



Peanut meal extract fermented with *Bacillus natto* attenuates physiological and behavioural deficits in a D-galactose-induced ageing rat model

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Abstract

Our previous studies have shown that the nutritional properties of peanut meal after fermentation are markedly improved. In this study, in order to facilitate the further utilisation of peanut meal, we investigated the effects of its fermentation extract by *Bacillus natto* (FE) on cognitive ability, antioxidant activity of brain and protein expression of hippocampus of ageing rats induced by D-galactose. Seventy-two female SD rats aged 4–5 months were randomly divided into six groups: normal control group, ageing model group, FE low-dose group, FE medium-dose group, FE high-dose group and vitamin E positive control group. Morris water maze (MWM) test was performed to evaluate their effects on learning and memory ability in ageing rats. Superoxide dismutase (SOD) activity and malondialdehyde (MDA) content of brain, HE staining and the expression of γ -aminobutyric acid receptor 1 (GABA_BR1) and N-methyl-D-aspartic acid 2B receptor in the hippocampus of rats were measured. The results show that FE supplementation can effectively alleviate the decrease of thymus index induced by ageing, decrease the escape latency of MWM by 66.06 %, brain MDA by 28.04 %, hippocampus GABA_BR1 expression by 7.98 % and increase brain SOD by 63.54 % in ageing model rats. This study provides evidence for its anti-ageing effects and is a research basis for potential nutritional benefits of underutilised food by-products.

Key words: Peanut meal: *Bacillus natto*: Fermentation: Antioxidants: Learning and memory ability

In the contemporary ageing society, ageing and related diseases have become the most serious issues that need to be urgently addressed. The incidence and mortality of Alzheimer's disease, Parkinson's disease and other major diseases that endanger the health and life of the elderly also increase with age⁽¹⁾, placing a heavy burden on society and families. Almost all ageing brains exhibit characteristic changes associated with neurodegeneration⁽²⁾. Therefore, the prevention and improvement of various potential risk factors brought about by ageing via non-pharmacological means have become a research hotspot. Studies have demonstrated that supplementation of natural products with high antioxidant activity can increase antioxidant enzyme activities *in vivo*, reduce oxidative protein damage⁽³⁾, reverse memory impairment⁽⁴⁾ and delay brain ageing in the ageing mice⁽⁵⁾.

The annual production of peanuts in China currently ranks first worldwide, with the planting area and peanut production growing every year. More than 55 % of peanuts are used for oil production, and a large number of peanut meal by-products are produced during the pressing and refining process⁽⁶⁾. It is found that peanut meal is rich in a variety of nutrients, such as protein, fiber, minerals, vitamins, polyphenols and so on⁽⁷⁾, of which the content of protein is as high as 47–55 %⁽⁸⁾. However, due to the imperfect existing peanut oil processing technology, the protein denaturation in peanut meal after high temperature squeezing, the essential amino acid content is unbalanced and easy to be contaminated by aflatoxin⁽⁹⁾, thus peanut meal is mainly utilised as animal feed or even fertilizer, with low added value and massive waste of resources. In reaction to the phenomenon, many new processing technologies

Abbreviations: FL, FE low-dose group; FM, FE medium-dose group; FH, FE high-dose group; GABA_BR1, γ -aminobutyric acid receptor 1; MDA, malondialdehyde; MWM, Morris water maze; SOD, superoxide dismutase; NMDAR2B, N-methyl-D-aspartic acid 2B receptor; N, normal control group; M, ageing model group; Y, vitamin E positive control group.

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have emerged to improve the utilisation rate of peanut meal. Among them, the fermentation technology can take advantage of the abundant protein and soluble sugar in peanut meal to provide nutrition for microorganisms and produces plenty of proteins, amino acids small molecular peptides⁽¹⁰⁾ and polyphenols that are more easily to be absorbed and utilised by the human body through fermentation decomposition⁽¹¹⁾, which has been proved to be a low-cost and high-benefit processing technology.

Bacillus natto (*B. natto*) is a subspecies of *Bacillus subtilis*. It belongs to Gram-positive aerobic species, with long rod-shaped cells, acid and heat resistance and strong pathogenic bacteria inhibition ability^(12,13) and is one of the forty probiotics announced by the USA FDA⁽¹⁴⁾. *B. natto* can secrete enzymes such as protease, amylase, lipase and phytase and can decompose macromolecules including protein, fat and carbohydrates into small peptides, amino acids, organic acids, oligosaccharides and other components that are easily absorbed by the human body⁽¹⁵⁾, thereby improving the utilisation rate of fermentation products. More importantly, the food source of nattokinase has the advantages of convenient oral administration and excellent gastrointestinal stability compared with other commercial drugs^(16,17) and has been widely used in the fields of medicine, health food, agriculture and animal husbandry so far⁽¹⁸⁾. It has been shown that the fermentation products of *B. natto* have high antioxidant activity⁽¹⁹⁾, which can help eliminate the excessive free radicals in the body⁽²⁰⁾, and have the ability to improve or enhance cognitive function⁽²¹⁾. Therefore, using *B. natto* to ferment peanut meal can make reasonable utilisation of oil production waste and achieve improvements of nutritional composition.

Our previous studies have shown that the supplementation of the peanut meal extract fermented by *B. natto* remarkably improved the cognitive function of the mice during growth and development⁽²²⁾ and can effectively improve hyperlipidaemia caused by a high-fat diet in mice⁽²³⁾, but it is still unclear whether it has beneficial effects on cognitive function in ageing rats. Herein, based on the aforementioned beneficial potentialities of *B. natto* and fermented peanut meal, the D-galactose was used to establish ageing models, and the rats were fed with the extracts prepared from peanut meal fermented by *B. natto*. By employing the behavioural and histological methods, from the aspects of hippocampal tissue structure, oxidative stress homeostasis and learning and memory abilities, the anti-ageing effect of the extract on the rats have been studied, aiming to provide theoretical and experimental basis for the development of functional foods based on the rational utilisation of peanut by-product resources.

Materials and methods

Preparation of peanut meal extracts fermented with *Bacillus natto*

Peanut meal (Qingdao Kerry Vegetable Oils Co., Ltd., Qingdao, China) – soyabean meal – bran (7:2:1) was utilised as the fermentation medium, and then the seed liquid of *B. natto* (preserved in Food Engineering Laboratory, Qingdao University, Qingdao,

China) was inoculated. The mixture was cultured at 37°C for 38 h, and subsequently, the ferments were centrifuged, filtered and freeze-dried for reservation.

Determination of the main components of fermentation extract by *Bacillus natto*

The Lowry–Folin phenol method was implemented to measure the Natto kinase activity⁽²⁴⁾, free amino acids were determined via the ninhydrin method⁽²⁵⁾, peptides were detected by the biuret method⁽²⁶⁾, total flavonoids were tested by the NaNO₂-Al(NO₃)₃-NaOH chromogenic method⁽²⁷⁾, polysaccharide was measured by the phenol-sulfuric acid method⁽²⁸⁾ and the number of *B. natto* was calculated by the plate counting method⁽²⁹⁾.

Animals and materials

All the animal experiment protocols and procedures were approved by the Ethics Committee of Qingdao University (QYFY WZLL 25879), and the experiments followed the National Guidelines for the Care and Use of Laboratory Animals and adhered to the ARRIVE Guidelines for reporting animal research⁽³⁰⁾. Seventy-two specific pathogen free (SPF) Sprague-Dawley (SD) rats (female, 4–5 months old, about 240 g) were purchased from Experimental Animal Center of Shandong Lukang (license number SCXK Lu 20130001). The rats were housed in the laboratory animal facilities (School of Public Health, Qingdao University, Qingdao, China), placed under a 12-h light/dark cycle, controlled at 22 ± 2°C and allowed free access to food and water. After 1 week of adaptation, rats were divided into six groups by the random number table according to the experimental instructions⁽³¹⁾ as following: normal control group (N), ageing model group (M), FE low-dose group (FL), FE medium-dose group (FM), FE high-dose group (FH) and vitamin E positive control group (Y), each with 12 animals. Except for the group N (treated with the same dose of normal saline), other groups were injected intraperitoneally with 400 mg/ (kg/d) of D-galactose every day for 8 weeks to induce an ageing rat model⁽³²⁾. Besides the D-galactose treatment, different ingredients were given by gavage: groups N and M with 0.2 ml/10 (g/d) of normal saline, group Y with 100 mg/ (kg/d) of vitamin E, groups FL, FM and FH with FE at doses of 60 mg/ (kg/d), 300 mg/ (kg/d) and 1500 mg/ (kg/d), respectively. The experimental design is depicted in Fig. 1. Lastly, after intervention for 8 weeks, rats were sacrificed after pentobarbital anaesthesia, the blood samples were taken from abdominal aorta and centrifuged at 644 g for 10 min to obtain the serum, while organs of each tissue were taken and placed at –80°C for storage. The viscera indices were calculated according to the following formula: Index = weight of viscera (mg)/body weight (g).

Morris water maze tests

On the eighth week of intervention, learning and memory skills were measured by the Morris water maze (MWM) tests⁽³³⁾. The experiment was performed as previously described^(34,35). The rats were trained for 5 d, daily training was consisted of four stages in which the rats were put into the water from four different quadrants of the tank and the time from entering the water to



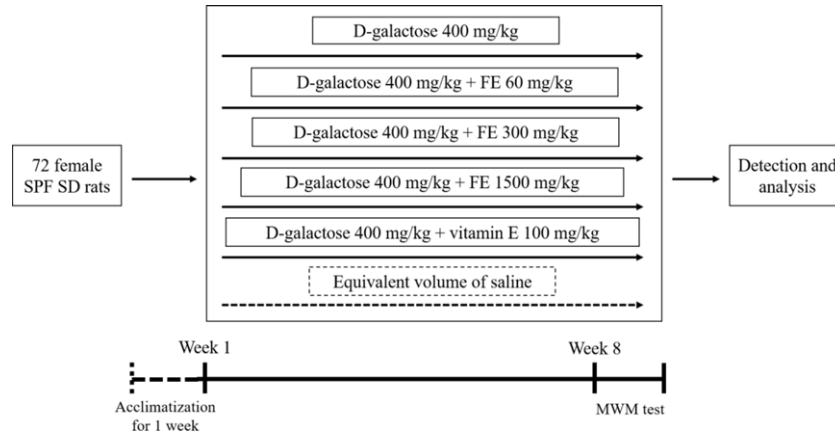


Fig. 1. Experimental protocol and design.

climbing the platform was recorded, namely, the escape latency. If the rats did not find the platform within 60 s, it was induced to the platform with a rod and kept for 10 s. In this case, the escape latency was recorded as 60 s. After 5 d, the platform was removed, and the rats were put into the water in the opposite side of the original platform quadrant. Then, the time ratio (the ratio of total swimming time in the target quadrant), distance ratio (the ratio of total swimming distance in target quadrant), swimming speeds and numbers of crossing over the platform were recorded by an MWM image analysis system (Mobile Datum).

Determination of antioxidant indexes of brain tissue

The brain tissues of the rats were finely minced and homogenised in 50 mM normal saline and centrifuged at 10 000 *g*, 4°C for 15 min in a Beckman Type 5430 refrigerated ultracentrifuge (Beckman Instruments). Afterwards, the supernatants were used for the antioxidant indexes assays.

The superoxide dismutase (SOD) activity was assessed by the total superoxide dismutase (T-SOD) assay kit (Hydroxylamine method, A001-1-2, Nanjing Jiancheng Bioengineering Institute), assayed using the xanthine/xanthine oxidase method based on the production of O²⁻ anions. The developed blue colour was measured at 550 nm. The activities of T-SOD were expressed as units per milligrams of protein (U/mgprot).

Malondialdehyde (MDA) content was determined by the MDA assay kit (TBA method, A003-1-2, Nanjing Jiancheng Bioengineering Institute). The optical density at 532 nm was measured via a spectrophotometer (UV-2000, Unicor Instrument, Shanghai, China). 1,1,3,3-Tetramethoxypropane was employed as an external standard. The level of MDA was expressed as nanomoles per milligram protein (U/mgprot).

Histological analysis of hippocampus

Cerebral hemispheres of the rats were taken out and fixed with 4% formaldehyde, embedded with paraffin, subjected to sectioning on microtome (CUT 5062, SLEE medical, Mainz, Germany) and the coronal sections with thickness of 6 mm were

obtained. The serial sections (six sections per hippocampus) at an interval of 200 mm between 1.8 and 3.2 mm from bregma were collected. The mounted sections were rinsed with distilled water and stained with alum haematoxylin followed by differentiating with 0.3% acid alcohol. After rinsing in distilled water, the sections were stained with eosin for 2 min and dehydrated for mounting, which were observed under a microscope (BX53, Olympus).

Expression detection of hippocampus γ -aminobutyric acid receptor 1 and N-methyl-D-aspartic acid 2B receptor

Expressions of GABA_BR1 and N-methyl-D-aspartic acid 2B receptor (NMDAR2B) were detected by the immunohistochemical techniques. The hippocampus sections were deparaffinised to water, washed with PBS for three times, sealed by dropping serum and placed at room temperature for 20 min. Mouse anti-mouse GABA_BR1 (Abcam) antibody and rabbit anti-mouse NMDAR2B (Abcam) antibody were added dropwise, placed overnight at 4°C and washed with PBS for three times; Cy3-Goat anti-mouse (Boster) and FITC-Goat anti-rabbit IgG (Boster) were added dropwise, incubated at 37°C for 1 h and rinsed with PBS for three times, while the staining was performed with diaminobenzidine. After the reaction was terminated, the film was dried, mounted and observed by a microscope with the IPP6-0 analysis system (BX53; Olympus Corp.), and the average optical density of positive cells was used as the quantitative expression result.

Statistical methods

All data were expressed as mean \pm standard deviation (sd). The data sets were first assessed for equal variance via Bartlett's test and for normality using the Kolmogorov-Smirnov test. The group means were compared by ANOVA followed by Fisher's least significant difference post hoc test. The body weight was compared by mixed model analysis. The difference was recognised as statistically significant when $P < 0.05$. Data analysis was performed by the SPSS 26.0 software (IBM Corp.) and Origin Pro 9.1 (OriginLab Corp.).

Results

Main components of fermentation extract by *Bacillus natto*

The tested results of the main composition of FE are listed in Table 1.

Evaluation of the ageing model

It can be observed that the rats in group N were in good physical condition, with white and shiny hair, elastic skin, normal diet and rapid movement, while the rats in group M exhibited prominent signs of ageing: emaciation, yellow and dull coat colour, skin laxity, slow movement and listlessness.

Effects of fermentation extract by *Bacillus natto* on growth performance in the ageing rats

The changes in average body weight of each group during the trial period are shown in Fig. 2(a). As seen, the body weights of the rats in group N were increased gradually from 241.67 g to 275.42 g in the whole experiment period, and the average body weight of the rats in group M showed a rising trend in the first 2 weeks as well, while from the third week onwards, the body weights were decreased gradually with the extension of modelling time which were declined from 240.33 to 235.73 g. The mean body weight of the rats in group FL was not notably changed, while those of group FM, group FH, and group Y were increased gradually by 7.99%, 12.18% and 12.75%, respectively.

There was no significant difference can be observed in liver index among all groups ($P > 0.05$). Compared with group N, the spleen index of group M was decreased from 2.35% to 2.11%, which was markedly decreased by 10.21% ($P < 0.05$), while that of group FM was increased by 9.19% ($P < 0.05$), in comparison with group M. The thymus index of group M was declined from 1.71% to 1.41%, compared with group N, which was remarkably decreased by 17.54% ($P < 0.05$), while those of groups FL, FM and FH were increased by 10.29%, 16.18% and 16.67%, respectively, compared with group M ($P < 0.05$). It can be inferred from the growth and organ indexes of rats that FE has no harmful effects on the health of the experimental animals.

Effects of fermentation extract by *Bacillus natto* on cognitive function in the ageing rats

Morris water maze test. The MWM was performed to evaluate the effects of FE on the learning and memory skills of rats. The swimming trajectory of each experimental group during probe tracking is shown in Fig. 3(a), and the last day of the trajectory is displayed in Fig. 3(b). Escape latencies, percentage of time and distance in target quadrant, numbers of crossing over the platform and average swimming speeds (Fig. 3(c)–(g)) were also observed.

As can be seen from Fig. 3(a) and (b), there were no notable differences in the swimming trajectories between all groups on the first day of training, while group M showed a significantly higher proportion of figure '8', large circling movements and longer times than those of the other groups on the last day, indicating that the rats in group M retained a less accurate spatial memory. However, the rats in group FL, FM and FH could

quickly find the platform position and exhibited strong spatial memory ability.

To remove the baseline difference, the slope change of escape latency during training of each rat was selected to evaluate the difference between each group, as seen in Fig. 3(c). Compared with group N, the escape latency of the rats in group M was prominently extended by 28.59%, while those of groups FM and FH were significantly shortened by 66.06% and 33.46%, compared with group M ($P < 0.05$). In terms of time ratio, distance ratio and times of crossing over the platform in the target quadrant, the rats in group M showed markedly different degrees of decline, compared with the rats in group N, which were 52.79, 55.07 and 75.82%, respectively ($P < 0.05$). Compared with group M, the time ratios of the rats in groups FL, FM and FH were remarkably risen by 35.43, 85.27 and 123.10%, respectively ($P < 0.05$), and the distance ratios of the rats in groups FL, FM and FH were notably elevated by 28.10, 94.48 and 106.19%, respectively ($P < 0.05$). As for crossing over the platform, groups FL, FM and FH were significantly increased by 75.19, 288.72 and 363.91%, respectively, compared with group M ($P < 0.05$). The swimming speed of the rats in groups FM and FH was significantly ascended by 9.52 and 12.45%, compared with that in group M ($P < 0.05$).

Effect of fermentation extract by *Bacillus natto* on antioxidant activity of the brain tissue in the ageing rats.

As shown in Fig. 4, the SOD activity in group M was significantly decreased by 30.22% from 137.19 U/mgprot to 95.73 U/mgprot, compared with that of group N ($P < 0.05$), while the activity was increased by 46.25% and 63.54% in groups FM and FH, respectively, compared with group M ($P < 0.05$). The MDA content in group M was markedly raised by 32.76% from 4.06 U/mgprot to 5.39 U/mgprot ($P < 0.05$), in comparison with group N. However, compared with group M, the MDA content in groups FM and FH was remarkably declined by 20.39% and 28.04%, respectively ($P < 0.05$).

Histomorphological observation of hippocampus. It can be observed from Fig. 5 that, in group N, the hippocampal neurons exhibited a clearly hierarchical and tight array, with large and round light staining nuclei, in which the nucleoli were clear. Comparatively, in group M, the neurons showed a disorganised pattern while its number was reduced, and the outline of nuclei was blurred with a large number of pyknotic degenerated cells, which were shuttle-shaped or triangle-shaped and deeply stained purple blue. In contrast with group M, groups FL, FM and FH displayed a more regular neuronal arrangement, clearer nuclear structure and a reduced number of nuclear pyknotic cells, which was most pronounced in group FH. These results indicate that FE can inhibit D-galactose-induced neuronal degeneration, thereby reducing neuronal damage, and helping to improve the learning and memory ability of the ageing model rats.

Expressions of γ -aminobutyric acid receptor 1 and N-methyl-D-aspartic acid 2B receptor in the hippocampus.

As seen in Fig. 6(a) and (b), positive expressions of GABA_BR1 and NMDAR2B can be observed from the results of



Table 1. Determined main composition in FE

Composition	NK (U/g)	Free amino acid (mg/ml)	Polypeptide (mg/ml)	Polysaccharide (mg/ml)	Total flavonoids (mg/ml)	Number of viable bacteria (CFU/ml)
Content	1360	54.89	10.32	10.36	10.28	4.2×10^{16}

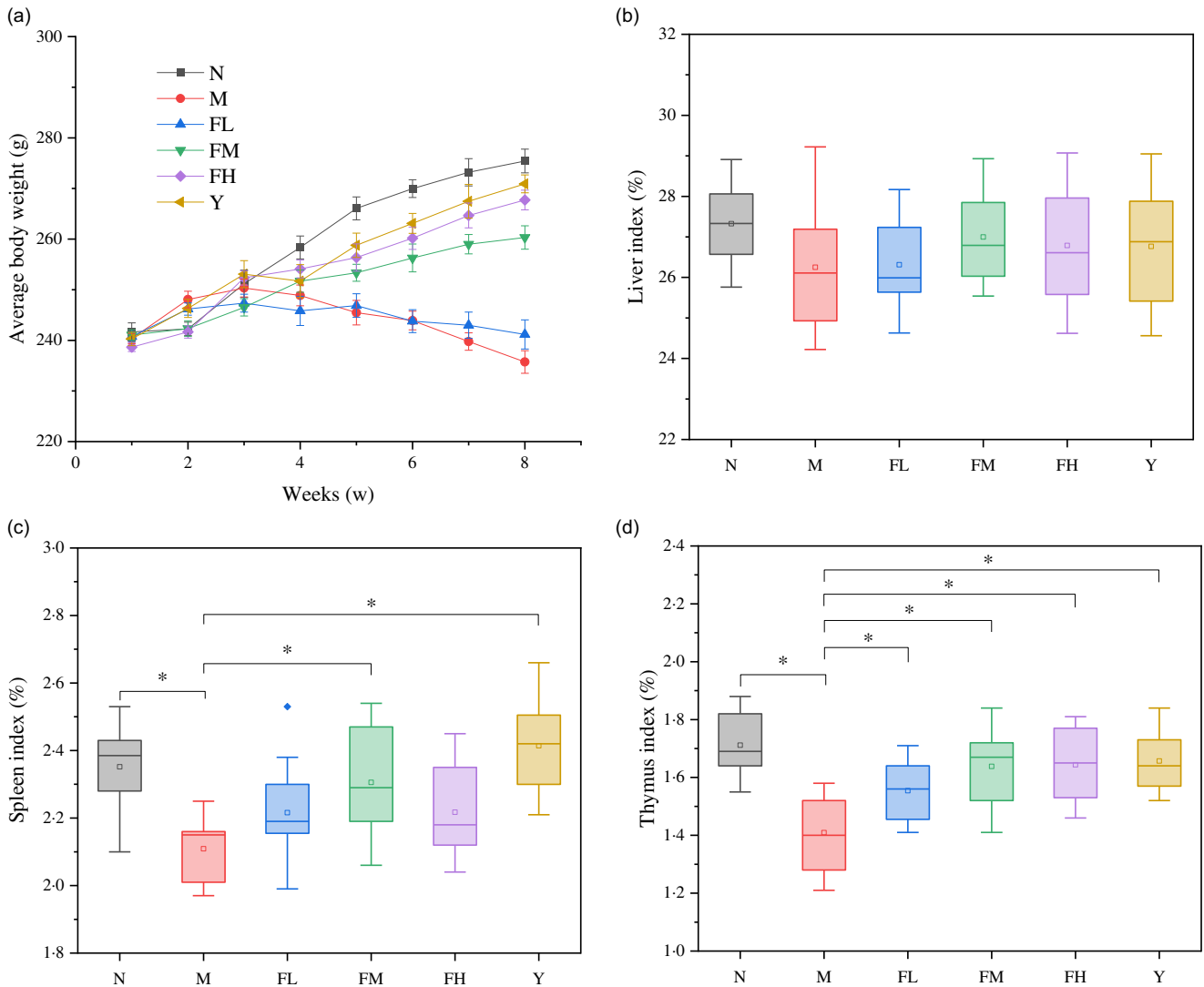


Fig. 2. (a) Weight gain of rats in each group; (b) liver index of rats in each group; (c) spleen index of rats in each group; (d) thymus index of rats in each group. N: normal group; M: model group; FL: fermented peanut meal extracts (FE) low-dose group; FM: FE medium-dose group; FH: FE high-dose group; Y: positive control group (VE).

immunohistochemistry and image analysis, and the cell membrane was stained in yellow.

It can be noticed from Fig. 6(c) and (d) that the expression of GABA_BR1 was elevated from 157.40 to 173.00 in group M, in comparison with group N, which was significantly increased by 9.91% ($P < 0.05$). And in group FH, the expression of GABA_BR1 was remarkably descended by 7.98%, compared with group M ($P < 0.05$). In contrast with group N, the expression of NMDAR2B was prominently declined by 10.31%, which was from 157.20 to 141.00 in group M ($P < 0.05$), and the expression in group FM was markedly raised by 3.83%, compared with group M ($P < 0.05$).

Discussion

Ageing is a progressive process in which the function of tissues and organs declines. Its physiological manifestations are mainly reflected in three aspects: morphological changes, such as decrease of cell number and changes in overall appearance; decline of physiological function and function reduction of major sensory organs^(36,37). Therefore, the evaluation of ageing is multidimensional. Among the hypotheses of ageing theory, the metabolic theory and free radical theory are generally accepted⁽³⁸⁾. These two mechanisms are involved in the ageing model prepared by D-galactose, which accumulate in animals by

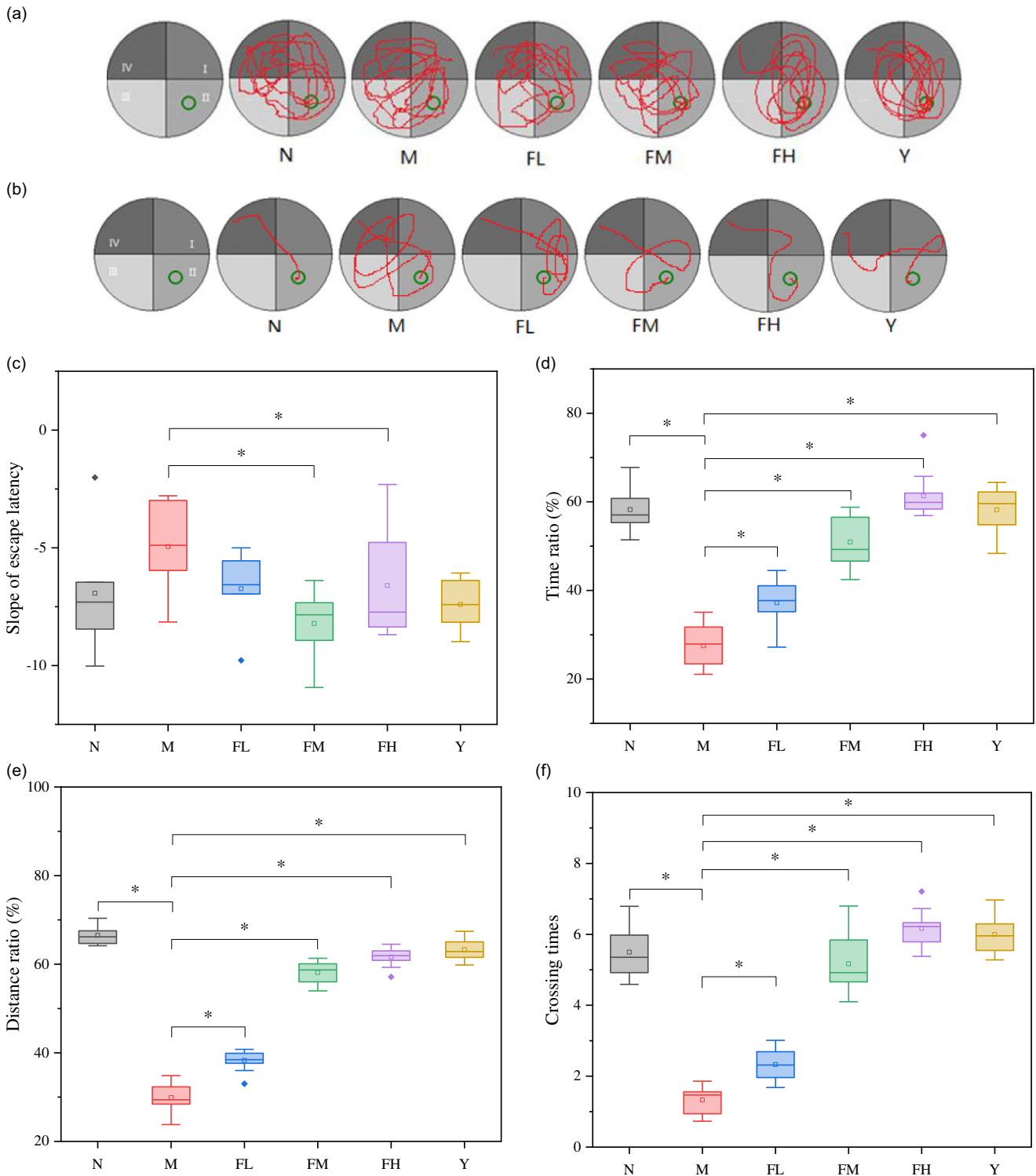


Fig. 3. (a) Swimming trajectory of the rats from each group on the first day; (b) swimming trajectory of the rats in each group on the last day; (c) escape latencies of each group in 5 d training trials; (d) percentage of time in target quadrant on the last day; (e) percentage of distance in target quadrant on the last day; (f) numbers of crossing over the platform on the last day; (g) average swimming speeds on the last day. N: normal group; M: model group; FL: fermented peanut meal extracts (FE) low-dose group; FM: FE medium-dose group; FH: FE high-dose group; Y: positive control group (VE).

continuous injection of D-galactose and increase intracellular osmotic pressure, eventually causing cell swelling, metabolic disorders and functional disorders⁽³⁹⁾. Meanwhile, a large

number of superoxide radicals can be generated during D-galactose metabolism, resulting in oxidative damage, reducing the immune function of the body, causing degenerative changes

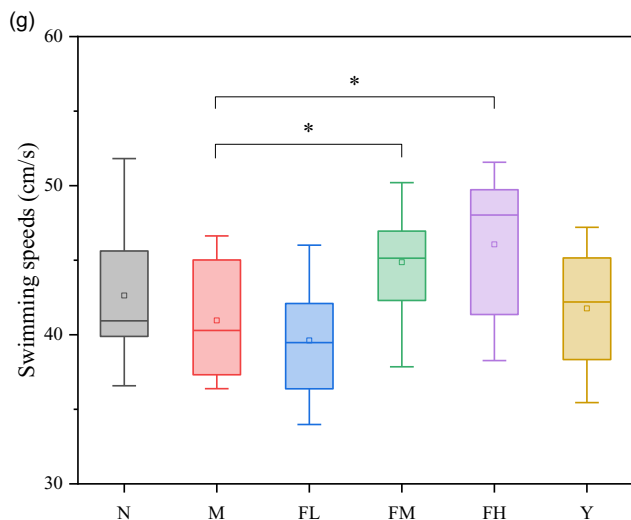


Fig. 3. Continued

in the central nervous system of animals and better simulating the natural ageing situation⁽⁴⁰⁾. Thus, it is widely used in the pharmacological study of neurodegenerative diseases⁽⁴¹⁾. In this study, the female rats were given subcutaneously 400 mg/(kg/d) of D-galactose daily for eight consecutive weeks. The rats in group M showed obvious signs of ageing, including mental fatigue, hair loss, weight loss and significant decrease of thymus index. Oxidative stress injury was observed in the brain, and the SOD activity was markedly dropped ($P < 0.05$), while the MDA content, which can reflect the degree of peroxidation, was significantly raised ($P < 0.05$). The results of MWM experiment show that the spatial learning and memory were declined, and neuronal damage occurred from the HE staining in hippocampal tissue. The immunohistochemical results reveal that the expression of GABA_BR1 in the hippocampus was remarkably increased ($P < 0.05$), while the expression of NMDAR2B was notably decreased ($P < 0.05$). It can be inferred from the above results that the ageing rat model prepared with D-galactose in this experiment was successfully replicated.

The immune system plays an important role in maintaining the stability of the internal environment of the body, ageing is one of the important triggers for reduced function of this system⁽⁴²⁾. With the growth of age, the immune function declines gradually, which is mainly manifested as the atrophy of immune organs, and the reduction of immune factor level⁽⁴³⁾, etc. Thymus is the main immune organ of the body, and calculation of thymus index can reflect the immune status to a certain extent⁽⁴⁴⁾. In this study, the thymus index of the rats in group M was significantly lower than that in group N, indicating that D-galactose could cause degenerative changes in the thymus of the ageing model rats, which was consistent with the literature reports^(45,46). According to the experimental data, the thymus index of groups FM and FH was notably higher than that of group M. It can be seen that a certain dose of FE can delay the ageing-induced thymic atrophy and exert a certain protective effect on the decline of immune function.

Ageing is an important risk factor for the development of many diseases, and neuroageing is a neurodegenerative disease closely related to age. Neuronal atrophy and neurotransmitter

reduction occur during brain ageing, in turn leading to sensory and unresponsiveness, cognitive decline and impaired brain function in the elderly, among which cognitive function is most severely affected by brain ageing⁽⁴⁷⁾. Studies have shown that impaired cognitive function due to brain ageing is found to be positively correlated with functional changes in brain regions of the hippocampus and prefrontal cortex by electron tomography⁽⁴⁸⁾. The main function of hippocampal brain regions is to regulate episodic memory and spatial memory, which plays an important role in memory consolidation. However, it is very fragile, and the structural and functional changes caused by ageing occurred earlier than other brain regions⁽⁴⁹⁾. Previous studies have shown that the hippocampal-related spatial learning and memory function are impaired with ageing⁽⁵⁰⁾. The MWM test is a classic behavioural experiment to examine the spatial memory ability of animals⁽⁵¹⁾. In this study, the spatial association and recall exploration ability of the rats in group M were significantly weakened, while the escape latency was markedly prolonged ($P < 0.05$), indicating that ageing could blur the spatial orientation of the rats and reduce their learning ability, which was similar to the results reported in related literature⁽⁵²⁾. At the same time, the swimming ability of the rats supplemented with FE was also compared. The escape latency of group FM and FH was remarkably shortened ($P < 0.05$), and the crossing times, time and distance ratio were notably increased ($P < 0.05$) compared with group M, indicating that the medium and high dose of FE can improve the learning and memory ability of the D-galactose-induced ageing rats. Moreover, the HE staining of the hippocampus showed that the nerve cells of the ageing rats supplemented with FE exhibited a tendency of recovery, and improved the expression changes of GABA_BR1 and NMDAR2B in the hippocampus. These results suggest that FE can exert a protective effect on D-galactose-induced neuronal injury in the rat hippocampus, which in turn enhances its learning and memory ability.

The brain has the highest oxygen demand in body and a weak antioxidant mechanism; therefore, it is highly susceptible to oxidative stress. Excessive reducing sugars can be converted into aldose and hydrogen peroxide in the body, producing reactive oxygen species and thus inducing oxidative stress⁽⁵³⁾. Normally, the body can ensure homeostasis by maintaining the generation of reactive oxygen species and the balance of antioxidant enzymes such as SOD; however, the decrease in the activity of antioxidant enzymes in the brain and the generation of excessive oxidative products may lead to cell tissue damage and physiological dysfunction of the brain, thus affecting learning and memory function^(54,55). Oxidative stress plays a key role in the ageing process of tissues and organs, thus the detection of oxidative stress markers *in vivo* can indirectly reflect the ageing degree of the body⁽⁵⁶⁾. SOD is the most important antioxidant enzyme in organisms, which has the effects of scavenging superoxide anion-free radical, anti-inflammatory and anti-ageing, etc., and the level of SOD activity can directly reflect the antioxidant capacity of tissues⁽⁵⁷⁾. When the activity of SOD decreases, the ability of body to eliminate oxygen-free radicals is weakened, which can induce the damage of DNA, protein and enzyme, thereby accelerating the ageing of various tissues and organs. Excessive reactive oxygen species production by

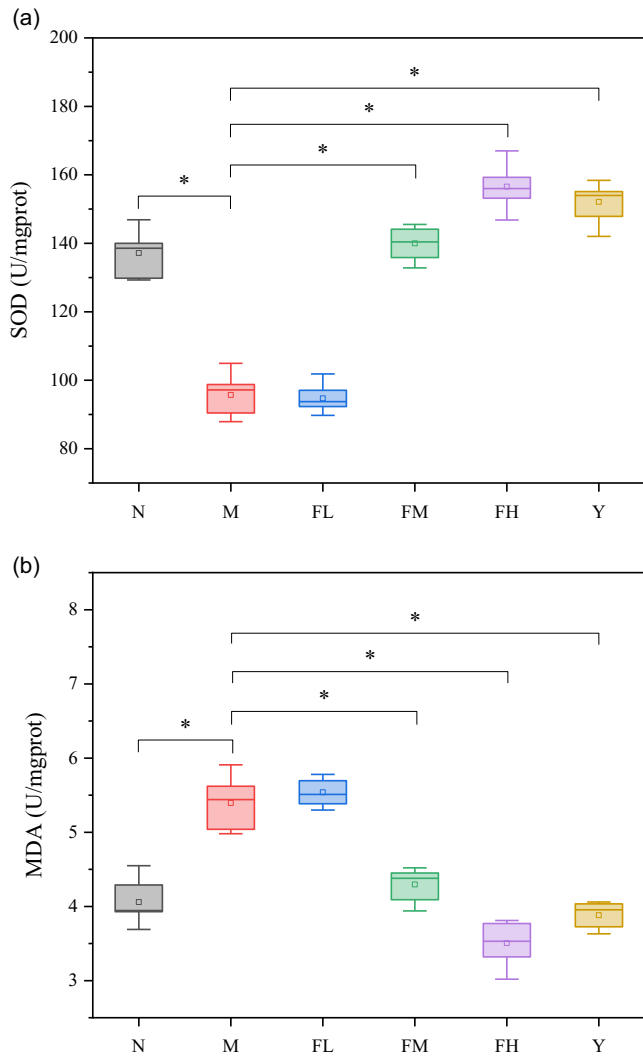


Fig. 4. Superoxide dismutase (SOD) (a) and malondialdehyde (MDA) (b) of ageing rats brain tissue. N: normal group; M: model group; FL: fermented peanut meal extracts (FE) low-dose group; FM: FE medium-dose group; FH: FE high-dose group; Y: positive control group (VE).

D-galactose can also accelerate the decomposition of biological tissues, leading to the increase of MDA concentration in the body. MDA is also an important marker of oxidative damage⁽⁵⁸⁾, and the measurement of MDA in tissues can objectively reflect the content of lipid peroxide in the body. Excess MDA accumulation has been shown to cause impaired cognitive function⁽⁵⁹⁾. Therefore, in this study, SOD and MDA, the biomarkers most closely related to oxidative stress, were detected to evaluate the anti-ageing effect of *B. natto* fermented peanut meal on the D-galactose-induced ageing model rats. The results showed that the antioxidant capacity of the rats in group M was declined. However, the SOD activity was elevated, while the MDA content was decreased in the brains of the rats supplemented with FE, indicating that FE could enhance the activity of antioxidant enzymes in the brains of the ageing model rats, and slow down the damage of free radicals to the brain cells, showing the anti-

ageing effect. Natural antioxidant substances can raise the SOD activity and enhance the oxygen-free radical scavenging and antioxidant damage ability. It can be speculated from the experimental data that FE can slow down the damage of free radicals to the brain cells by enhancing the SOD activity in the brain tissues of the ageing rats, the mechanism of which may be lied in the polyphenols, peptides and polysaccharides contained in FE.

In summary, FE can delay the ageing process of the D-galactose-induced female ageing model rats, which may be related to two aspects: on the one hand, it can improve the body's immunity and reduce the atrophy of the immune organs; on the other hand, it can enhance the antioxidant capacity of the body. By suppressing the oxidative stress response caused by the D-galactose intervention, FE enhances the antioxidant level of the body and reduces the damage of the brain tissue, which may be a key factor in preventing cognitive decline in the rats. Nonetheless, in-depth study still should be done to clarify the exact components of FE that can delay ageing and improve the learning and memory abilities *in vivo* and further explore their possible mechanisms of action.

Conclusion

Based on the above experimental results, we can infer that FE helps to improve the growth performance of the ageing rats, enhance the learning and memory ability and delay ageing. Its mechanism of action may be due to the delay of brain ageing by reducing the expression of GABA_BR1, increasing of the antioxidant activity for the brain, prevention of neuronal degeneration and protection of the hippocampus. This study provides evidence for the anti-ageing effect of FE and a reference for the study of the potential nutritional value of by-products of peanut processing industry.

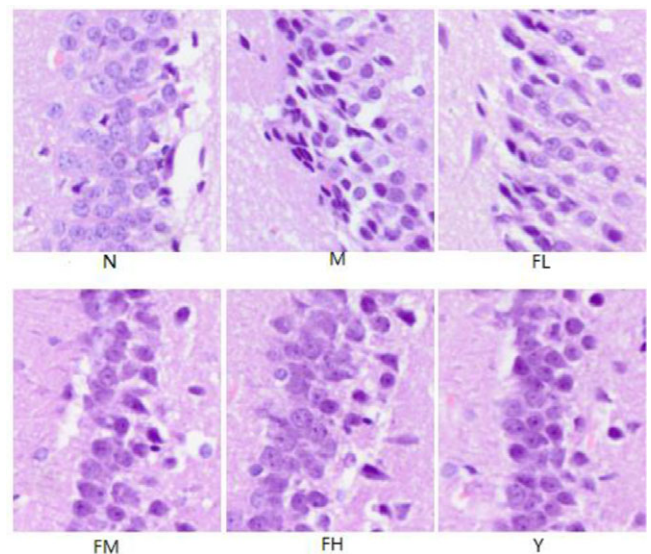


Fig. 5. Histological features of H&E staining in hippocampus (original magnifications, x400). N: normal group; M: model group; FL: fermented peanut meal extracts (FE) low-dose group; FM: FE medium-dose group; FH: FE high-dose group; Y: positive control group (VE).

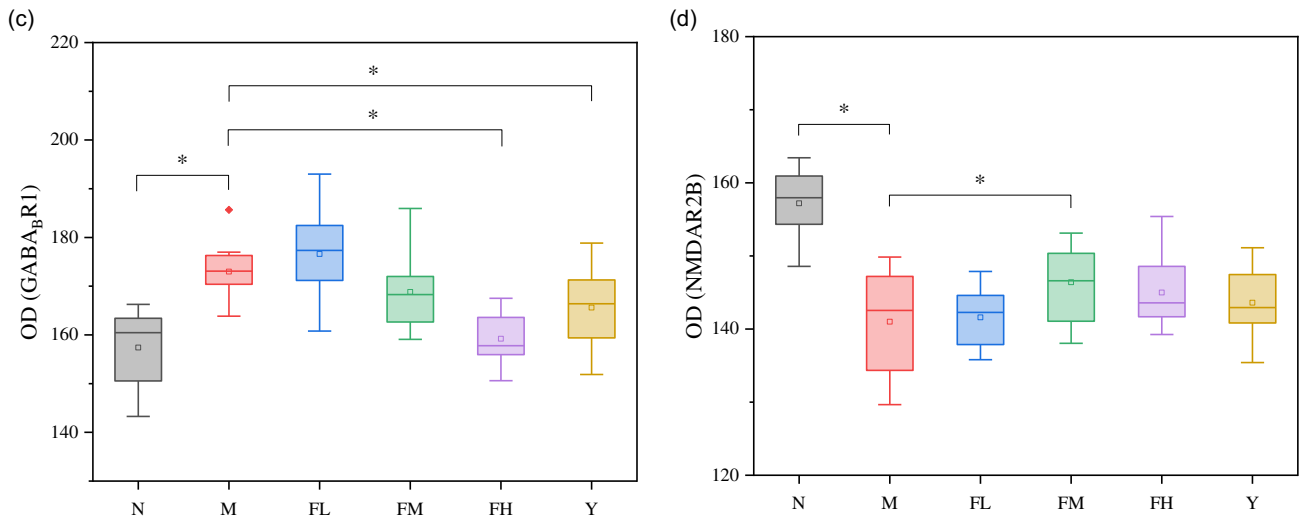
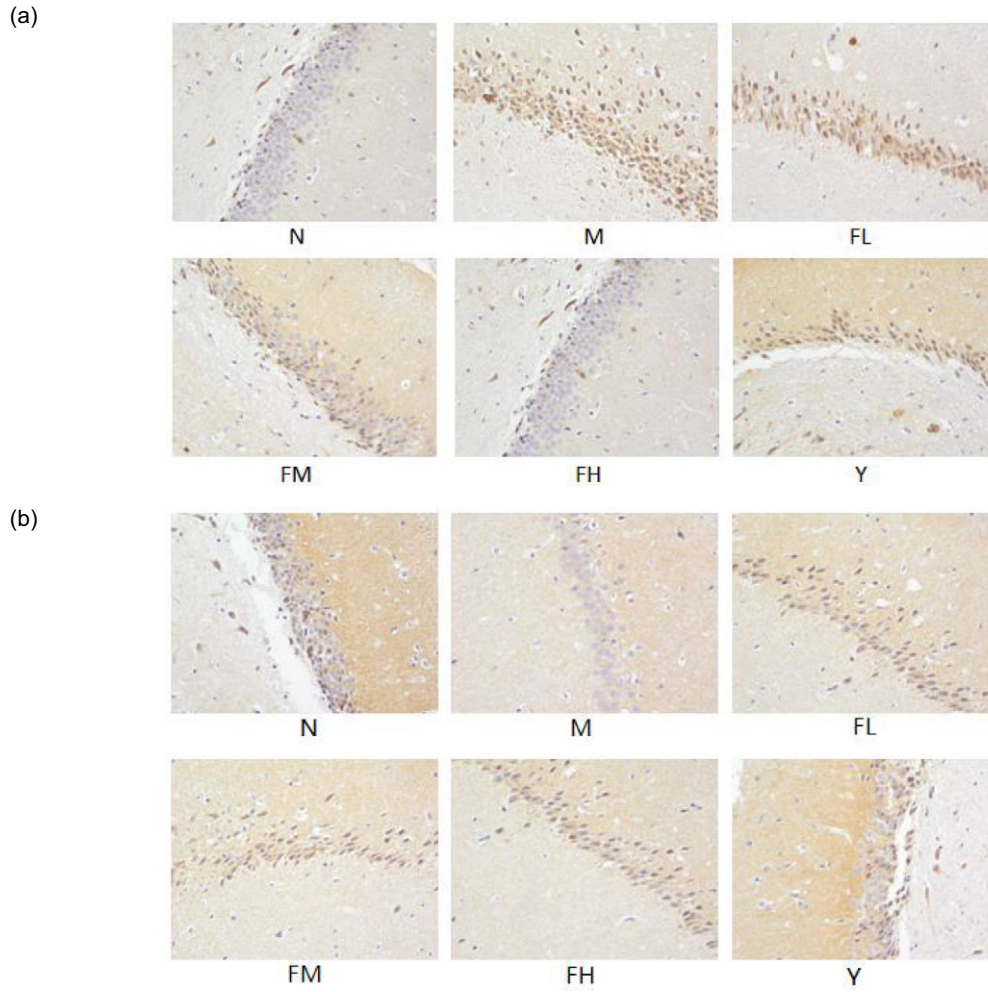


Fig. 6. (a) Immunohistochemical staining of γ -aminobutyric acid receptor 1 (GABA_BR1) in hippocampus (×400); (b) Immunohistochemical staining of N-methyl-D-aspartic acid 2B receptor (NMDAR2B) in hippocampus (×400); (c) expression of GABA_BR1; (d) expression of NMDAR2B. N: normal group; M: model group; FL: fermented peanut meal extracts; (FE) low-dose group; FM: FE medium-dose group; FH: FE high-dose group; Y: positive control group (VE).

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The authors declare that there are no conflicts of interest.

References

- Hou Y-J, Dan X-L, Babbar M, *et al.* (2019) Ageing as a risk factor for neurodegenerative disease. *Nat Rev Neurol* **15**, 565–581.
- Wyss-Coray T (2016) Ageing, neurodegeneration and brain rejuvenation. *Nature* **539**, 180–186.
- Chauhan A & Chauhan V (2020) Beneficial effects of walnuts on cognition and brain health. *Nutrients* **12**, 550.
- Unno K, Ishikawa Y, Takabayashi F, *et al.* (2008) Daily ingestion of green tea catechins from adulthood suppressed brain dysfunction in aged mice. *Biofactors* **34**, 263–271.
- Liu H, Zhang X-G, Xiao J, *et al.* (2020) Astaxanthin attenuates d-galactose-induced brain aging in rats by ameliorating oxidative stress, mitochondrial dysfunction, and regulating metabolic markers. *Food Funct* **11**, 4103–4113.
- Jiang N, Liu H-Z, Liu L, *et al.* (2013) Structure characterization and bioactivity of polysaccharide from peanut. *J Chin Cereal Oil Assoc* **28**, 119–123.
- Mei N, Zhou W-M, Hu X-Y, *et al.* (2007) Analysis on the nutrient composition of peanut meals. *Acta Agriculturae Boreali-occidentalis Sin* **16**, 96–99.
- Reddy N, Chen L & Yang Y (2013) Thermoplastic films from peanut proteins extracted from peanut meal. *Ind Crops Prod* **43**, 159–164.
- White BL, Oakes AJ, Shi X, *et al.* (2013) Development of a pilot-scale process to sequester aflatoxin and release bioactive peptides from highly contaminated peanut meal. *LWT Food Sci Technol* **51**, 492–499.
- Couto SR & Sanromán MÁ (2006) Application of solid-state fermentation to food industry – a review. *J Food Eng* **76**, 291–302.
- Xiong K, Zhou L-Y, Wang J-Y, *et al.* (2020) Construction of food-grade pH-sensitive nanoparticles for delivering functional food ingredients. *Trends Food Sci Technol* **96**, 102–113.
- Li A-Y, Wang Y-P, Li Z-X, *et al.* (2019) Probiotics isolated from yaks improves the growth performance, antioxidant activity, and cytokines related to immunity and inflammation in mice. *Microb Cell Fact* **18**, 112.
- Song YH, Yu J, Song JL, *et al.* (2021) The antihypertensive effect and mechanisms of bioactive peptides from Ruditapes philippinarum fermented with Bacillus natto in spontaneously hypertensive rats. *Journal of Functional Foods* **79**, 104411.
- Wu F-C, Chang C-W & Shih I-L (2013) Optimization of the production and characterization of milk clotting enzymes by Bacillus subtilis natto. *Springerplus* **2**, 33.
- Dong M-Z, An J-Y, Wang L-T, *et al.* (2020) Development of fermented chestnut with Bacillus natto: functional and sensory properties. *Food Res Int* **130**, 108941.
- Amadou I, Le G-W, Shi Y-H, *et al.* (2011) Optimized lactobacillus plantarum lp6 solid-state fermentation and proteolytic hydrolysis improve some nutritional attributes of soybean protein meal. *J Food Biochem* **35**, 1686–1694.
- Weng Y-Q, Yao J, Sparks S, *et al.* (2017) Nattokinase: an oral antithrombotic agent for the prevention of cardiovascular disease. *Int J Mol Sci* **18**, 523–535.
- Kimura K & Yokoyama S (2019) Trends in the application of bacillus in fermented foods. *Curr Opin Biotechnol* **56**, 36–42.
- Chu J-X, Zhao H-Z, Lu Z-X, *et al.* (2019) Improved physicochemical and functional properties of dietary fiber from millet bran fermented by Bacillus natto. *Food Chem* **294**, 79–86.
- Chen Y-Y, Gao X, Wei Y-X, *et al.* (2018) Isolation, purification and the anti-hypertensive effect of a novel angiotensin I-converting enzyme (ACE) inhibitory peptide from Ruditapes philippinarum fermented with Bacillus natto. *Food Funct* **9**, 5230–5237.
- Jeong D-Y, Ryu MS, Yang H-J, *et al.* (2021) γ -PGA-Rich Chungkookjang, short-term fermented soybeans: prevents memory impairment by modulating brain insulin sensitivity, neuro-inflammation, and the gut-microbiome-brain axis. *Foods* **10**, 221.
- Jiang X-Y, Ding H-Y, Liu Q, *et al.* (2020) Effects of peanut meal extracts fermented by Bacillus natto on the growth performance, learning and memory skills and gut microbiota modulation in mice. *Br J Nutr* **123**, 383–393.
- Lu Y-Q, Ding H-Y, Jiang X-Y, *et al.* (2021) Effects of the extract from peanut meal fermented with Bacillus natto and Monascus on lipid metabolism and intestinal barrier function of hyperlipidemic mice. *J Sci Food Agric* **101**, 2561–2569.
- Lu M-H, Lin D-Q, Wu Y-C, *et al.* (2005) Separation of nattokinase from Bacillus subtilis fermentation broth by expanded bed adsorption with mixed-mode adsorbent. *Biotechnol Bioprocess Eng* **10**, 128–135.
- Jones D, Owen AG & Farrar JF (2002) Simple method to enable the high resolution determination of total free amino acids in soil solutions and soil extracts. *Soil Biol Biochem* **34**, 1893–1902.
- Xiao LR, Ren GP & Zhang S-W (2011) Study on the effect of precipitation of milk protein by trichloroacetic acid analyzing with biuret spectrophotometry method. *Sci Technol Food Ind* **32**, 415–417.
- Hu Y-F, Ding H-Y, Wei Y-X, *et al.* (2017) Extraction of flavonoids from peanut meal and its antioxidant activity. *China Oil Fats* **42**, 141–144.
- Cuesta G, Suarez N, Bessio MI, *et al.* (2003) Quantitative determination of pneumococcal capsular polysaccharide serotype 14 using a modification of phenol-sulfuric acid method. *J Microbiol Meth* **52**, 69–73.
- Massa S, Caruso M, Trovattelli F, *et al.* (1998) Comparison of plate count agar and R2A medium for enumeration of heterotrophic bacteria in natural mineral water. *World J Microbiol Biotechnol* **14**, 727–730.
- Gao X, Zhang HJ, Li KL, *et al.* (2021) Sandalwood seed oil improves insulin sensitivity in high-fat/high sucrose diet-fed rats associated with altered intestinal microbiota and its metabolites. *Food & Function* **12**, 9739–9749.
- Festing MFW & Altman DG (2002) Guidelines for the design and statistical analysis of experiments using laboratory animals. *ILAR J* **43**, 244–258.
- Ho S-C, Liu J-H & Wu R-Y (2003) Establishment of the mimetic aging effect in mice caused by D-galactose. *Biogerontology* **4**, 15–18.
- Tomas Pereira I & Burwell RD (2015) Using the spatial learning index to evaluate performance on the water maze. *Behav Neurosci* **129**, 533–539.
- Li X-Y, Men W-W, Zhu H, *et al.* (2016) Age- and brain region-specific changes of glucose metabolic disorder, learning, and memory dysfunction in early alzheimer's disease assessed in

- APP/PS1 transgenic mice using (18)F-FDG-PET. *Int J Mol Sci* **17**, 1707.
35. Subash S, Essa MM, Braidy N, *et al.* (2016) Consumption of fig fruits grown in Oman can improve memory, anxiety, and learning skills in a transgenic mice model of Alzheimer's disease. *Nutr Neurosci* **19**, 475–483.
 36. Khan SS, Singer BD & Vaughan DE (2017) Molecular and physiological manifestations and measurement of aging in humans. *Aging Cell* **16**, 624–633.
 37. Hernandez-Segura A, Nehme J & Demaria M (2018) Hallmarks of cellular senescence. *Trends Cell Biol* **28**, 436–453.
 38. Balaban RS, Nemoto S & Finkel T (2005) Mitochondria, oxidants, and aging. *Cell* **120**, 483–495.
 39. Azman KF & Zakaria R (2019) D-Galactose-induced accelerated aging model: an overview. *Biogerontology* **20**, 763–782.
 40. Sadigh-Eteghad S, Majidi A, McCann SK, *et al.* (2017) D-galactose-induced brain ageing model: A systematic review and meta-analysis on cognitive outcomes and oxidative stress indices. *PLOS ONE* **12**, e0184122.
 41. Shwe T, Pratchayasakul W, Chattipakorn N, *et al.* (2018) Role of D-galactose-induced brain aging and its potential used for therapeutic interventions. *Exp Gerontol* **101**, 13–36.
 42. Müller L & Pawelec G (2014) Aging and immunity – impact of behavioral intervention. *Brain Behavior Immun* **39**, 8–22.
 43. Rezzani R, Franco C, Hardeland R, *et al.* (2020) Thymus-pineal gland axis: revisiting its role in human life and ageing. *Int J Mol Sci* **21**, 8806–8845.
 44. Wang Z-H, Lin Y, Jin S, *et al.* (2020) Bone marrow mesenchymal stem cells improve thymus and spleen function of aging rats through affecting P21/PCNA and suppressing oxidative stress. *Aging* **12**, 11386–11397.
 45. Sun K-Y, Yang P-Y, Zhao R, *et al.* (2018) Matrine attenuates D-galactose-induced aging-related behavior in mice via inhibition of cellular senescence and oxidative stress. *Oxid Med Cell Longevity* **2018**, 7108604.
 46. Li J-H, Wei T-T, Guo L, *et al.* (2021) Curcumin protects thymus against D-galactose-induced senescence in mice. *Naunyn-Schmiedeberg's Arch Pharmacol* **394**, 411–420.
 47. Li Q, Wang L, Li X-Y, *et al.* (2018) Total salvianolic acid balances brain functional network topology in rat hippocampi overexpressing miR-30e. *Front Neurosci* **12**, 448–448.
 48. Persson J, Sylvester C-YC, Nelson JK, *et al.* (2004) Selection requirements during verb generation: differential recruitment in older and younger adults. *NeuroImage* **23**, 1382–1390.
 49. Bartsch T & Wulff P (2015) The hippocampus in aging and disease: from plasticity to vulnerability. *Neurosci* **309**, 1–16.
 50. Voss MW, Soto C, Yoo S, *et al.* (2019) Exercise and hippocampal memory systems. *Trends Cognitive Sci* **23**, 318–333.
 51. Vorhees CV & Williams MT (2006) Morris water maze: procedures for assessing spatial and related forms of learning and memory. *Nat Protoc* **1**, 848–858.
 52. Lee B, Sur B-J, Han J-J, *et al.* (2010) Krill phosphatidylserine improves learning and memory in Morris water maze in aged rats. *Prog Neuro-Psychopharmacol Biol Psychiatr* **34**, 1085–1093.
 53. Cogley JN, Fiorello ML & Bailey DM (2018) 13 reasons why the brain is susceptible to oxidative stress. *Redox Biol* **15**, 490–503.
 54. Haider S, Liaquat L, Shahzad S, *et al.* (2015) A high dose of short term exogenous D-galactose administration in young male rats produces symptoms simulating the natural aging process. *Life Sci* **124**, 110–119.
 55. Li G-W, Yu J-M, Zhang L, *et al.* (2018) Onjisaponin B prevents cognitive impairment in a rat model of D-galactose-induced aging. *Biomed Pharmacother* **99**, 113–120.
 56. Moloney JN & Cotter TG (2018) ROS signalling in the biology of cancer. *Semin Cell Dev Biol* **80**, 50–64.
 57. Tu D-G, Chang Y-L, Chou C-H, *et al.* (2018) Preventive effects of taurine against d-galactose-induced cognitive dysfunction and brain damage. *Food Funct* **9**, 124–133.
 58. Zhang X-Y, Xu Z-P, Wang W, *et al.* (2018) Vitamin C alleviates LPS-induced cognitive impairment in mice by suppressing neuroinflammation and oxidative stress. *Int Immunopharmacol* **65**, 438–447.
 59. Liu Z-H, Liu Y-T, Tu X-J, *et al.* (2017) High serum levels of malondialdehyde and 8-OHdG are both associated with early cognitive impairment in patients with acute ischaemic stroke. *Sci Rep* **7**, 9493.