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Effect of dietary *Ginkgo biloba* leaf on the growth performance and nonspecific immunity of red swamp crayfish *Procambarus clarkii*

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Abstract

This trial investigated the effect of dietary *Ginkgo biloba* leaf (GBL) on the growth performance and nonspecific immunity of red swamp crayfish *Procambarus clarkii*. 180 Crayfishes were randomly divided into three groups. One group was fed with basic diet, whereas the other two groups were fed with diets containing 1% and 3% GBL. After 32 days of feeding, GBL addition tended to increase the body weight gain rate compared with control. In 3% GBL group, the bodyweight gain rate of male crayfish was higher than that of female crayfish. While female crayfish were advantageous in terms of meat yield. Liver-related indexes were influenced by GBL addition and 3% GBL could reduce glutamic pyruvic transaminase and glutamic oxaloacetic transaminase as well as total cholesterol in male crayfish, showing its function in liver protection. Moreover, GBL addition effects on liver protection was better in male crayfish than female crayfish.

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Introduction

Ginkgo biloba is an endemic species in China, which has experienced over 200 million years. It is highly tolerant to industrial and urban pollution and repellent for viruses, bacteria, fungi and insects. In China, people use its extracts as traditional Chinese medicine for different purposes. Ginkgo extracts contain 24% ginkgo-flavone glycosides and 6% terpenoids and have medical effects on a series of human diseases, such as diabetic cardiomyopathy and myocardial lesion (Martinez-Solis et al., 2019; Oken et al., 1998), neurodegenerative diseases, neurodegenerative retinal diseases (Martinez-Solis et al., 2019), hippocampus neuronal lesions (Li et al., 2019), cancer (Li et al., 2019), obesity, and liver damage (Hirata et al., 2019). Medicinal extracts are made from dried leaves (GBL). Besides, GBL is used as feed additives for disease control in aquaculture (Bao et al., 2019). When added to feed, it can improve the growth, hepatic and intestinal health status, hepatic antioxidant status, and immunity of *Nile tilapia* (Abdel-Latif et al., 2021), increase *Cyprinus carpio*'s body growth and feed utilization and red blood cell levels, white blood cells, hematocrit, hemoglobin, total protein, albumin, and globulin (Bao et al., 2019), and elevate the rainbow trout (*Oncorhynchus mykiss*)'s plasma total immunoglobulin, lysozyme activity, and peroxidase activity caused by organic phosphorus pesticide diazinon (Hajirezaee et al., 2019).

The red swamp crayfish, *Procambarus clarkii*, is one of the world's most important freshwater crayfish species. Due to its delicious taste and rich nutrition, crayfish is favored by consumers and has broad market prospects (Manfrin et al., 2015). However, with the rapid expansion of the scale of farming, the polluted environment and high-density aquaculture often lead to frequent diseases and reduce crayfish production (Du et al, 2016). In recent years, the frequent outbreaks of spot syndrome virus (WSSV) have caused great losses to crayfish aquaculture (Zhu et al., 2009). Meanwhile, Aeromonas species commonly appear in crayfish culture, a pathogenic bacterium that can cause hemorrhagic disease of crayfish (Qin et al., 2018). In previous studies, native compounds, e. g. galactooligosaccharide (Nedaei et al., 2019), fulvic acid (Zhang, 2018), chitosan (Sun et al., 2016), and dietary *Rhodiola rosea* polysaccharide (Cheng, 2019), have been used to resolve the challenges in diseases of crayfish. However, the study about the effects of *G. Biloba* on the immunity of *P. clarkia* is rarely reported.

The working mode of the immune system in invertebrates is different from those in vertebrates. Most crustaceans, including crayfish, rely mainly on nonspecific immunity other than specific immunity, called humoral immunity. In this study, lysozyme (LYZ) (Zhang et al., 2021), alkaline phosphatase (AKP) (Chen et al., 2020), glutamic pyruvic transaminase (GPT) and glutamic oxaloid transaminase (GOT), which can reflect the liver health of crustaceans (Cheng et al., 2020), and superoxide dismutase (SOD) were chosen to evaluate the nonspecific immunity of *P. clarkia* (Liu et al., 2020). In addition, the metabolic rates of crustaceans can be different from gender (Colpo and Lopez-Greco, 2018), so this experiment will also consider comparing the different effects of dietary GBL on male and female *P. clarkia*.

In this study, to explore the role of GBL in the immunity of *P. clarkia*, we used GBL as the feed additive for crayfish. The effect of GBL on the immune level of crayfish was judged by measuring the growth, immune function, and blood indicators of crayfish, to provide theoretical reference for the artificial culture and breeding technology of crayfish.

Materials and Methods

Crayfish preparation

All crayfish handling followed the guidelines on the care and use of animals for scientific purposes established by the Institutional Animal Care and Use Committee (IACUC) of Yancheng Institute of Technology, China. Red swamp crayfish were collected from Jiangsu Jinfeng Agricultural Technology Co., Ltd. A commercial feed (Tongwei (Dafeng) Feed Co., Ltd., Jiangsu, China) was used to feed the crayfish twice every day (7:00 and 19:00), and they were temporarily cultured to acclimate to the experimental conditions for two weeks (average body weight: (9.08 ± 1.06) g). The conditions for temporary culture were temperature ((25 ± 1) °C), pH 7.60–8.40, NH_3 and H_2S less than 0.04 mg L^{-1} and 0.03 mg L^{-1}, respectively, and dissolved oxygen (DO) kept more than 6.0 mg L^{-1} throughout the experiment.

Diet preparation

Basal feed was purchased from Nantong Haida Biotechnology Co., Ltd.; the ingredients are presented in **Table 1**. Dry GBL was ground and passed through 60 mesh sieves. Then GBL was added to the basal diet at two concentrations (1% and 3%), expressed as 1% group and 3% group throughout the rest of this paper. A basal diet without GBL was set as a control. All diets were individually blended in a mixer and then homogenized with a strong adhesive (starch and cellulose). Then diets were stirred with proper water, extruded by a pelletizer (F-26, South China University of Technology, Guangzhou, China), cut into pellets (4 mm), dried at 50 °C, sealed in separate bags, and stored at −20 °C for further use.

Table 1 Composition of the basal diets of red swamp crayfish

Ingredients	%
Fish meal	43
Soybean meal	6.5
Yeast meal	2.5
crayfish shell meal	7
Wheat flour	34.5
Cellulose	0.6
Gluten	2.5
Fish oil	0.8
Mineral mixture[a]	2
Vitamin mixture[b]	0.6
Fish meal	43
Soybean meal	6.5

[a] Vitamin mixture (g/kg): β-Carotene, 3 M.I.U.; Cholecalciferol, 0.6 M.I.U.; Thiamin, 3.6; Riboflavin, 7.2; Pyridoxine, 6.6; Cyanocobalamine, 0.02; α-Tocopherol, 16.5; Menadione, 2.4; Niacin, 14.4; Pantothenic acid, 4; Biotin, 0.02; Folic acid, 1.2; Inositol, 30; Ascorbic acid, 100.

[b] Mineral premix (g/kg): P, 120; Ca, 120; Mg,15; Fe, 1.5; Zn, 4.2; Cu, 2.1; K, 75; Co, 0.11; Mn, 1.6; Se, 0.01; Mo, 0.005; Al, 0.025; I, 0.4.

Crayfish culture

In the initial experiment, 180 crayfish were randomly assigned to 9 tanks, 3 tanks for each group and 10 males plus 10 females per tank. Two treatment groups were fed with GBL contained diets, whereas the control group had a basal diet. The crayfish were cultured in 10 L circular fiber-glass-reinforced plastic tanks, which was suitable, according to Cheng and Wu (2019).

The feeding amount was set to 3% body weight of crayfish in each tank and carried out twice daily at 08:30 and 20:30. The rest feed was cleared with a siphon the next day. The culture conditions were the same as those in the acclimation period. The culture water was refreshed daily with 30% fresh water. Experiments were performed in triplicate. The experiment lasted for 32 d.

Growth performance

After the crayfish were starved for 24 hours before and after the experiment, a towel was used to dry the crayfish's surface. Each crayfish was determined before the experiment (initial body weight), and determined again at the end of the experiment (final body weight, peeled meat). The parameters were calculated as follows:

Weight gain rate (WGR) = 100 × (final body weight – initial body weight)/initial body weight
Meat yield (MY) = 100 × (final meat weight/final body weight)

Blood biochemical indicator assay

After the 32-d experiment, six crayfish were randomly picked up from each tank. A sterile syringe was used to sample blood from the pericardial cavity of the crayfish's body. The blood was stored in a sterile centrifuge tube overnight at 4 °C and then centrifuged at 2500×g for 10 min. The obtained serum was used for the determination of glucose (GLU), total cholesterol (TC), triglyceride (TG), lysozyme (LYZ), alkaline phosphatase (AKP), glutamic pyruvic transaminase (GPT) and glutamic oxaloid transaminase (GOT). AKP activity was assayed according to the sodium phenylene phosphate colorimetric method. Other indicators were determined according to the kit method provided by Nanjing Jiancheng Biological Company Research Institute.

Hepatopancreatic antioxidant activity assay

After thawing, hepatopancreas samples were weighed. The hepatopancreas and saline were mixed at a volume ratio of 1:9, homogenized in an ice bath, centrifuged at 4000×g, and kept 4 °C for 10 min. The supernatant was used to determine superoxide dismutase (SOD) activity. The indicator was assayed using the kit and according to the method provided by Nanjing Jiancheng Biological Company Research Institute.

Statistical analysis

The experiment data was arranged using Microsoft Excel 2016, and the results were presented as Mean ± SD. After a normal distribution test, a one-way ANOVA test with LSD post-mortem multiple comparisons were conducted to test the significant difference among the different feed groups. The difference between male and female crayfish was tested using a t-test. The analysis was performed by IBM SPSS Statistics24. The significance level was set at $P<0.05$.

Results

Growth performance

At the end of the 32 d trials, GLB addition tended to increase WGR, and the effect on male crayfish was more pronounced. In the 3% group, the WGR of male crayfish was significantly higher than female crayfish ($P<0.05$, **Figure 1**). Conversely, the MY of female crayfish was significantly higher than female crayfish ($P<0.05$, **Figure 2**).

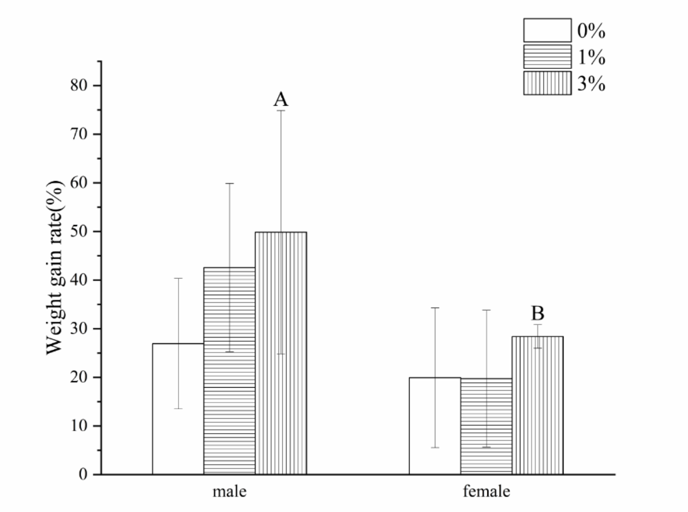

Figure 1 Effect of GBL on the weight gain rate of crayfish.

Note: Different capital letters show significant differences in parameters between male and female crayfish, and different lowercase letters show significant differences in parameters among feed groups (*P*<0.05), the same as follows.

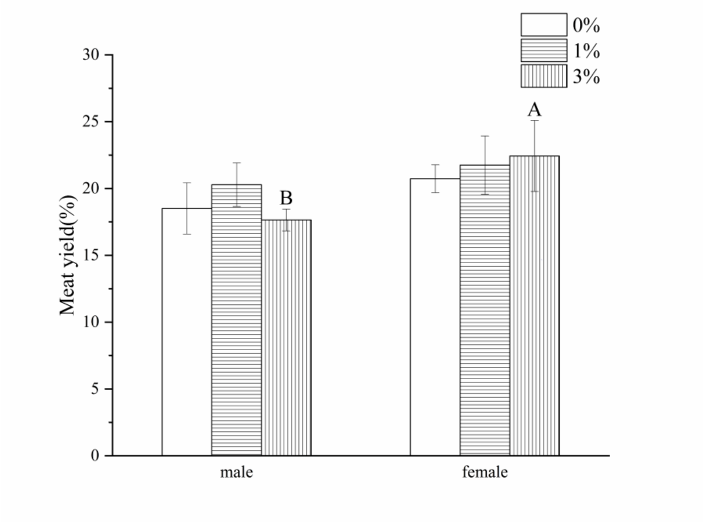

Figure 2 Effect of GBL on the meat yield of crayfish.

Serum biochemical parameters

For male crayfish, GPT activities ranged by order as 1% group>control>3% group (*P*<0.05). While for female crayfish, GPT activities ranged by order as 1% group>3% group>control, and GPT of 1% group was significantly higher than that of control (*P*<0.05). GPT of male crayfish was significantly higher than that of female crayfish in control and 1% groups (*P*<0.05). At the same time, the GPT of male crayfish was significantly lower than that of female crayfish in the control and 1% groups (*P*<0.05, **Figure 3**).

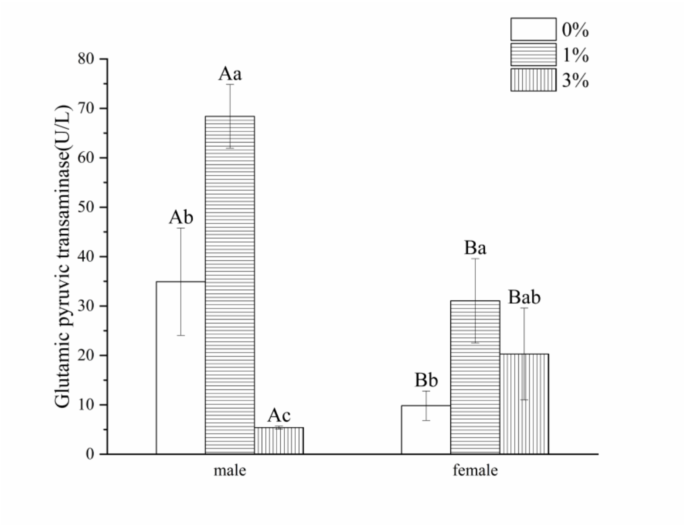

Figure 3 Effect of GBL on the GPT of crayfish.

For male crayfish, GOT activities ranged by order as control>1% group>3% group (*P*<0.05). While for female crayfish, GPT activities ranged by the same order as the control >1% group >3% group, and the GPT of the 3% group was significantly lower than that of the control (*P*<0.05). GPT of male crayfish was significantly higher than that of female crayfish in control and 1% groups (*P*<0.05, **Figure 4**).

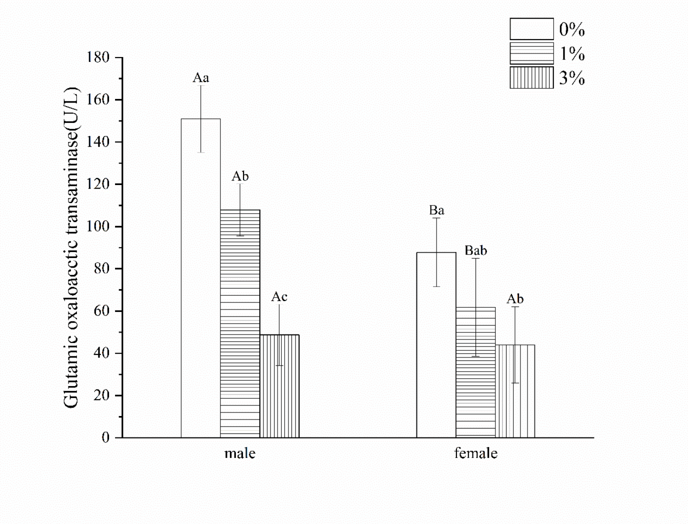

Figure 4 Effect of GBL on the GOT of crayfish.

For male crayfish, the TC level of the 1% group was significantly higher than that of the 3% group (*P*<0.05, **Figure 5**).

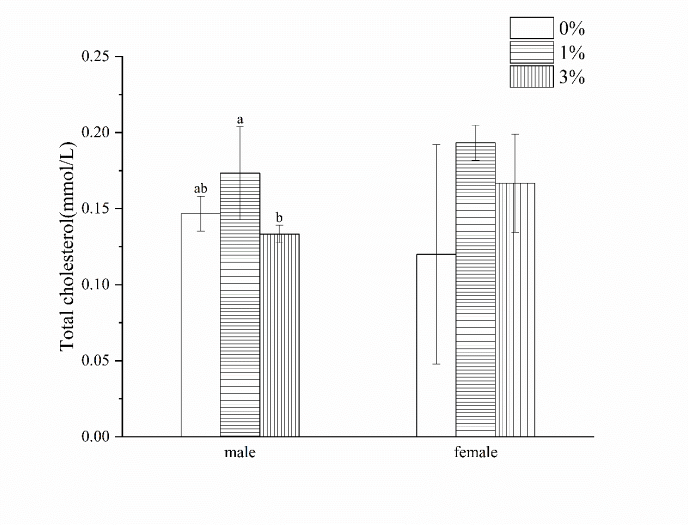

Figure 5 Effect of GBL on the TC of crayfish.

There was no significant difference in other serum biochemical parameters among the groups (P>0.05), including LYZ (**Figure 6**), GLU (**Figure 7**), and TG (**Figure 8**).

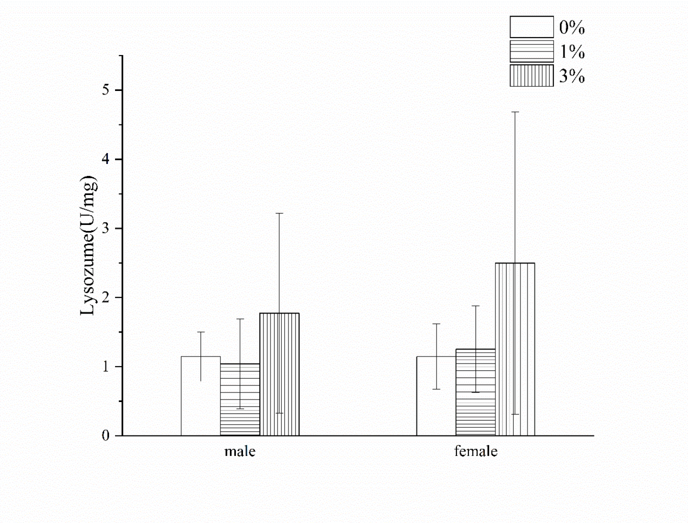

Figure 6 Effect of GBL on the LYZ of crayfish.

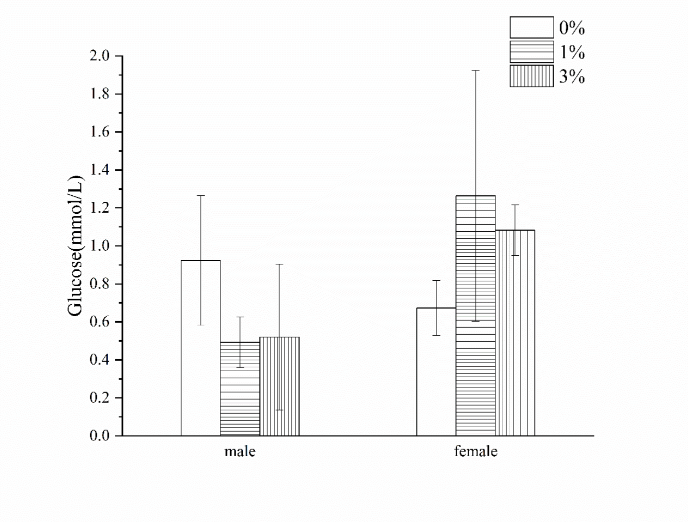

Figure 7 Effect of GBL on the GLU of crayfish

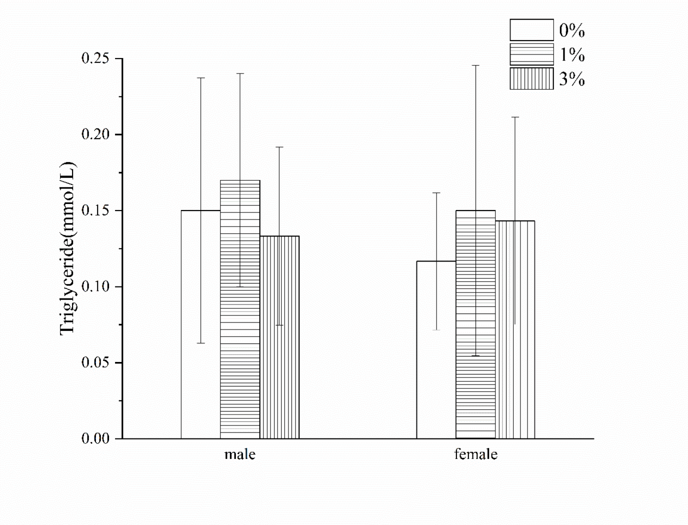

Figure 8 Effect of GBL on the TG of crayfish

Hepatopancreatic antioxidant activities

No significant difference was found among groups regarding SOD (*P*>0.05, **Figure 9**).

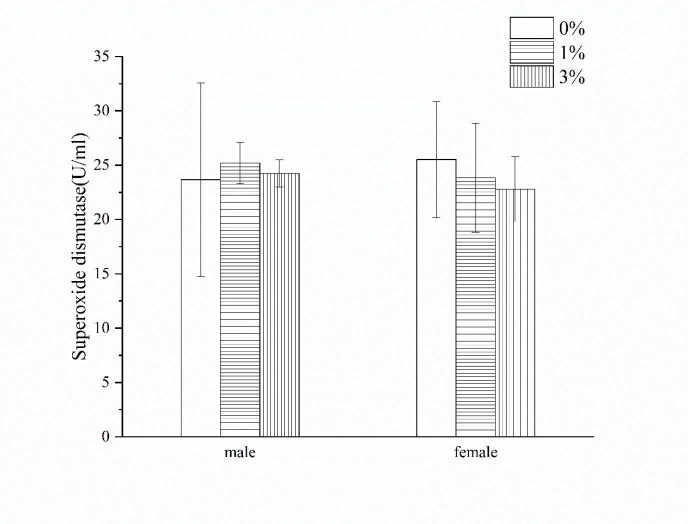

Figure 9 Effect of GBL on the SOD of crayfish

Discussion

Effects of GBL on the growth of crayfish

G. biloba is a botanical dietary supplement that contains hundreds of different bioactive compounds, including terpene trilactones, flavonoids, fatty acids, proanthocyanidins, and polysaccharides (Beck and Stengel, 2016; Ma et al., 2016). This explained the positive effects of GBL on growth, antioxidant capacity, immune responses, and blood biochemical reactions in crayfish. In this study, compared with the control group, the 1% and 3% GBL addition tended to increase the weight gain rate and meat yield of the crayfish, indicating that GBL could improve the growth performance of the crayfish. Many studies have shown that crustaceans and aquatic animals' growth performance improvements come from various medicinal herbs' extracts (Abdel-Latif et al., 2021; Cheng, 2019; Dong et al., 2018; Pan et al., 2018; Tan et al., 2018). For GBL, it can send out fragrance to stimulate the appetite of crayfish and improve the digestibility and absorption rate of feed in the digestive tract of the crayfish (Lange and Schultze, 2010; Jannathulla and Dayal, 2022). In addition, it is rich in protein. All the factors above improve the growth performance. However, due to the lack of experience, GBL and basal feed might not be thoroughly mixed, and the digestion and absorption of the crayfish for GBL needed to be more robust, which led to the effect not being obvious. we also studied the different growth performances of both genders. Brewis and Bowler (1982) state that male crayfish can maintain a higher growth rate than female crayfish, especially in large sizes. And female crayfish need to reserve more nutrient substances to prepare for future reproduction. So, in this study, a 3% GBL addition tended to increase the whole-body weight of female crayfish and the meat yield of female crayfish.

Effect of GBL on the antioxidant and immune measurements of crayfish

SOD is the only confirmed enzyme that can directly scavenge free radicals. SOD can decompose superoxide to oxygen and hydrogen peroxide with high specificity and efficiency (Borgstahl and Oberley-Deegan, 2018). In this study, no significant difference was found after GBL addition, which differed from the results that GBL affected the antioxidant status of *Oreochromis niloticus* and *Epinephelus lanceolatus* (Abdel-Latif et al., 2021; Tan et al., 2018). In another study, SOD activity in the hepatopancreas of Chinese mitten crabs (*Eriocheir* *sinensis*) increased significantly after dietary glyceryl monolaurate supplementation (Fu et al., 2022). The antioxidant capacity of crabs can be induced to a significantly higher level by a taurine diet. In addition, the addition of other nutrients in the feed can also promote the SOD activity of crayfish, Kong et al. (2021) found an increase in the SOD activity in crayfish fed with vitamin C (VC) supplemented diets. It is speculated that no severe stress occurs during the experiment under artificial conditions. GBL and basal feed might not be thoroughly mixed, resulting in insufficient digestion and absorption of the crayfish, which led to no significant effect.

LYZ plays an essential role in the control of microbial invaders (Misra et al., 2004). the activity of LYZ in crustaceans' hepatopancreas and blood cells is directly related to the immune function and indicates the health of crayfish so that LYZ can enhance the immunity of aquatic animals (Haug et al., 2002; F. Liu et al., 2020). In the present study, similar to the results of SOD activity, the LYZ activity of crayfish did not change, suggesting that GBL addition did not enhance the immunity of crayfish in this study. In contrast, vibriosis resistance in *L. vannamei* was improved by dietary inclusion of 0.5% or 1.0% *S. fusiforme* polysaccharide extracts, and this coincided with increased muscular LYZ activity of *L. vannamei* (Huang et al., 2006). For other crustaceans, the activity of LYZ in hemolymph was significantly increased by glyceryl monolaurate addition rate at 1000 and 2000 mg/kg (Fu et al., 2022). Meanwhile, *Angelica sinensis* exhibits immunostimulatory effects on Pacific white shrimps (*L. vannamei*) and may thus be used as a diet supplement (Pan et al., 2018). The less noticeable improvement can come from no microbial invasion occurring during the experiment under artificial conditions. GBL and basal feed might not be thoroughly mixed, resulting in insufficient digestion and absorption of the crayfish, leading to an insignificant effect.

Effect of GBL on the biochemical blood indexes of crayfish

The GPT and GOT are specific enzymes that play essential roles in cellular nitrogen metabolism, oxidation of amino acids, and liver gluconeogenesis (Richard et al., 2010). GPT and GOT are usually used as indicators of liver function (Peng et al., 2018). Previous Studies showed that ambient stressors could increase GPT and GOT levels in aquatic animals (Cheng et al., 2017). We found that the GPT first increased and then decreased, and the highest level was found in the 1% group. This suggests that a moderate GBL addition to the fed is the best inducer for the GPT production of crayfish, and a higher GBL addition cannot continue to promote the level.

Conversely, we found that the GOT level of both male and female crayfish decreased significantly. Protein and methionine intake by juvenile black tiger shrimp *Penaeus monodon* could increase GPT and GOT activities (Richard et al., 2010). Different results were also obtained in the present study. This may be due to the low protein content of feed. In addition, the GPT and GOT activities between male and female crayfish in control and 3% group showed significant differences. This may be related to the male crustaceans have a unique androgenic gland, which results in a different metabolic mode (Ventura et al., 2011).

Glucose is the most common form of sugar that animals can easily absorb. Crustaceans need to take much longer to clear glucose load than mammals, which are generally considered to be glucose intolerant (Liu et al., 2017). We found that the contents of GLU were not significantly affected by the addition of GBL. The TG and TC are integral components of blood fat, indicating the absorption, metabolism, and utilization of lipids. When their contents decrease, it means fat utilization happens (Zhang et al., 2018). In this study, the TC content in the male crayfish of 3% GBL group was significantly lower than that in male crayfish of 1% GBL It showed that GBL could reduce the blood fat of crayfish when it reached some threshold and its efficacy is affected by gender. The active ingredients in GBL for TG and TC need further study. In conclusion, 1% GBL addition is the better feed formula in this study. But the challenge assays with pathogenic microorganisms are advised to determine the curative effect from GBL for crayfish.

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