that DA receptor polymorphisms and lack of pleasure in individuals with depression are closely related (Juárez Olguín et al., 2016). Preventing

chronic social frustration and depression in mice may be achieved to some extent by increasing DA content.

There are 3 types of GABA receptors: GABA_A, GABA_B, and GABA_C. The B receptor is a G protein-coupled transmembrane-type metabolic receptor, which is distributed on the presynaptic and posterior membranes (Hirano, 2016). Activation of the GABA_B receptor causes changes in K⁺ and Ca²⁺ channels by regulating neurotransmitter and hormone release, and affects the production of the second messenger cyclic adenylylate (cAMP) in the brain (Cunningham and Enna, 1996; Nikoui et al., 2016). The cAMP signaling pathway was one of the earliest studied and it has been researched the most thoroughly in relation to the pathogenesis

Figure 6. Western blot analysis of the proteins related to the γ -aminobutyric acid (GABA) B receptor-cAMP-protein kinase A-cAMP-response element binding protein (GABA_B-cAMP-PKA-CREB) signaling pathway in mice hippocampus when treated with GABA-enriched fermented milk. (A) GABA_B, PKA, CREB, and phosphorylated (p)-CREB protein expression levels in mice hippocampus. Quantification of the expression of GABA_B (B), PKA (C), and p-CREB/CREB ratio (D) protein in the mouse hippocampus using Image-Pro Plus software (Media Cybernetics, Rockville, MD). Groups: (1) healthy mice administered 0.4 mL of saline (0.9% NaCl, “control”); (2) mild depression-like mouse model, with mice administered 0.4 mL of saline (0.9% NaCl, “model”); (3) mice administered common fermented milk (“normal,” 0.4 mL of fermented milk without GABA); (4) mice administered a low dose of adzuki bean sprout fermented milk (“low,” 0.1 mL of GABA-enriched fermented milk with 0.3 mL of saline per day); (5) mice administered half dose of adzuki bean sprout fermented milk (“medium,” 0.2 mL of GABA-enriched fermented milk with 0.2 mL of saline water sample per day); (6) mice administered full dose of adzuki bean sprout fermented milk (“high,” 0.4 mL of GABA-enriched fermented milk sample per day); and (7) mice treated with fluoxetine (20 mg/kg of BW per day; “fluoxetine”). Different letters (a–d) indicate significant differences between groups ($P < 0.05$), and error bars indicate the standard deviation (SD).

of depression. The GABA_B receptor can modulate p-CREB expression in the nucleolus through PKA with a cAMP-dependent signaling pathway. Additionally, as the activator of PKA, cAMP can be translocated into the nuclei along with the catalytic subunit of PKA to phosphorylate CREB, thus regulating neurological functions in the GABA_B-cAMP-PKA-CREB signaling pathway (Lee et al., 2011; Ali Shah et al., 2013). A variety of nervous system functions are regulated by CREB and it plays an important role in the central nervous system (Whitaker and Wei, 2009), although its regulatory transcription function is affected by its own phosphorylation (p-CREB; Saeki et al., 1999). Phosphorylation of CREB can regulate the activity of transcription factors, mediating cell function in the nervous system (Zamarbide et al., 2014). Compared with the model group, mice in the medium- and high-dose GABA-enriched adzuki bean sprout fermented milk groups showed significantly increased expression levels of GABA_B, PKA, and p-CREB proteins in the mouse hippocampus, which means that the GABA_B-cAMP-PKA-CREB signaling pathway was related to the symptoms of depression, and that GABA-enriched adzuki bean sprout fermented milk is a potential dietary approach to preventing mild depression in mice—and potentially in humans.

CONCLUSIONS

We prepared a functional fermented milk by combining a lactic acid bacterial strain *Lb. brevis* J1 that was shown to have high GABA production and an adzuki bean sprout fermented milk that was also rich in GABA. The obtained fermented milk displayed a significant effect in relieving mild depression-like symptoms in a mouse model. The results of this study suggest that GABA-enriched adzuki bean sprout fermented milk has potential as a new dietary therapy for the adjuvant treatment of chronic social stress.

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ORCIDS

- Zhen Wu <https://orcid.org/0000-0002-3607-6440>
 Pengyu Wang <https://orcid.org/0000-0002-8017-3045>
 Daodong Pan <https://orcid.org/0000-0002-5299-644X>
 Yuxing Guo <https://orcid.org/0000-0003-2935-9781>