RESEARCH ARTICLE

Effects of probiotic fermented soymilk on the activities of mouse intestinal digestive enzymes

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Received: December 28, 2022; **accepted:** March 12, 2023.

Digestive enzymes play the important roles in human food digestion. Normal gastrointestinal enzyme activities are the basis for ensuring the stable functioning of the digestive system and the human health. Probiotics are active microorganisms that promote human gastrointestinal motility and regulate intestinal enzyme activities. For the purpose of improving human intestinal digestive function, this study employed experimental animals to investigate the effects of probiotics on the activities of intestinal digestive enzymes. 60 specific pathogen-free male ICR mice were involved in this research. The probiotic fermented soymilk was prepared by mixing pure milk and soymilk in a ratio of 3:7 with the addition of *Lactobacillus plantarum* and *Bifidobacterium*. The probiotic fermented soymilk was then added into the basal food of mice in the concentration of 0%, 15%, and 30%, respectively. The animal feces were sampled after feeding to measure the enzyme activities of intestinal amylase, protease, and lipase. The effects of probiotic fermented soymilk on the regulation of intestinal digestion were then assessed. The results showed that the most ideal enzyme activities were observed by feeding animals with 15% probiotic fermented soymilk, while feeding less concentrated probiotic fermented soymilk would reduce the secretion of intestinal enzymes. However, although feeding animals with high concentrated probiotic fermented soymilk would increase the secretion of intestinal enzymes, it might cause a consequence of damaging and further destroying intestinal cells and reduce the intestinal digestive enzyme activities. This study confirmed that optimal concentrated, probiotic fermented soymilk could increase the activities of intestinal amylase, protease, and lipase of the mouse, which laid the foundation for further exploration of the effects of probiotic fermented soymilk on human intestinal digestive enzymes.

Keywords: probiotics; fermented soymilk; intestine; digestive enzymes.

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Introduction

With the fast development of biotechnology and genetic engineering in recent decades, the application of biogenic food additives such as probiotics in food science field has been widely promoted [1]. It has been well known that probiotics are active microorganisms that promote human gastrointestinal motility and digestive system health, while it has strong adaptability in the animal intestinal tract and external environment. Probiotics are not only tolerance to stomach acid and the protease-rich body fluid [2], but also multiplying rapidly in such environment [3]. Therefore, probiotics have great potential to improve the functions of gastrointestinal tract. Some previous studies combined probiotics with other chemicals to produce the newly created, compounded bacterial product. In addition, studies focused on the effects of probiotics on the intestinal digestive enzyme activities have been widely conducted. Some previous studies that investigated the potential mechanisms of fermented soybean meal with a compound probiotic to affect intestinal digestive enzyme activities in weaned pigs showed that probiotics could increase the activities of intestinal digestive enzymes in piglets, thereby, reducing the diarrhea rate of piglets [4, 5]. Those studies also verified that probiotics had a positive effect on improving intestinal function.

Soymilk is a vegetable protein drink that is popularly consumed by human being worldwide. Soymilk is enriched with amino acids that are closely related to the essential elemental requirements of the human body. Therefore, moderate intake of soymilk is benefit to human health. By adding probiotics to the soymilk to make probiotic fermented soymilk is a relatively innovative trial, which can not only maintain the microbial flora balance in the human intestinal tract, but also improve the activities of intestinal digestive enzymes.

Mouse has many similar physiological and anatomical features as humans [6, 7], and has been used as the animal mode in biomedical studies for many years. This study employed male mouse without specific pathogens as the experimental subjects to study the effects of probiotic fermented soymilk on the activities of mouse intestinal digestive enzymes. The results of this study might lay the foundation for further exploring the effects of probiotic fermented soymilk on human intestinal digestive enzyme activities.

Materials and methods

Experimental animal and probiotics

Total 60 pathogen-free, ICR male mice were acquired from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd, Beijing, China. All animals were 12 weeks old with the average weight of 23 ± 2 grams. The procedures of animal handling and animal experiments were approved by Institutional Ethics Committee of School of Biological and Environmental Engineering, Xi’an University (Xi’an, Shaanxi, China). The probiotics employed in this study were *Lactobacillus plantarum* and *Bifidobacterium*, which were acquired from Shanghai Biotechnology Co., Ltd. (Shanghai, China) and Beijing Municipal Hospital (Beijing, China), respectively. A single colony from each probiotic was inoculated into 50 mL of MRS medium (Peptone 10 g/L, Yeast extract 4 g/L, Beef extract 10 g/L, Glucose 20 g/L, Sodium acetate 5 g/L, Diammonium citrate 2 g/L, Magnesium sulfate 0.2 g/L, Manganese sulfate 0.05 g/L, Dipotassium hydrogen phosphate 2 g/L, Agar 15.5 g/L) (Beijing Aoboxing Biotechnology Co., Ltd., Beijing, China) in a 100 mL anaerobic bottle and incubated in PXY-190S-A Biochemical incubator (Guangdong Instrument Meter Co., Ltd. Guangzhou, Guangdong, China) at 37°C for 48 hours [8, 9], and then stored at -20°C for future experiments.

Preparation of probiotic fermented soymilk

Selected soybeans were soaked in 0.5% sodium bicarbonate solution at a ratio of 1 g soybeans in 4 mL of $NaHCO_3$ at 30°C for 14 hours before boiling in water for 3 mins at a ratio of 1 g soybeans in 46 mL of water. After filtering through a sieve with the mesh size of 180, sucrose (40 g/L) was added and thoroughly dissolved to make the pure soymilk with 12% solid content [10]. The soymilk was then mixed with sterilized, pure skimmed milk (Mengniu, Hohhot, Inner Mongolia, China) in a ratio of 7:3. The mixed milk was sterilized at 100°C for 15 mins, and then, cooled down to 45°C before adding 5% prepared active probiotics. The mixture of milk and probiotics was fermented at 42°C for 4 hours, and then, stored at 4°C for 24 hours before following experiments [11].

Animal treatment with probiotic fermented soymilk and fecal sample collection

The mice were divided into three groups including control, low-dose, and high-dose with daily basic diet of 40% corn, 26% bran, 29% bean cake, 1% salt, 1% bone meal, 3% vitamin, and water. The probiotic fermented soymilk was added to the experimental groups at the time of 9:00, 12:00, and 18:00 daily for 14 consecutive days. The low-dose group was fed with the addition of 15% probiotic fermented soymilk, while the high-dose group was fed with the addition of 30% probiotic fermented soymilk. The control group was on basic diet only. The fresh stool samples of experimental animals were collected starting at 2 hours after the end of 14 days probiotic fermented soymilk treatment, and then, every two hours at the time points of 4, 6, 8, 10 hours [12-13].

Determination of different index values of probiotic fermented soymilk

1. Probiotic determination

0.1 g fresh stool sample was diluted in 0.9 mL of glass beads solution (1:10 dilution) with shaking for 30 mins to homogenize the sample. The samples were then applied to eosin methylene blue (EMB) and *E. coli*/Coliform Count (EC) plates and dried for 1 hour. Those obligate anaerobic plates were incubated for 24-48 hours before titration with EMB and EC media (1 µL per drop) in 3 dilutions per plate from high dilution to low dilution for the bacteria determination.

(1) Probiotic growth curves:

Identified live *Lactobacillus plantarum* and *Bifidobacterium* were seedling in the liquid media to determine the probiotic growth curves. The samples were taken in every two hours to be measured by using colorimetric tube turbidimetry (Hangzhou Trian Technology Co., Ltd, Hangzhou, Zhejiang, China). The probiotic growth curves were then determined [14-15].

(2) Conductivity of probiotic fermentation broth:

The electrical conductivities of probiotic cultural media were detected by inserting two electrodes into the probiotic fermentation broth for resistance (R) measurement to determine the integrity of probiotic cells.

(3) Probiotic cell structure:

The cell structures and characteristics were observed by using atomic force microscopy (AFM) (Kaigonas Instrumen, Shanghai, China) [16].

2. Determination of intestinal enzyme activities

The activities of mouse intestinal amylase, protease, and lipase were inspected in this study. 50 gram of mouse feces was dissolved in 5 mL of saline as the test sample for each enzymatic experiment.

(1) Amylase activity:

The amylase activity was measured by mixing 5 mL of 0.6% soluble starch solution with 5 mL of probiotic fermented soymilk treated animal feces. The reaction mixture was fully reacted at 30°C for 2 mins before 5.0 mL of phosphate buffer (pH 6.8) was added. The reaction was incubated at 30°C water bath with the addition of 1 mL of 0.5% dilute iodine. After 48 hours incubation, the color of the reaction mixture was measured by using pH color test paper (Hangzhou Trian Technology Co., Ltd, Hangzhou, Zhejiang, China). The dilute iodine solution was used as the control. The enzyme activity was determined according to the color difference comparing to the standard color scale [17].

(2) Protease activity:

Casein was used to detect the protease activity in this study. Briefly, 5 mL of 0.5% casein solution was mixed with 5 mL of probiotic fermented soymilk treated animal faces and incubated at 30°C for 2 mins before adding 10 mL of 20% trichloroacetic acid [18]. After 10 mins incubation, the complete precipitate was then filtered by using filter paper. The filtrate was then added to the mixture of 1 mL of 0.25% sodium carbonate and 1 mL of 0.25% Alin solution. The reaction was incubated at 100°C for 2 mins to develop the color. The control reaction was prepared by mixing trichloroacetic acid with probiotic fermented soymilk first, and then, adding casein solution. The enzyme activity was determined by comparing the color of tested pH strip to the standard color scale.

(3) Lipase activity:
The content of polyvinyl alcohol (PVA) was used to determine the lipase activity. Briefly, 2 mL of olive oil was added to 5 mL of probiotic fermented soymilk treated animal feces and processed with a high-speed chewing processor (Zhuzhou Jinxin Cemented Carbide Group Co., Ltd, Zhuzhou, Hunan, China). 5 mL of milky white solution was mixed with 5 mL of phosphate buffer [19], which was preheated in water bath at 20°C. The reaction was incubated at 20°C for 10 mins, and then, 5 g of mixed sediment was removed for color detection by using pH test paper. The control experiment was prepared by mixing 0.05 mL of ethanol and 50 mL of test solution, and then, incubating for 5 mins [20]. The color of the reaction was compared to the standard color scale and recorded. The lipase activity was then calculated by using the following formula:

$$X = (V - V_0) \times C / 0.05 \times 50 \times n \times 1/15$$

where X was the lipase activity, V was the standard solution volume consumed during titration, V_0 was the standard solution volume consumed when titrating blank, C was the concentration of standard solution, n was the dilution factor.

Statistical analysis
SPSS (version 11.0) software (IBM, Armonk, New York, USA) was used for statistical analysis in this study. The experimental data were expressed as mean ± standard deviation (SD). One-way analysis of variance was performed with the *P* < 0.05 as the significant difference.

Results and discussion

Features of probiotics
The growth curves of *Lactobacillus plantarum* and *Bifidobacterium* were shown in Figure 1. The results demonstrated that both *Lactobacillus plantarum* and *Bifidobacterium* were grown properly. The conductivities of the probiotic fermentation broths were shown in Figure 2, which reflected the ionic capacity in the probiotic fermentation booth. The probiotic cell structure was also examined by using atomic force microscopy (Figure 3). Those results confirmed that the probiotic cells used for this study demonstrated the normal structure and growth patten with a certain degree of integrity.

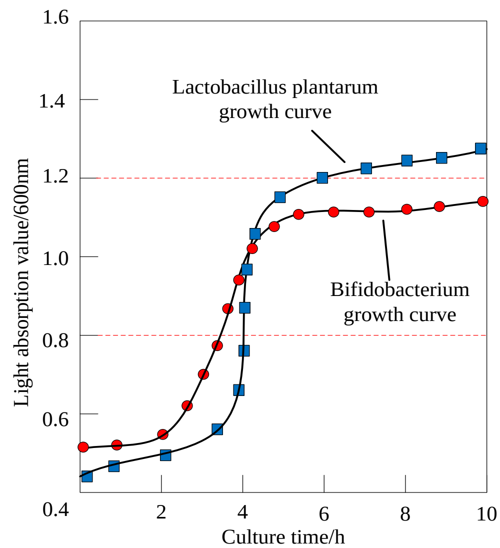

Figure 1. Growth curve of probiotics.

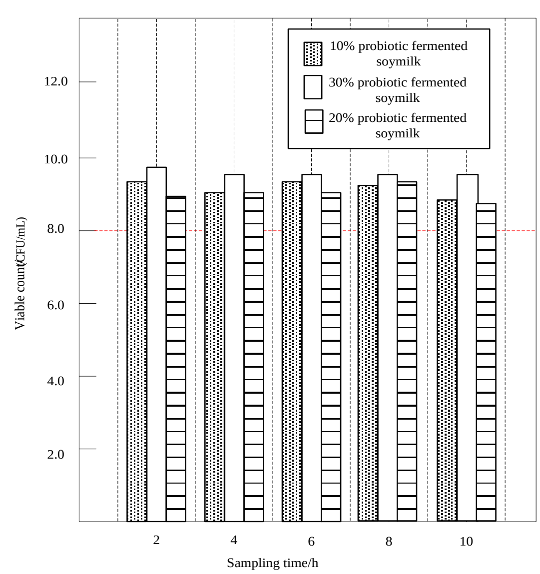

Figure 2. Conductivities of probiotic fermentation broths.

Figure 3. Probiotic cell structure.

The effects of probiotic fermented soymilk on intestinal enzyme activities

The digestive enzymes, mainly proteases, play an important role in gastroenteric system function regulations. This study inspected the three major intestinal enzymes' activities including amylase, protease and lipase and applied them as the indicators to evaluate the effects of different concentrated probiotic fermented soymilk on the activities of intestinal digestive enzymes. The activities of intestinal amylase, protease, and lipase from the three tested animal groups fed with different concentrations of probiotic fermented soymilk were shown in Figure 4. The results showed that the amylase activity of the control group was the lowest one comparing to that of experimental groups. However, the amylase activity of 15% probiotic fermented soymilk feeding group demonstrated a gradually increasing trend and even higher than that of 30% probiotic fermented soymilk feeding group after 4 hours end of feeding. On the other hand, the 30% probiotic fermented soymilk feeding group showed gradually increased amylase activity in the first 4 hours and then turned to stabilized level (Figure 4a). The results demonstrated that 15% probiotic fermented soymilk in the basal diet exhibited the optimal amylase activity. The results indicated that probiotic fermented soymilk might impact the activity of intestinal amylase of experimental animals, and 15% probiotic fermented soymilk group demonstrated the highest amylase activity at the sampling time of 10 hour after ending the treatment.

The intestinal protease activities were also increased in 15% and 30% probiotic fermented soymilk treated animals comparing to that in the control group. There was no significant difference between the two treatment groups. However, the protease activity of 15% probiotic fermented soymilk treated group was still higher than that of 30% probiotic fermented soymilk group. In addition, the protease activity gradually increased with the prolongation of sampling time (Figure 4b). The 15% probiotic fermented soymilk group demonstrated the highest protease activity at the sampling time of 10 hour after ending the treatment.

The intestinal lipase activities in both treatment groups were greater than that of control group and demonstrated the similar significant increase trend as the other two enzymes with 15% probiotic fermented soymilk group higher than 30% probiotic fermented soymilk group, especially during 2 - 6 sampling hours (Figure 4c). The result showed that probiotic fermented soymilk could also affect the activity of intestinal lipase. It was observed that mice fed with 15% probiotic fermented soymilk had the continuously increased lipase activity during the 10 hour sampling period.

The enzymatic activity results showed that the addition of 15% probiotic fermented soymilk in the basic diet increased amylase, protease, and lipase activities significantly. The improvement of enzyme activities was possibly related to the increase of enzyme secretion and/or the decrease of enzyme degradation. Digestive enzymes are mainly derived from the liver, pancreas, and intestinal juice, while intestinal digestive enzymes are mainly derived from the

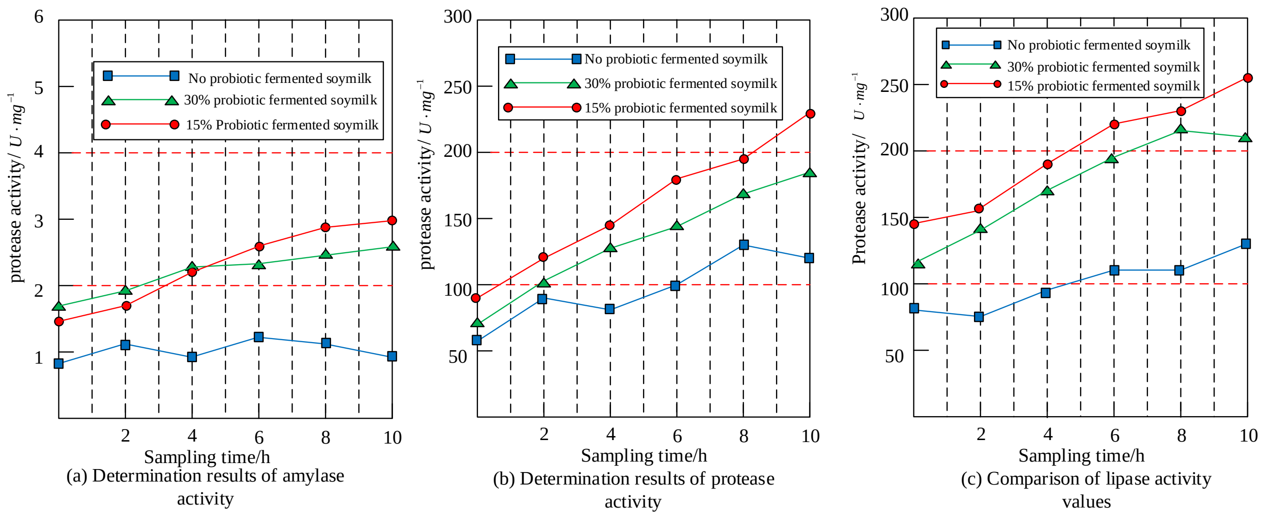

Figure 4. The enzymatic activities of experimental animal feces. (a) amylase activities, (b) protease activities, (c) lipase activities.

brush border of intestinal villi epithelial cells. When the brush border epithelial cells were shed, the enzymes inside the cells entered the intestinal lumen. The probiotic fermented soymilk may have the similar impacts on the activities of intestinal enzymes as the regular administration of probiotics. The probiotic fermented soymilk could also promote the growth of beneficial bacteria such as *Bifidobacteria* and lactic acid production bacteria. Those bacteria and their metabolites would then stimulate the intestinal peristalsis and the secretion of digestive enzymes, and therefore, increased the activities of intestinal enzymes. On the other hand, the proliferation of probiotics in probiotic fermented soymilk inhibited the proliferation of harmful bacteria such as *Escherichia coli* and *Clostridium*. The reduction of harmful bacteria would lead to the reduction of intestinal lysate, which would reduce the enzyme degradation, thereby, increasing the activity of digestive enzymes. Probiotic fermented soymilk might regulate the environment of experimental animal intestinal tract, thereby, laying a physiological foundation for the intestinal absorption of nutrients. Administration of sufficient quantity of probiotic fermented soymilk containing probiotics might effectively boost the activity of digestive enzymes in the intestinal tract. However, excessive intake of probiotic fermented soymilk was not recommended because, based on the experimental results, 15% probiotic fermented soymilk was in the more optimal concentration than 30%. The results suggested that, if too little probiotic fermented soymilk was fed, the secretion of intestinal enzymes would be low, which would not promote the intestinal enzyme activities. On the other hand, if too much probiotic fermented soymilk was fed, the secretion of intestinal enzymes would be high and might damage some intestinal cells and, thereby, affecting the activities of total intestinal digestive enzymes.

Conclusion

Digestive enzymes play an important role in the process of chemical digestion. This study examined the effects of probiotic fermented soymilk on the activities of intestinal amylase, protease, and lipase in experimental mice. The results confirmed that probiotic fermented soymilk might enhance and regulate the intestinal health by increasing the activities of digestive enzymes. However, it was necessary to control the intake amount of probiotic fermented soymilk to maximize the intestinal digestive enzyme activities.

Acknowledgments

The research was supported by National Natural Science Foundation for Youth (No. 32100389)

References

1. Dai S, Pan M, El-Nezami H, Wan J, Wang M, Habimana O, *et al*. 2019. Effects of lactic acid bacteria-fermented soymilk on isoflavone metabolites and short-chain fatty acids excretion and their modulating effects on gut microbiota. J Food Sci. 84(7):1854-1863.
2. Liu H, Liu Z, Lou K, Chao Q, Zeng J. 2020. Effects of different probiotics on body weight gain, intestinal enzyme activity and immune indexes of Liuzhou Ma chicken. J Agri. 33(01):198-205.
3. Hu T, Chen R, Qian Y, Ye K, Long X, Park K, *et al*. 2022. Antioxidant effect of *Lactobacillus fermentum* HFY02-fermented soy milk on D-galactose-induced aging mouse model. Food Sci Human Wellness. 11(5):1362-1372.
4. Yue X, Fu X, Hu L, Han X. 2016. Effects of soybean meal fermented with probiotics on intestinal morphology and digestive enzyme activities of weaned piglets. Chin J Anim Husb. 52(11):49-54.
5. Zhong X, Li X, Cai W, Xu C, Li Q, Huang J, *et al*. 2018. Effects of fermented feed on growth performance, digestive enzyme activity, fillet quality and immunity of juvenile carp. J Nanjing Agri Univ. 41(1):154-162.
6. Li C, Liu H, Yang J, Mu J, Wang R, Zhao X. 2020. Effect of soybean milk fermented with *Lactobacillus plantarum* HFY01 isolated from yak yogurt on weight loss and lipid reduction in mice with obesity induced by a high-fat diet. RSC Adv. 10(56):34276-34289.
7. Pan F, Yang M, Liu M, Hu W, Wang Y. 2019. Growth-stimulating effects of pea protein hydrolysates on probiotics. J Chin Inst Food Sci Technol. 19(2):32-41.
8. Zhu Y, Thakur K, Feng J, Cai J, Zhang J, Hu F, *et al*. 2020. B-vitamin enriched fermented soymilk: A novel strategy for soy-based functional foods development. Trends Food Sci Technol. 105:43-55.
9. Kumari M, Kokkiligadda A, Dasriya V, Naithani H. 2022. Functional relevance and health benefits of soymilk fermented by lactic acid bacteria. J Appl Microbiol. 133(1):104-119.
10. Abd Rahim M, Hazrin-Chong N, Harith H, Wan-Motar W, Sukor R. 2023. Roles of fermented plant-, dairy-and meat-based foods in the modulation of allergic responses. Food Sci Human Wellness. 12(3):691-701.
11. Zhu Y, Thakur K, Feng J, Zhang J, Hu F, Cespedes-Acuña C, *et al*. 2022. Riboflavin bioenriched soymilk alleviates oxidative stress mediated liver injury, intestinal inflammation, and gut microbiota modification in B2 depletion–repletion mice. J Agri Food Chem. 70(12):3818-3831.
12. Jiang XL. 2021. Application of probiotics in the digestive system. Chin J Dig Dis Imagin (El Ed). 11(02):49-53.
13. Xu R. 2019. Observation on the effect of compound digestive enzyme capsules combined with probiotics in the treatment of children with functional dyspepsia. Drug Eval. 16(04):56-58+66.
14. Tian L, Zhao R, Xu X, Zhou Z, Xu X, Luo D, *et al*. 2023. Modulatory effects of *Lactiplantibacillus plantarum* on chronic metabolic diseases. Food Sci Human Wellness. 12(4):959-974.
15. Rasika D, Vidanarachchi J, Rocha R, Baltuazar C, Gruz A, Sant'Ana A, *et al*. 2021. Plant-based milk substitutes as emerging probiotic carriers. Curr Opin Food Sci. 38:8-20.
16. Zendeboodi F, Khorshidian N, Mortazavian A, Cruz A. 2020. Probiotic: conceptualization from a new approach. Curr Opin Food Sci. 32:103-123.
17. Li X, Jiang L, Xia Q, Zeng X, Wang W, Pan D, *et al*. 2021. Effects of novel flavonoid-enriched yogurt on the diversity of intestinal microbiota in mice. Braz J Microbiol. 52(4):2287-2298.
18. Zhao W, Liu Y, Latta M, Ma W, Wu Z, Chen P. 2019. Probiotics database: A potential source of fermented foods. Int J Food Prop. 22(1):198-217.
19. Wang Y, Meng X, Wang C, Yang C, Qian J, Li J. 2019. The influence of probiotics and synbiotics on intestinal inflammation and microbiota in mice with acute colitis. Chin J Intern Med. 58(8):584-591.
20. Liu B, Xiao X, Zhou X, Zhou J, Lan L, Long X, *et al*. 2019. Effects of *Lactobacillus plantarum* CQPC01-fermented soybean milk on activated carbon-induced constipation through its antioxidant activity in mice. Food Sci Nutr. 7(6):2068-2082.