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Beneficial effects of functional ingredients in feed on immunity improvement and growth promotion of aquaculture animals

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Beneficial effects of functional ingredients in feed on immunity improvement and growth promotion of aquaculture animals

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Editorial: Beneficial effects of functional ingredients in feed on immunity improvement and growth promotion of aquaculture animals

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Editorial on the Research Topic

Beneficial effects of functional ingredients in feed on immunity improvement and growth promotion of aquaculture animals

Aquaculture now supplies an increasing proportion of food fish, 49% (1.226 billion tons) of the total in 2020, and is the fastest growing food sector with production as the per capita food fish supply has reached 20.2 kilograms (FAO, 2022). With capture fisheries declining in worldwide and the expansion of the farming industry, fish meal, the critical protein sources in feed, is obviously inadequate to support the requirement, and use levels will still have to be declined. The application of plant protein sources and non-protein energy sources are increasingly widespread. Consequently, the poor feed palatability, decreased food intake, reduced health and growth performance of farmed animal induced by receiving low-fishmeal diets have recently gained increasing attention (Lazzarotto et al., 2018; Niu et al., 2019; Wattanakul et al., 2019). On the other hand, Due to the rapid expansion of farming scale, the increased rearing densities and deteriorated aquatic environment leads to an outbreak in disease frequency (Li et al., 2020; Jahangiri et al., 2022). Given the use of antibiotics is being restricted, it is particularly important to develop safe and effective ingredients to improve both the growth and health of aquaculture animals.

Here, we inspire a Research Topic including 10 papers on dietary ingredients influencing the growth performance, meat quality, ovarian development, intestinal histology, antioxidant activity and immunity of economic aquatic animals, aiming to highlight the strategies and fundamental mechanisms for promoting the sustainable development of aquaculture based on both the experiments and theory.

To alleviate growth reduction and enteritis caused by low fish meal diets, Yao et al. evaluate the different inclusion level of *Schizochytrium limacinum* in diets, and demonstrated that 0.6% *S. Limacinum* supplementation can improve the growth performance, promote hepatopancreas lipid metabolism, reduce apoptosis, promote

autophagy and improve intestinal health of *Litopenaeus vannamei* fed low fish meal diets. The study of Yue et al. revealed that limonene, allicin and betaine supplementation in low fish meal diets had a time effect on the growth performance, and could improve antioxidant capacity, meat quality and intestinal health of largemouth bass (*Micropterus salmoides*). Meanwhile, Ke et al. reported that that dietary sodium butyrate intervention could enhance growth and feed utilization of orange-spotted grouper (*Epinephelus coioides*) with high soybean meal-induced enteritis by promoting intestinal digestive enzyme activities, reducing mucosa permeability, maintaining the integrity of intestinal morphology and attenuating the intestinal inflammatory response.

Plant-based additives have been attracting growing attention in the last two decades. Studies in *M. salmoides* demonstrated that mulberry leaf extract could improve antioxidant capacity, immune function, and glycolipid metabolism, thereby alleviating the negative effects of a high-starch diet (Tingsen et al.). An addition of 2% fermented tea residue can also improve the growth and the liver antioxidant capacity, and enhance the resistance to *Aeromonas hydrophila* in largemouth bass (Jiang et al.). Moreover, Chien et al. reported that the appropriate level (250-500 ppm) of dietary grape extract could improve the growth performance and antioxidant activity of *L. vannamei*.

Other additives were also involved in this Research Topic. An increase of growth performance and antioxidant status were observed in juvenile yellow catfish (*Pelteobagrus fulvidraco*) fed diets supplied with pyrroloquinoline quinone (Shi et al.). Additionally, SKL17-2 peptide, a 17-amino acids (aa) short peptide synthesized based on interferon-g related protein in large yellow croaker (*Larimichthys crocea*), could destroy *Pseudomonas plecoglossicida* and was considered to be a potential feed additive used for prevention and treatment of visceral white nodules disease (Lin et al.).

Beside feed supplements, several ingredients were also investigated in this Research Topic. The effects of cholesterol on ovarian development of *Eriocheir sinensis* were well studied by Guo et al., in which 0.4% of dietary cholesterol can promote ovarian maturation *via* improving the estradiol level of the organism. Moreover, the impact of the dietary inclusion of cellulose with different viscosities on the growth, nutrient digestibility, serum biochemical indices, and the hepatic and gut morphology of largemouth bass indicated that viscosity is the main anti-nutritional effect of dietary carboxymethyl cellulose and soluble non-starch polysaccharides (Liu et al.).

The Research Topic summarized some of the latest advancement on the recent development and achievement in revealing growth and immune regulation of functional dietary ingredients on aquaculture animals. We are appreciated to all authors for their innovative works and all reviewers for their helpful comments. We sincerely thank the Editorial Team of Frontiers in Marine Science for their hard work to get the publication of Research Topic.

Author contributions

XW: Writing-original draft. JS: Writing-review. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Fermented tea residue improved growth performance, liver antioxidant capacity, intestinal morphology and resistance to *Aeromonas hydrophila* infection in juvenile largemouth bass (*Micropterus salmoides*)

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The study aimed to evaluate the effects of fermented tea residue (FT) on growth performance, intestinal morphology, liver antioxidant capacity and Aeromonas hydrophila infection in juvenile Largemouth bass. A total of 240 fish were randomly distributed in 12 tanks with 20 fish per tank (4 treatments with 3 replications) and fed with diets FT at the rate of 0 (control), 2, 4 and 6%. The weight gain rate (WGR), specific growth rate (SGR) and intestinal villi height (VH) of juvenile largemouth bass were significantly higher than those of the control group after feeding FT ($P< 0.05$); meanwhile, the liver superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) and catalase (CAT) activities of juvenile largemouth bass were significantly higher and the malondialdehyde (MDA) levels were significantly lower than those of the control group after feeding FT ($P< 0.05$). Mortality occurred in all groups of largemouth bass after the injection of *A.hydrophila*, but feeding FT reduced the cumulative mortality compared with the control group ($P< 0.05$). In juvenile largemouth bass infected with *A.hydrophila*, the relative mRNA expression of the intestinal anti-inflammatory factors IL-10 and TGF-α was significantly higher and that of the pro-inflammatory factors IL-1, IL-15, IL-8, and TNF-α was significantly lower ($P< 0.05$). In summary, it can be seen that a 2% FT addition can improve the liver antioxidant capacity of juvenile largemouth bass, enhance the resistance to *A.hydrophila* and increase the growth of largemouth bass.

KEYWORDS

tea residue, largemouth bass, growth performance, immune capacity, *A. hydrophila*

1 Introduction

Largemouth bass (*Micropterus salmoides*) is a carnivorous warm-water fish from California, U.S.A. It has become one of the main species of freshwater aquaculture in China because of its strong adaptability, fast growth rate and tender flesh (Bureau 2021). In 2020, the farmed production reached 619,519 tons, an increase of 29.66% compared with 2019; (Bureau, 2021). However, under intensive farming, disease outbreaks are more frequent in largemouth bass, which are mainly caused by parasites, bacteria and fungi, The producers need to use a lot of antibiotics and disinfectants to treat these diseases, which leads to economic losses (ValladãO et al., 2015). However, the misuse of antibiotics or chemicals can cause many negative effects on the environment, animals and humans (Cabello, 2010; Rico and Brink, 2014). Therefore, the use of antibiotics is slowly being restricted. It has become a trend in the global aquaculture industry to use positive immune stimulants to enhance the innate immune mechanism of fish to increase their disease resistance (Fuchs et al., 2015; ValladãO et al., 2015).

Studies have shown that preventive treatments promote innate immune responses in fish and reduce disease outbreaks in aquaculture (Ma et al., 2020; Tadese et al., 2020). The use of micro-ecological preparations, such as fermented feed, and plant-based additives can better enhance the immunity of aquaculture animals (Burr et al., 2010; Newaj-Fyzul and Austin, 2015; Dawood et al., 2018; Niu et al., 2020). The addition of Aqualase® (a yeast-based commercial probiotic composed of Saccharomyces cerevisiae and Saccharomyces ellipsoidal) in the feed can moderate the intestinal microbiota of rainbow trout and improve immunity and growth (Adel et al., 2017). The inclusion of 2.5 - 2.61*10^7 CFU/kg of two probiotics (*Lactococcus lactis* and *weissella confuse*) in the diet can improve the growth performance of fingerling great sturgeon, improve the immune index, and increase the height of intestinal villi (Yeganeh Rastekenari et al., 2021). The fermented dragon fruit in the diet improves the growth performance and feed utilization of *Platax pinnatus* and increases antioxidant enzyme activity to some extent (Chu et al., 2021). Feeding fermented feed by *Bacillus subtilis* to Penaeus monodon is shown to improve the growth performance, feed digestibility, survival rate and immunity of spot prawns (De et al., 2018).

After soaking goldfish infected with *A. hydrophila* for a certain period of days with 1% compound herbal water, the damaged primary gill flaps, liver, heart and muscle tissue structure of goldfish are restored (Ramasamy et al., 2010). Immunostimulant (mixture of Chinese herbs and Bacillus), could significantly upregulate the expression of NADPH oxidase genes and antioxidant genes in tilapia spleen neutrophils, thus improving the immunity of tilapia (Abarike et al., 2019).

Tea residue is a by-product of tea refining, which is rich in tea polyphenols, theanine and other active ingredients, with natural antioxidants, immune promotion and other functions (Rietveld and Wiseman, 2003; Hamer, 2007). There are more types of fermented feed, but the result of synergistic fermentation of bacteria and enzymes is better than the result of bacteria and enzymes alone (Xie et al., 2015). Because the combination of bacteria and enzymes can make the degradation of macromolecules more complete, the fermentation efficiency is higher (Sun et al., 2021). The addition of green tea powder to the diet could increase the serum TP content and SOD activity of rainbow trout, upregulate the mRNA expression levels of anti-inflammatory factors and downregulate the mRNA expression levels of pro-inflammatory factors in the spleen and kidney (Nootash et al., 2013). Green tea could improve the growth performance and feed utilization of *Paralichthys olivaceus*, and effectively reduce serum glutathione transaminase (GPT) and low-density lipoprotein (LDL) in fish (Cho et al., 2007). And it improves the growth performance and health of Nile Tilapia against infection by *A.hydrophila* (Abdel et al., 2010). Tea and fermented feed have positive effects on growth performance, immune system, pathogen protection and immunity in different fish species. The application of fermented tea residue on largemouth bass is limited. In this experiment, we investigated the effects of FT with different addition ratios on the growth performance, intestinal tissue structure, antioxidant capacity and infection of *A.hydrophila* of largemouth bass.

2 Materials and methods

2.1 Ethical statement

This study was conducted in strict accordance with the Experimental Animal Management Regulations of Southwest University of Science and Technology. All of the procedures were performed following the Declaration of Helsinki and relevant policies in China.

2.2 Experimental diets and design

Four test diets were prepared. Basal diets and rations with 2%, 4% and 6% fermented tea residue (FT) were added to the basal diet. The composition of the base diet is shown in Table 1, and the nutritional composition of the diets is shown in Table 2. After the base diet was prepared, we added different proportions of FT to the base diet and then mixed and kneaded them into soft pellets (1.2-mm diameter) for feeding. FT is made by crushing tea residue and adding 4% corn flour, 0.8% glucose, 35% water, 0.1% probiotics and enzyme preparations to ferment thoroughly at 36°C for 3 days. FT is the tea residue produced

after Fuxuan 9 processing, from Sichuan, China. Probiotics and enzyme preparations were purchased from a biological company; the product label number is Q/12JX 4450-2019. The fermentation bacteria include *Lactobacillus Plantarum* ≥ 1.0×10^9 CFU/g, *Bacillus subtilis* ≥ 1.5×10^9 CFU/g, *Saccharomyces cerevisiae* ≥ 1.0×10^9 CFU/g; the enzyme preparation contains cellulase ≥ 1000 U/g, xylanase ≥ 500 U/g, β-glucanase ≥ 3000 U/g, β-mannanase ≥ 50 U/g.

2.3 Experimental fish and breeding management

Healthy juvenile largemouth bass (average weight of about 5 g) were obtained from Meishan City, Sichuan Province, China. Upon arrival, all fish were tamed in a test environment for one week. In the formal trial, after all fish were starved for 24 hours, 240 largemouth bass were randomly divided into 4 treatments (groups T0 - T3). T0 was the control group receiving the basal diet, and T1-T3 were fed with FT added to the basal diet at 2%, 4% and 6%, respectively. Each treatment was stocked with three replicates stocked with 60 fish (20 fish/tank) in the tank (1m * 50cm * 1m). During the experiment, fish were hand-fed with experimental diets twice a day at a rate of 3% of body weight (8:30 am and 4:30 pm). We changed 1/5 - 3/5 of the water every 2 - 3 days and used the pump to remove the bottom feces. The water temperature was natural and the dissolved oxygen content was greater than or equal to 6.0 mg/L, pH 7.0 ± 0.2, ammonia nitrogen ≤ 0.02 mg/L. The rearing experiment lasted for 56 days.

TABLE 1 Composition and nutrient levels of the basal diet (air-dry basis).

Items	Content(%)
Ingredients	
fish meal	44
chicken powder	10
cassava starch	8
flour	11
gluten	2
soybean meal	12
soybean oil	6
squid ointment	4
$Ca(H2PO4)_2$	1.5
premix	1.5
Total	100

1) Premix (per kilogram of premix): VA 8000000 IU, VD 2000000 IU, VE 5000 UI, VK 1000 mg, VB_1 1500 mg, VB_2 1500 mg, VB_6 800 mg, VB_{12} 20 mg, nicotinamide 400 mg, calcium pantothenate 25 mg, folic acid 25 mg, biotin 8 mg, inositol 100 mg; $MnSO_4{\cdot}H_2O$ 50 mg, KI 100 mg, $CoCl_2$ (1%) 100 mg, $CuSO_4$ ·$5H_2O$ 20 mg, $FeSO_4$ ·H_2O 260 mg, $ZnSO_4{\cdot}H_2O$ 150 mg, Na_2 SeO_3 (1%) 50 mg.

TABLE 2 Nutrient composition of the basal and experimental diets.

Groups	EE	CP	Ash	Moisture
T0	8.8	49.7	15.71	11.1
T1	8.6	48.2	15.8	39.1
T2	8.6	47.5	15.7	37.1
T3	8.5	48.5	15.7	37.5

1) Nutrient levels were measured in values.

2.4 *A.hydrophila* challenge test

At the end of the feeding trial, 30 fish of each group (10 fish/tank) were selected to be injected with *A.hydrophila* for infection, and previously we derived from pre-experiment that the LC_{50} of *A.hydrophila* on juvenile largemouth bass was 1.65×10^6 CFU/ml at an injection dose of 0.2 ml/tail. The trial fish were anesthetized with an appropriate amount of 50 ppm MS-222 for 3-5 min, and then 0.2 ml of *A.hydrophila* liquid with a concentration of 1.65×10^6 CFU/ml was slowly injected from the abdominal cavity using a 1 ml injector, and the breeding environment was kept unchanged.

2.5 Sample collection

For statistical analysis of growth performance, we fasted for 24 h at the end of the breeding test. We weighed the total weight of each test tank and counted the number of surviving fish for statistical analysis. Nine fish were randomly selected from each replicate, anesthetized with 100 ppm MS-222, and their body weight and length were measured. Subsequently, the midgut of three fish was randomly selected from each group of nine fish and fixed with 4% paraformaldehyde for intestinal histological observation. Take the remaining fish viscera to measure the body index. collected livers and intestines were snap frozen in liquid nitrogen (-196°C) and then transferred to -80°C refrigerator for storage. On the third day after the *A.hydrophila* infection treatment, all fish were fasted for 24 h, anesthetized with 100 ppm MS-222, and the whole intestines were taken into sterile tubes, snap-frozen in liquid nitrogen (-196°C), and then transferred to -80°C refrigerator storage for intestinal inflammatory factor expression assay. Growth indicators and cumulative mortality were calculated as follows:

$$\text{WGR (weight gain rate, \%)} = 100 \times (W_t - W_0)/W_0$$

$$\text{SR (survival rate, \%)} = 100 \times (N_t/N_0)$$

$$\text{SGR(specific growth rate, \%/}d\text{)} = 100 \times (lnW_t - lnW_0)/t$$

$$\text{HSI (Hepatosomatic index, \%)} = 100 \times W_h/W$$

$$\text{CF}\left(\text{Condition factor g/cm}^3\right) = W/L^3$$

$$\text{Cumulative mortality rate}\ (\%) = (N_d/N_a) \times 100$$

Where N_t and N_o represent the total number of fish samples at the beginning and end of the experiment, respectively; W_t and W_0 are the initial and final weight data at the beginning and end of the experiment (g); W is the body weight per fish, W_h is the liver weight of per fish. t represents the number of experimental days (day); L is the length of the fish (cm). N_d is the cumulative number of fish dead; N_a is the initial number of fish after injection of *A.hydrophila*.

2.6 Sample measurement

2.6.1 Histomorphology of the intestine

The midgut of juvenile largemouth bass was rinsed with saline and fixed with 4% paraformaldehyde. After processing, sections (5 μm) were sectioned using a paraffin slicer, followed by hematoxylin-eosin (HE) staining and image acquisition by light microscopy, with the observed sections first observed under low magnification and the appropriate areas selected for image acquisition under high magnification. The data for villi height, width, and thickness of the muscular layer were measured by Image-Pro Plus software.

2.6.2 Analysis of antioxidant enzyme activities and immune enzyme activities in liver

The liver tissues stored at -80 °C were thawed on ice, and the tissues were homogenized with saline 1:9 according to the kit instructions, centrifuged at 4 °C and 2,500 r/min for 10 min, and then the supernatant was taken as the tissue homogenate. Total protein (TP), superoxide dismutase (SOD), total antioxidant capacity(T-AOC), glutathione peroxidase (GSH-Px), malondialdehyde (MDA) and catalase (CAT) were measured in the liver using a spectrophotometer or enzyme marker according to the steps of the kit (Nanjing Jiancheng Institute of Biological Engineering) instructions.

2.6.3 Measurement of intestinal inflammatory factor expression after *A.hydrophila* infection

Intestinal tissues stored at -80°C were placed in RNAase-free centrifuge tubes and ground using a microtissue homogenizer. Total RNA was extracted from the intestinal tissues by the Trizol method (TaKaRa, Japan). The concentration of RNA was measured using a micro ultraviolet spectrophotometer. The first-strand cDNA was synthesized using the kit (product number RR047A, TaKaRa) according to the instructions. Protocol for reverse transcription: 37°C for 15 minutes; 85°C for 5 seconds. The primers were used to refer to the study of Xv (Xv et al., 2021). Table 3 shows the PCR primers used in this study for the coding sequences of *IL-1β*, *IL-8*, *IL-10*, *IL-15*, *TNF-α* and *TGF-β* genes in the largemouth bass genome. And *β-actin* was used as an internal reference gene, the specific primers for *β-actin* and target genes were synthesized by Tsingke Biotechnology Co., Ltd. Quantitative real-time PCR (qPCR) was performed using NovoStart SYBR qPCR SuperMix Plus (Novoprotein) on Bio-Rad CFX96 (Bio-Rad) in a total volume of 20μL.

2.7 Calculations and statistical methods

All data are expressed as mean± SD. Significance levels were determined by one-way analysis of variance (ANOVA) with

TABLE 3 Primer sequences for real-time PCR.

Genes	Primers	Sequence 5′–3′	TM (°C)	Accession number
IL–8	F	CGTTGAACAGACTGGGAGAGATG	64.9	RNA-seq by (Xv et al., 2021)
	R	AGTGGGATGGCTTCATTATCTTGT		
IL–10	F	CGGCACAGAAATCCCAGAGC	62.1	RNA-seq by (Xv et al., 2021)
	R	CAGCAGGCTCACAAAATAAACATCT		
IL–15	F	GTATGCTGCTTCTGTGCCTGG	62	RNA-seq by (Xv et al., 2021)
	R	AGCGTCAGATTTCTCAATGGTGT		
IL–1β	F	CGTGACTGACAGCAAAAAGAGG	59.4	RNA-seq by (Xv et al., 2021)
	R	GATGCCCAGAGCCACAGTTC		
TGF-β	F	GCTCAAAGAGAGCGAGGATG	59	RNA-seq by (Xv et al., 2021)
	R	TCCTCTACCATTCGCAATCC		
TNF-α	F	CTTCGTCTACAGCCAGGCATCG	63	RNA-seq by (Xv et al., 2021)
	R	TTTGGCACACCGACCTCACC		
β-actin	F	AAAGGGAAATCGTGCGTGAC	60	RNA-seq by (Xv et al., 2021)
	R	AAGGAAGGCTGGAAGAGGG		

IL-1β, interleukin-1β; IL-8, interleukin-8; IL-10, interleukin-10; IL-15, interleukin-15; TNF-α, tumor necrosis factor-α; TGF-β1, transforming growth factor-β.

IBM SPSS Statistics 23. Multiple comparisons were performed using the Tukey multiple range test. The statistical significance level was set at p<0.05. Graphs were drawn using GraphPad Prism6 (GraphPad Software, Inc., USA).

3 Results

3.1 Growth performance and morphometric parameters

As the growth performance and morphological indices are shown in Table 4, FT can significantly improve the weight gain rate and specific growth rate of juvenile largemouth bass compared with T0 ($P< 0.05$). Compared with T3, T1 and T2 also significantly increased the weight gain and specific growth rate of juvenile largemouth bass ($P< 0.05$). The condition factor of T1 was significantly higher than the rest of the other groups, and FT could significantly reduce the HSI index of juvenile largemouth bass ($P< 0.05$). However, there was no significant difference in the mortality rate among all groups ($P > 0.05$).

3.2 Cumulative mortality of juvenile largemouth bass after injection of *A.hydrophila*

In this trial, largemouth bass were infected with *A.hydrophila* and mortality was counted for 3 consecutive days, as shown in Figure 1. After the injection of *A.hydrophila*, all groups showed mortality on the first day, but the cumulative mortality rate of the control group was higher than that of the other groups ($P< 0.05$), and the cumulative mortality rate rapidly increased with time. The cumulative mortality rate for T0 was 43.33%, while T1 was 23.33%, T2 was 20.00%, and T3 was 26.67%, respectively.

3.3 Morphological observation of the intestinal tract of juvenile largemouth bass

The tissue structure of the midgut of juvenile largemouth bass is shown in Figure 2, and the characteristics of the midgut villi are shown in Table 5. The intestinal villi of juvenile largemouth bass in the control group were shorter and sparser, with fewer villi and goblet cells ($P< 0.05$). The height of intestinal villi was significantly higher in the test group than in the control group ($P< 0.05$).

3.4 Largemouth bass liver antioxidant index

The antioxidant indices in the livers of juvenile largemouth bass are shown in Table 6. Compared with the control group, the activities of SOD, GSH-PX, CAT and the level of T-AOC in the livers showed a significant increase as well as a significant decrease in MDA content in juvenile largemouth bass after 8 weeks of FT feeding ($P< 0.05$).

3.5 Expression of intestinal inflammatory factors in juvenile largemouth bass after *A.hydrophila* infection

Infection with *A.hydrophila* caused an inflammatory response in the intestine characterized by increased expression of pro-inflammatory factors and decreased expression of anti-inflammatory factors. In this experiment, we measured the expression of intestinal inflammatory factors in largemouth bass after infected by *A.hydrophila*. And the results are shown in Figure 3. In juvenile largemouth bass fed FT for 8 weeks, the relative mRNA expressions of intestinal pro-inflammatory factors IL-1β, IL-15, IL-8 and TNF-α decreased significantly, while the relative mRNA expressions of anti-inflammatory factors IL-10 and TGF-β increased significantly ($P< 0.05$). The relative mRNA expression of anti-inflammatory factors IL-10 and TGF-β1 was significantly higher in T2 compared with those of T1 and T3. In contrast, the relative mRNA expression of pro-inflammatory factors IL-1β and IL-15 was significantly lower in T2 compared with T1 and T3 ($P<0.05$).

4 Discussion

Tea residue is rich in tea polyphenols, theanine, tea saponin and other active substances, which can improve the production

TABLE 4 Growth performance and morphological indicators.

Items	T0	T1	T2	T3
WGR	137.73 ± 11.16^{C}	219.27 ± 7.77^{A}	204.83 ± 18.71^{A}	175.93 ± 2.84^{B}
SGR	1.53 ± 0.06^{C}	2.07 ± 0.06^{A}	2.00 ± 0.10^{A}	1.80 ± 0.00^{B}
SR	98.33 ± 2.89	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
CF	1.73 ± 0.21^{B}	2.03 ± 0.06^{A}	1.70 ± 0.10^{B}	1.80 ± 0.00^{B}
HSI	3.23 ± 0.50^{A}	2.00 ± 0.2^{B}	1.97 ± 0.45^{B}	$2.20 \pm 0.0.36^{B}$

Different letters indicate significant differences (P< 0.05); Values are presented as mean ± SD (n = 3); WGR, weight gain rate; SR, survival rate; SGR, specific growth rate; HSI, Hepatosomatic index; CF, Condition factor.

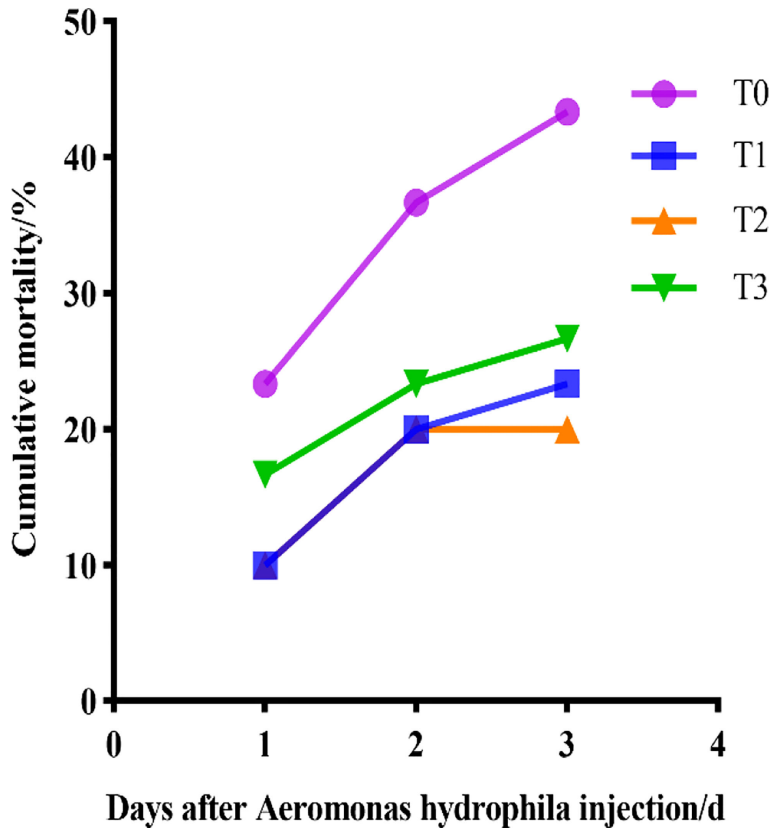

FIGURE 1
Cumulative mortality of juvenile largemouth bass after injected with *A.hydrophila*.

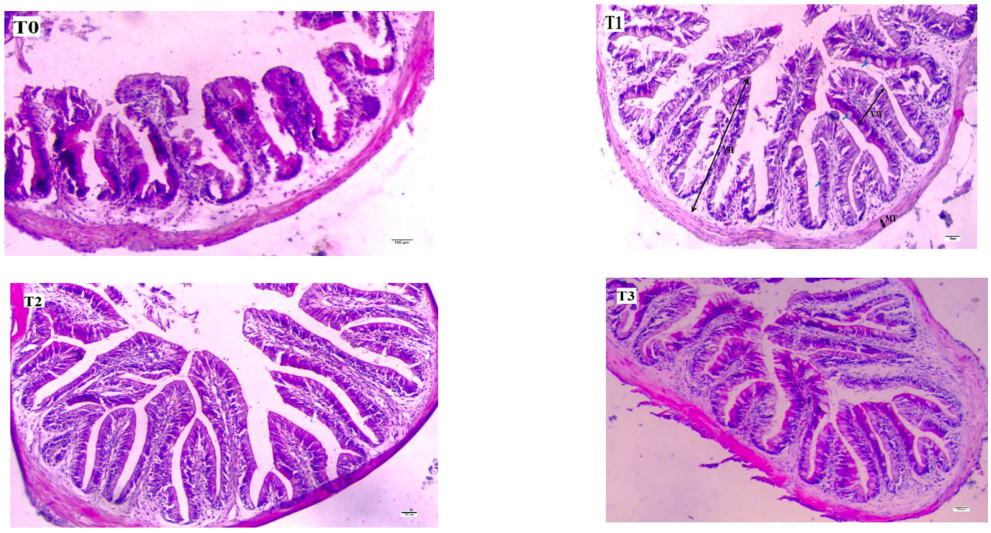

FIGURE 2
Effect of different scales of FT on the morphology of the mid-gut of the juvenile largemouth bass (X100, H&E staining, scale bar = 100(μm). Villi height (VH), villi width (VW), muscular layer thickness (MT), and the blue arrows indicated are goblet cells.

TABLE 5 Characteristics of midgut villi of largemouth bass.

Items	VH/μm	VW/μm	MT/μm
T0	582.47 ± 30.15^{C}	216.14 ± 14.10^{A}	74.59 ± 3.85
T1	793.92 ± 24.30^{A}	199.57 ± 11.64^{B}	71.72 ± 4.05
T2	725.50 ± 23.74^{B}	216.16 ± 15.11^{A}	76.37 ± 3.95
T3	720.64 ± 56.81^{B}	181.69 ± 14.05^{C}	73.75 ± 4.56

Different letters indicate significant differences (P< 0.05); Values are presented as mean ± SD (n = 5); VH, Villi height; VW, villi width; MT, muscular layer thickness.

performance of livestock and meat quality (Hamer, 2007). It has been shown that the addition of appropriate amounts of tea to diets can improve the activity of digestive enzymes, reduce anti-nutritional factors, and improve the use of nutrients, thus promoting the growth of fish (Zhang et al., 2015; Zheng et al., 2017). Similar to this study, plant extracts such as Common Sage (*Salvia officinalis*), Coneflower (*Echinacea angustifolia*), Cornelian cherry (*Cornus mas L.*), Rose hip and Safflower can stimulate the innate immune response and feed intake, thus improving fish growth performance (Dadras et al., 2016; Dadras et al., 2019; Ahmadifar et al., 2022). Fermented feeds can reduce the anti-nutritional factors in feeds and increase the digestion and absorption capacity of feeds, thus promoting the growth of the organism (Ilha et al., 2017; Wang et al., 2017). The addition of fermented tea residue to the diet can improve the fattening performance and digestive performance of fattening pigs (Ding et al., 2020). In this study, FT was able to improve the weight gain rate and specific growth rate of juvenile largemouth bass, but the weight gain rate and specific growth rate were lower than the actual production values, which was probably because the experiment was conducted during the seasonal change in autumn and winter, the temperature may have affected the growth performance of largemouth bass.

The intestine is an important site for digestion and absorption of nutrients as well as protection against pathogens in fish. Intestinal histological assessment is an effective method to assess the effect of dietary components on the intestinal health of fish (Chauhan and Singh, 2018; Ding et al., 2020). Some studies found that tea polyphenols and fermented feed could increase the height of intestinal villi and thickness of the muscle layer in fish, and improve the intestinal histology of fish (Mamauag et al, 2019; Ma et al., 2021; Zhuo et al., 2021). It was found that the height and width of intestinal villi of juvenile largemouth bass were higher than those of the control group after feeding FT, which indicated that the integrity and stability of the intestinal tract were enhanced, thus strengthening the digestion and absorption of nutrients by juvenile largemouth bass and promoting their growth, this might be one of the reasons for their better growth performance compared to the control group.

Oxidative stress is a state in which there is an imbalance between oxidation and antioxidant action in the body, a negative effect produced by free radicals in the body, which predisposes the body to age and disease (Bai et al., 2017). The most common enzymatic antioxidants present in animals are CAT, SOD and GSH-Px, which mainly serve to scavenge peroxides in the body to protect it from damage caused by oxidative stress (Yuan et al., 2019; Chen et al., 2020). The antioxidant enzyme activity in animals determines the antioxidant capacity of their bodies, which can be used to assess the health of fish (Tovar-Ramírez et al., 2010). The level of T-AOC is one of the indicators of antioxidant capacity in animals, which is an important indicator of the antioxidant capacity of fish directly (Cui et al., 2014; Yu et al, 2021). This study found that FT could enhance the activity of SOD, GSH-Px and CAT as well as the level of T-AOC in the liver of juvenile largemouth bass, which indicated that FT could enhance the antioxidant capacity of juvenile largemouth bass and slow down the oxidative damage to the organism. MDA is a peroxidation metabolite generated by lipids in the body under the influence of free radicals. The level of MDA content can directly reflect the damage to the body by free radicals, and higher levels of MDA reflect higher peroxidation reactions in the

TABLE 6 Analysis of antioxidant enzyme activities and immune enzyme activities in liver.

Items	T0	T1	T2	T3
TP (mgprot/mL)	3.09 ± 0.19	3.55 ± 0.17	3.53 ± 0.22	3.50 ± 0.33
SOD (U/mgprot)	115.22 ± 1.79^{C}	176.41 ± 4.35^{A}	152.72 ± 9.70^{B}	148.64 ± 3.63^{B}
GSH-PX (U/mgprot)	18.04 ± 1.18^{D}	51.96 ± 2.01^{A}	40.68 ± 2.67^{C}	46.76 ± 0.86^{B}
T-AOC (U/mgprot)	2.64 ± 0.30^{C}	5.65 ± 0.35^{A}	3.58 ± 0.26^{B}	3.57 ± 0.39^{B}
MDA (nmol/mgprot)	5.65 ± 0.31^{A}	4.07 ± 0.01^{B}	3.55 ± 0.12^{C}	3.57 ± 0.14^{C}
CAT (U/mgprot)	144.51 ± 3.30^{C}	229.84 ± 7.10^{B}	241.40 ± 14.16^{B}	273.59 ± 9.61^{A}

Different letters indicate significant differences (P< 0.05); Values are presented as mean ± SD (n =5); TP, total protein; SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde; T-AOC, total antioxidant capacity; GSH-PX, glutathione peroxidase.

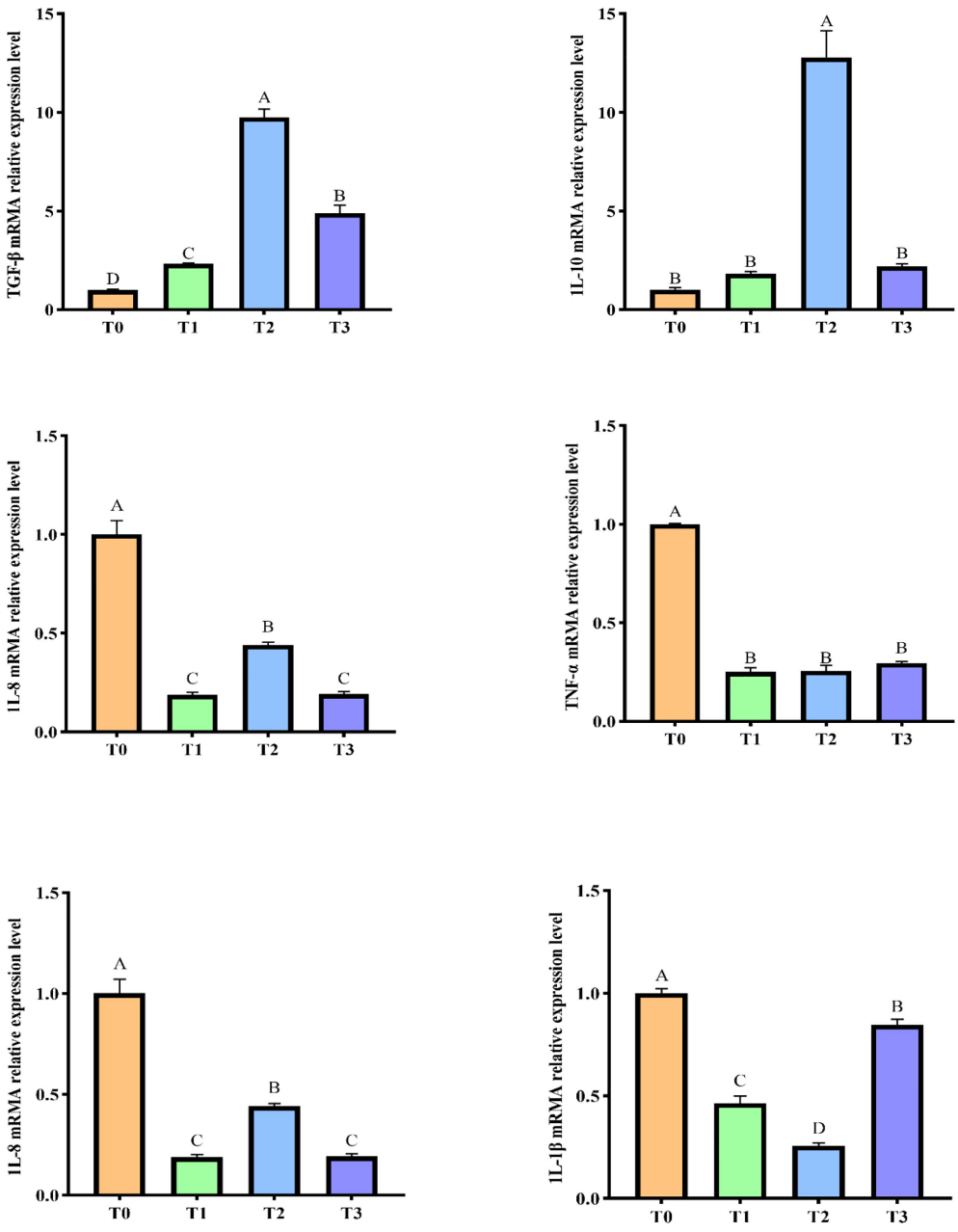

FIGURE 3
Effect of feeding FT on the expression of inflammatory factors in the intestine of largemouth bass after infection with *A.hydrophila*. Different letters indicate significant differences ($P < 0.05$); Values are presented as mean ± SD (n =5). IL-1β: interleukin-1β; IL-8: interleukin-8; IL-10: interleukin-10; IL-15: interleukin-15; TNF-α: tumor necrosis factor-α; TG F-β1: transforming growth factor-β.

body (Janero, 1990; Yu et al., 2019). In this study, we found that feeding FT could reduce the liver MDA content of juvenile largemouth bass. The results were generally consistent with other studies that dietary medical plants or fermented tea residue could enhance the activity of SOD, GSH-Px and CAT as well as the level of T-AOC and reduce the content of MDA in rainbow trout (Ghafarifarsani et al., 2022), Holstein heifers (Xie et al., 2020), common carp (Ahmadifar et al., 2022), Siberian sturgeon (Hasanpour et al., 2019), Tilapia (Qian et al., 2021), Sea bream (Pérez-Jiménez et al., 2012) and juvenile Wuchang bream (Guo et al., 2020).

Bacterial infection is often used as a final indicator of fish health status after nutrient analysis (Wang et al., 2015; Li et al., 2020). Bacterial enteritis is the most common intestinal disease

of freshwater fish. Among many pathogenic bacteria, *A.hydrophila* is usually considered one of the main pathogens causing intestinal inflammation in fish (Cascón et al., 2000; Macpherson et al., 2012). In our study, the test fish fed FT showed a higher survival rate after infected with *A.hydrophila*, which may be due to the probiotic bacteria used in tea residue with fermentation to promote the immune response of largemouth bass. This is in agreement with previous studies that found green tea or probiotics can enhance the resistance of Nile tilapia (Abdel et al., 2010; Cavalcante et al., 2020), Cyprinus carpio (Chandravanshi et al., 2020), Lates calcarifer (Lin et al., 2017) and Labeo rohita (Rai et al., 2015) to *A.hydrophila*. It showed that feeding FT improved the survival of juvenile largemouth bass and protected the intestine from damage by *A.hydrophila*.

Intestinal immune regulatory molecules (cytokines) expression is positively correlated with the immune status of fish (Gil, 2002; Reda et al., 2018). Inflammation occurs as an important component of the innate immune responses. Therefore, inflammatory cytokines are often used as biomarkers of immune regulation (Safari et al., 2016). Fish cytokines can be classified into anti-inflammatory factors (such as IL-10 and TGF-β1) and pro-inflammatory factors (such as TNF-α, IL-1β, IL-15 and IL-8), which have important functions in the immune response. Enterocolitis decreases the expression of anti-inflammatory factors and increases the expression of pro-inflammatory factors, so they can indicate inflammatory damage at the molecular level (Song et al., 2014; Fcab et al., 2019). Dietary medical plants and probiotics can regulate fish intestinal innate immunity by promoting anti-inflammatory factors and reducing the expression of pro-inflammatory factors, thus strengthening the resistance of fish to disease-causing agents and thus slowing down inflammation (Panigrahi et al., 2007; Nootash et al., 2013; Feng et al., 2019; Vazirzadeh et al., 2019; Ahmadifar et al., 2022). In this study, it was found that feeding FT can reduce the mRNA relative expression levels of pro-inflammatory factors IL-1β, IL-15, TNF-α and IL-8, and increase the mRNA relative expression levels of anti-inflammatory factors IL-10 and TGF-β1 in juvenile largemouth bass. Therefore, the appropriate amount of FT can regulate the inflammatory state in the intestine of juvenile Largemouth bass after infection with *A.hydrophila*, thereby reducing the intestinal damage caused by *A.hydrophila*.

5 Conclusion

In this research, we found that the addition of a certain amount of FT could improve the growth performance and antioxidant capacity of juvenile largemouth bass, improve intestinal health, and increase resistance to *A.hydrophila*. A comprehensive analysis of this experiment showed that 2% FT addition was more effective.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

This trial was approved by the Southwest University of Science and Technology in China, Institutional Animal Care and Use Committee. All of the procedures were performed by the Declaration of Helsinki and relevant policies in China.

Author contributions

LJ: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. XZ: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. JY: Conceptualization, Methodology, Investigation, Writing – review and editing. SB: Formal analysis, Data curation, Investigation. JL: Data curation, Investigation. QW: Data curation, Investigation. MW: Investigation. YW: Investigation, Supervision, Funding acquisition. BL: Investigation, Writing – review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effects of dietary pyrroloquinoline quinone on growth performance, serum biochemical parameters, antioxidant status, and growth-related genes expressions in juvenile yellow catfish, *Pelteobagrus fulvidraco*

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This study aimed to evaluate the impacts of dietary pyrroloquinoline quinone (PQQ) supplement on growth performance, serum biochemical parameters, antioxidant status, and growth-related genes expressions in juvenile yellow catfish, *Pelteobagrus fulvidraco*. Triplicate groups of fish (n = 40) with an average weight of 5 g were fed with five gradient levels PQQ-incorporated diets (0 (basal), 1.5 mg/kg; 3.0 mg/kg; 4.5 mg/kg, 6.0 mg/kg) for 56 days. Our findings revealed that fish fed with the diets containing PQQ at the level of 3.0-6.0 mg/kg showed significantly higher final body weight, weight gain rate, and specific growth rate than those of that in the control group ($P < 0.05$). The activities of protease were observed significantly increased in fish fed with diets containing 4.5 mg/kg and 6 mg/kg PQQ ($P < 0.05$). Meanwhile, fish in 4.5 mg/kg PQQ group showed significantly lower levels of serum total cholesterol, triglycerides, and low-density lipoprotein cholesterol, and significantly higher level of the high-density lipoprotein cholesterol ($P < 0.05$). The antioxidant-related parameters of superoxide dismutase and total antioxidant capacity were markedly elevated ($P < 0.05$), while malondialdehyde content was significantly reduced in 3.0-6.0 mg/kg PQQ group ($P < 0.05$). Meanwhile, the mRNA expression levels of growth-related genes (*growth hormone*, *insulin-like growth factor 1*, and *insulin-like growth factor 2*) were dramatically up-regulated in the liver of fish fed with the diets containing 3-6 mg/kg PQQ in comparison with the control group ($P < 0.05$). In conclusion, dietary PQQ could improve the growth performance, serum biochemical parameters, antioxidant status, and growth-related genes expressions in juvenile yellow catfish, and the

optimal dietary PQQ level was evaluated to be 4.92 mg/kg of dry diet for juvenile yellow catfish.

KEYWORDS

pyrroloquinoline quinone, growth, antioxidant status, gene expression, *Pelteobagrus fulvidraco*

Introduction

Yellow catfish, *Pelteobagrus fulvidraco* is an important aquaculture species in China and its production has greatly improved over the past 10 years due to its suitability for aquaculture, marketability, good taste, and high nutritional value (Shi et al., 2021). Unsurprisingly, this kind of fish has been largely cultured to meet the increasing market demands. As a result, lots of outbreaks of infectious diseases that caused by microorganisms (such as viruses, bacteria fungi, or parasites) are commonly seen and which usually leads to large economic losses (Zhang et al., 2014; Jiang et al., 2018). Antibiotics have been commonly used as a traditional strategy to control the outbreak of various infectious disease (Ramesh and Souissi, 2018). However, the over and continuous application of antibiotics may cause the emergence of antimicrobial resistance, environmental hazards, and food safety problems (Hollis and Ahmed, 2014). Meanwhile, the interest in the safety and quality of aquatic products of customers is obviously increasing with the growing problems of contaminants, antibiotics, and carcinogens in aquatic industry (Rama and Manjabhat, 2014). Therefore, antibiotics have been banned or restricted for utilization in aquaculture and which has encouraged researchers to develop alternative strategies. Thus far, many research teams in this field have devoted themselves to evaluating the positive effects of eco-friendly bio-active components as functional feed supplements on the growth, feed utilization, and enzymatic profiles in different fish including yellow catfish (Gabriel et al., 2017; Safari et al., 2020; Park et al., 2021; Fu et al., 2022).

Pyrroloquinoline quinone (PQQ), a water-soluble thermo-stable triglyceride-quinone (Zhang et al., 2006), is initially identified in methylotrophic bacteria and characterized as a redox cofactor of bacterial dehydrogenases, such as alcohol and glucose dehydrogenases (Killgore et al., 1989). PQQ is an essential nutrient for animals, and intake of PQQ-deficient diet usually leads to multifarious illnesses (Akagawa et al., 2016). PQQ has caused considerable attention, as it is exactly important for mammalian growth, development, reproduction, and immune function (Steinberg et al., 2003; Ikemoto et al., 2017). PQQ is also an effective antioxidant that can protect mitochondria from oxidative stress-induced lipid peroxidation, protein carbonyl formation, and mitochondrial respiratory chain inactivation (Hwang and Willoughby, 2018). On a molar basis, PQQ exhibited 15-fold effects than ascorbic acid in reducing chemiluminescence from xanthine-xanthine oxidase reaction and 7-fold effects than alpha-tocopherol in preventing lipid peroxidation in rat brain preparations (Hamagishi et al., 1990). In addition, PQQ inhibits the apoptosis of cardiomyocytes under conditions of oxygen/glucose deprivation (Xu et al., 2014). Because of its versatile functions, PQQ-containing products have been certified by authorities in Canada as a Natural Health Product (Health Canada, 2012) and have also been authorized as a new type of food for use in food supplement by the European Commission in 2018. Until now, no published studies concerning the physiological responses to PQQ-supplemented diets has been reported in yellow catfish. Meanwhile, it is also unclear whether PQQ can be used as a supplement in aquaculture. According to studies in broilers (Samuel et al., 2015; Liu et al., 2020; Zheng et al., 2020) and pigs (Zhang et al., 2019; Yin et al., 2019), we hypothesized that PQQ may benefit aquaculture by affecting growth, plasma parameters, and antioxidant status of fish. To verify this hypothesis, the present study evaluate the effects of PQQ supplementary diets on the growth performance, serum biochemical parameters, antioxidant status, and growth-related gene expression such as *growth hormone* (*GH*), *insulin-like growth factor 1* (*IGF-1*), and *insulin-like growth factor 2* (*IGF-2*) in juvenile yellow catfish.

Materials and methods

Experimental diets and feeding trials

PQQ (purity, ≥98 mg/kg; Shanxi Boke Biological Technology Co., Ltd., Xian, China) was diluted with wheat flour to a concentration of 1 g/kg mixture before being mixed into the diet. Five experiment diets containing 0 (control), 1.5, 3.0, 4.5, and 6 mg per kg of PQQ in this experiment were produced at Neijiang Normal University, Neijiang, China, as described by Shi et al. (2021). The amount of cellulose was reduced in compensation. The dried experimental diets were stored at −20 °C for further use. The composition of the experimental diets was shown in Table 1.

The current study was performed in polyvinyl chloride round aquarium (Diameter × High: 100 × 120 cm) located at Neijiang Normal University (Sichuan, China). Fish provided by a private fishery farm (Meishan, China) were transported to the rearing facilities with air pumps, acclimated for two weeks and fed with the basal diet during this period. After two weeks of acclimatization to aquarium conditions, a total of 600 fish (average body weight of 5 g) were randomly divided into five treatment groups with three replicates (40 fish per replicate). During the feeding period, fish were fed with the designed diet twice daily (7:30 and 18:30) for 56 days. Daily feeding rates were 4-6% of the total body weight for each aquarium. The detailed food intake was recorded. Uneaten pellets were collected at 30 min after feeding, gathered, dried, and weighted in turn, and the data were used to calculate the actual food intake. This trial was carried out under natural photoperiod. About 25-30% of the water in the aquarium was replaced per day and the water temperature, dissolved oxygen, and pH were maintained at 25.0 ± 2.5°C, 7.3 ± 0.2 mg/L, and 7.6 ± 0.2, respectively. Furthermore, the level of ammonia was kept below 0.3 mg/L.

Samples collection

At the end of the feeding trial, fish were fasted for 24 h, weighed and counted to calculate their growth performance. Blood samples (9 fish per replicate) were drawn from the caudal vein, separated by centrifugation after clotting (10 min at 4000 rpm). The supernatant was stored at −80°C for plasma biochemical and antioxidant enzyme activity analysis. After that, the liver and intestine (6 fish per replicate) were removed immediately using sterile forceps, frozen rapidly by dipping in liquid nitrogen, and then stored at −80 °C until for analysis.

Digestive enzyme activity

The intestine samples were removed onto the ice, homogenized in a 1:9 (m/v) ratio of physiological saline solution, and then centrifuged with 3000 rpm at 4 °C for 10 min. After that, the supernatant comprising enzymes was stored at −80 °C until further utilization. The amylase and lipase activities were determined in triplicate using commercial assay kits supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The protease activity of each sample were analyzed in triplicate was detected using Folin method with reference to the professional standards of People' s Republic of China to determine protease activity SB/T 10317-1999 (SB/T 10317-1999, (1999)). The total amount of protein in the intestine was determined by using the Bradford method (Bradford, 1976). All enzyme activities were measured as the change in absorbance using a Microplate Reader (UV-2802S; Unico, Shanghai, China).

Serum biochemical parameters

The serum biochemical parameters, including alanine aminotransferase (ALT), aspartate aminotransferase (AST),

TABLE 1 Diet formulation and proximate analysis.

Ingredients (g kg^{-1})	Control	1.5 mg/kg PQQ	3.0 mg/kg PQQ	4.5 mg/kg PQQ	6.0 mg/kg PQQ
Fish meal	280	280	280	280	280
Soybean meal	230	230	230	230	230
Wheat flour	310	310	310	310	310
Soybean oil	25	25	25	25	25
Chicken meal	80	80	80	80	80
Lecithin	20	20	20	20	20
Mineral premix[1]	10	10	10	10	10
Vitamin premix[2]	10	10	10	10	10
Choline chloride	5	5	5	5	5
CaH_2PO_4	10	10	10	10	10
Cellulose	20	18.5	17	15.5	14
PQQ premix[3]	0	1.5	3.0	4.5	6.0
Proximate compositions (% dry weight)					
Crude protein	43.36	43.25	43.30	43.38	43.27
Crude lipid	8.67	8.61	8.58	8.64	8.65
Moisture	5.73	5.72	5.69	5.76	5.74
Crude ash	9.35	9.38	9.33	9.32	9.36

[1]Mineral premix (mg/kg per premix): Mg 26 g; Fe 8 g; Mn 2 g; I 500 mg; Cu 1 g; Zn 5 g; Se 35 mg; Co 100 mg.
[2]Vitamin premix (mg/kg per premix): VA 200000 IU; Vitamin D_3 150000 IU; Vitamin C 11000 mg; VE 4500 mg; Vitamin K_3 480 mg; Vitamin B_1 500 mg; Vitamin B_2 750 mg; Vitamin B_6 650 mg; Vitamin B_{12} 2 mg; Inositol 3000 mg; Nicotinamide 3200 mg; D-calcium pantothenate 1500 mg; Folic acid 130 mg; D-biotin 15 mg.
[3]PQQ was diluted with corn starch to a concentration of 1.0 g/kg mixture.

total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and blood urea nitrogen (BUN) were measured with commercial kits producted by Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instructions.

Antioxidant parameters

The antioxidant parameters including superoxide dismutase (SOD), catalase (CAT), total antioxidant capacity (T-AOC), glutathione peroxidase (GPX), and malondialdehyde (MDA) were all determined using kits from the same manufacturer as described by an our previous study (Shi et al., 2019).

RNA isolation and gene expression

The RNA isolation and detection process were performed according to the method described by Shi et al. (2019). In brief, the total RNA was extracted from liver sample (approximately for 50 mg) using 1 mL Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. Thereafter, RNA quality and purity were verified by agarose gel (1 mg/kg) electrophoresis and UV-spectroscopic analysis. A fixed concentration of RNA (2 μg) was used for cDNA synthesis using Prime Script II 1st Strand cDNA Synthesis Kit (Tiangen, Beijing, China) based on the manufacturer's protocol. For qRT-PCR, specific primers for *GH*, *IGF-1*, and *IGF-2* genes were designed with online Primer 5 software (PREMIER Biosoft International, San Francisco, CA, USA), based on an our transcriptome data of yellow catfish (Table 2). The SYBR Green qPCR Master Mix Kit (Glpbio, USA) was used for qRT-PCR analysis on a Bio-Rad CFX Connect System (Bio-Rad, Hercules, CA, USA). The qRT-PCR program was designed as follows: 95°C for 5 min, followed by 95°C for 15 s, annealing at specific temperatures (Table 1) for each gene for 30 s, a total of 40 cycles, and 72°C for 30 s. The reaction volume was 20 μL. Each transcript was analyzed in triplicate (3 fish for each replicate). The $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expression levels of selected genes.

Statistical analysis

The weight gain rate (WGR), the specific growth rate (SGR), the feed conversion ratio (FCR), the survival rate (SR), hepatosomatic index (HSI), and condition factor (CF) were calculated as follows according to the report by Park et al. (2021):

$$WGR = (final\ body\ weight - initial\ body\ weight) / initial\ body\ weight \times 100$$

$$SGR = [100 \times (Ln(final\ body\ weight) - Ln(initial\ body\ weight)] / time\ interval\ (days)$$

$$FCR = feed\ consumed\ (g,\ dry\ weight) / weight\ gain\ (g,\ wet\ weight)$$

$$SR = (initial\ fish\ individuals - dead\ fish\ individuals) / initial\ fish\ individuals \times 100$$

$$HSI = 100 \times liver\ weight\ (g) / whole\ body\ weight\ (g) \quad ;$$

$$CF = 100 \times bodyweight\ (g) / (body\ length,\ cm)^3$$

All data were presented as the mean ± standard error, analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests using SPSS 22.0 software (IBM Corp., Armonk, NY, USA). Data in different groups was considered to be significant if $P < 0.05$.

Results

Growth performance

The effect of PQQ-supplemented diets on growth parameters of juvenile yellow catfish is displayed in Table 3. Compared with the control group, dietary PQQ supplementation at 3-6 mg/kg significantly increased the growth parameters such as FBW, WGR, and SGR of the juvenile yellow catfish ($P < 0.05$), and the maximum

TABLE 2 The oligonucleotide sequences of primers for quantitative real-time PCR analysis in this study.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
IGF-1	5′-GTACGAGAGCAACGGCACACAG-3′	5′-GGCTTGAGTTCTTCTGATGGACCTC-3′
IGF-2	5′-GATATGAGCAGTGGCAACGGATAGC-3′	5′-TTTGAACTTTCTGGAGCGGAGGATG-3′
β-actin	5′-GATTCGCTGGAGATGATGCT-3′	5′-CGTGCTCAATGGGGTACTTC-3′
GH	5′-GCGAGTTTGCTCTTTAGT-3′	5′-CGATGGAGTCCGAGTTG-3′

TABLE 3 Effects of PQQ on growth performance of yellow catfish.

Groups[1]	Control	1.5 mg/kg	3.0 mg/kg	4.5 mg/kg	6.0 mg/kg
FBW (g)	27.52 ± 6.20^{a}	30.17 ± 5.59^{ab}	32.09 ± 4.36^{b}	36.47 ± 3.64^{c}	33.12 ± 2.07^{bc}
WGR (%)	452.31 ± 29.56^{a}	521.66 ± 34.68^{b}	553.62 ± 38.97^{b}	625.84 ± 21.76^{c}	578.56 ± 25.42^{bc}
SGR (% day^{-1})	3.03 ± 0.27^{a}	3.19 ± 0.14a^{b}	3.32 ± 0.36^{b}	3.57 ± 0.09^{c}	3.38 ± 0.18^{bc}
FCR (%)	1.31 ± 0.03^{c}	1.22 ± 0.06^{bc}	1.16 ± 0.11^{b}	0.96 ± 0.12^{a}	1.07 ± 0.14^{ab}
SR (%)	96.67± 0.03	95.83± 0.05	98.33 ± 0.01	95.00 ± 0.03	96.67 ± 0.03
HSI (%)	1.43 ± 0.03	1.46 ± 0.07	1.44 ± 0.04	1.42 ± 0.02	1.50 ± 0.06
CF (g/cm^{-3})	1.89 ± 0.16	1.90 ± 0.20	1.86 ± 0.23	1.87 ± 0.09	1.92 ± 0.17

[1]Data are presented as the mean ± SD (n = 3 replicates).
Data in the same row with different superscripts show significant differences ($P < 0.05$).
FBW, final body weight, WGR, weight gain rate, SGR, specific growth rate, FCR, feed conversion rate, SR, survival rate, HSI, hepatosomatic index; CF, condition factor.

values of these parameters were appeared at 4.5 mg/kg group. On the contrary, FCR was decreased significantly with the administration of 3-6 mg/kg PQQ to the diet and the minimum value was observed in the 4.5 mg/kg group ($P < 0.05$). No significant change was detected in SR, HSI, and CF in all groups ($P > 0.05$). The relationship between FBW and dietary PQQ levels for yellow catfish juveniles can be well expressed by the following secondary curve equation: $y = -0.30286 x^2 + 2.98381 x + 27.01114$ ($R^2 = 0.83237$) (Figure 1).

Digestive enzyme activity

As shown in Table 4. Dietary PQQ supplementation had no significant effect on the amylase and lipase activities in the intestine of yellow catfish ($P > 0.05$). However, significantly higher protease activities were observed in fish fed with 4.5 mg/kg and 6 mg/kg PQQ groups compared with those of that in the control group ($P < 0.05$).

Serum biochemical parameters

Table 5 presents the effect of dietary PQQ supplementation on serum biochemical parameters in juvenile yellow catfish. The activity of AST in the 3 mg/kg PQQ group and the level of LDL-C in the 4.5 mg/kg PQQ group were all significantly lower than those of that in the control group ($P < 0.05$). Except for 6 mg/kg group, HDL-C level was significantly higher than the control group ($P < 0.05$), and the highest value was recorded in the 3 mg/

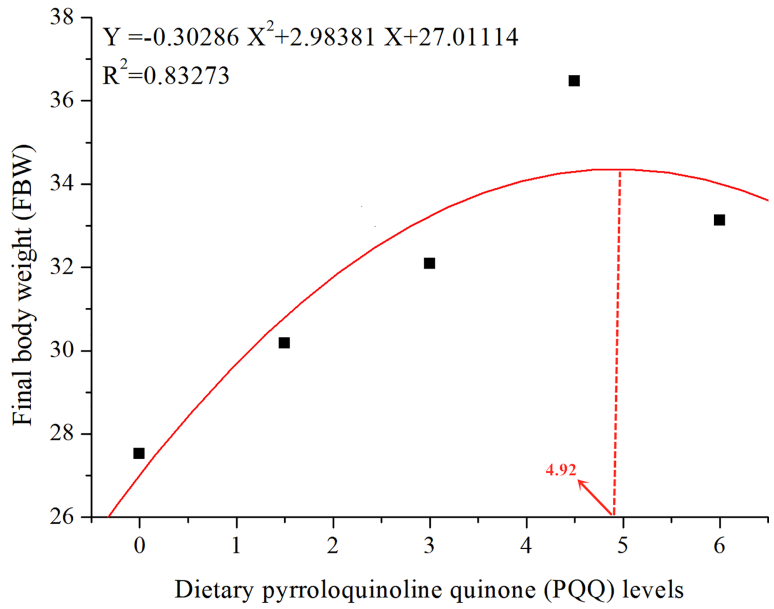

FIGURE 1
The relationship between the final body weight (FBW) of juvenile yellow catfish and different levels of PQQ supplemented diet after feeding for 8 weeks.

TABLE 4 Effects of PQQ on digestive enzyme activity in the intestine of yellow catfish.

Groups[1]	Control	1.5 mg/kg	3.0 mg/kg	4.5 mg/kg	6.0 mg/kg
Protease (U mg^{-1} prot)	88.35 ± 11.24^{a}	92.07 ± 15.36^{a}	90.18 ± 10.67^{a}	109.54 ± 16.73^{b}	113.89 ± 13.55^{b}
Lipase (U mg^{-1} prot)	127.58 ± 12.94	130.35 ± 10.50	138.72 ± 21.65	129.38 ± 17.74	136.35 ± 15.27
Amylase (U mg^{-1} prot)	99.32 ± 13.17	96.84 ± 15.26	98.54 ± 10.17	97.04 ± 12.38	101.35 ± 14.20

[1]Data are presented as the mean ± SD (n = 3 replicates).
Data in the same row with different superscripts show significant difference (P < 0.05).

kg PQQ group. Besides, the levels of TG and TC in the 4.5 mg/kg PQQ and 6 mg/kg PQQ groups were significantly lower in comparison with the control group ($P < 0.05$). However, dietary PQQ supplementation did not cause significant changes in the serum ALT and BUN contents ($P > 0.05$).

Serum antioxidant status

As displayed in Table 6, the activities of SOD in the serum significantly increased in fish fed with PQQ diets than that of those in the control group ($P < 0.05$), of which in 1.5 mg/kg PQQ group was highest among various groups ($P < 0.05$). The levels of T-AOC in fish fed with PQQ diets (except 1.5 mg/kg) were significantly increased ($P < 0.05$), but no significant difference was observed among the various PQQ diets ($P > 0.05$). Unlike SOD and T-AOC, remarkable decrease of MDA contents was found in yellow catfish fed with 3 mg/kg, 4.5 mg/kg, and 6 mg/kg of PQQ compared to the fish from the control group. However, no significant difference was observed regarding the serum GPX and CAT activities in the PQQ supplementation groups and the control group ($P > 0.05$).

Gene expression

Relative gene expression levels of growth-related genes *GH*, *IGF-1*, and *IGF-2* were shown in Figure 2. The mRNA expression levels of *GH* in the liver of fish fed with higher concentration of PQQ in diets (4.5 mg/kg and 6 mg/kg) were significantly increased than that of those fed with the lower concentration (1.5 mg/kg and 3 mg/kg) and control diet ($P < 0.05$). Except for 1.5 mg/kg group, relative mRNA levels of live *IGF-1* in various PQQ groups were significantly higher than that of those in the control group ($P < 0.05$). Dietary PQQ supplementation significantly increased the mRNA expressions of the *IGF-2* in the liver of yellow catfish ($P < 0.05$), especially in the 4.5 mg/kg PQQ group, with representing 12.06-fold higher in comparison with the control group.

Discussion

In the present study, dietary with PQQ (3-6 mg/kg) supplementation resulted in significantly higher FBW, WGR, and SGR, as well as significantly lower FCR. Thus, dietary SHE was advantageous for the growth of yellow catfish. Similarly, growth was enhanced by adding PQQ to the basal diet in mice (Steinberg et al., 2003). Meanwhile, PQQ was a feed additive that can effectively promote the utilization of nutrients and stimulate the development of breast muscle in broiler chicks (Samuel et al., 2015; Liu et al., 2020), and improved the growth of weaned pigs, feed efficiency, and reduces the incidence of diarrhea in weaned pigs (Yin et al., 2019). The promotion of growth by dietary PQQ might be attributed to the modulation of mitochondrial function

TABLE 5 Effects of PQQ on serum biochemical parameters of yellow catfish.

Groups[1]	Control	1.5 mg/kg	3.0 mg/kg	4.5 mg/kg	6.0 mg/kg
AST (U L^{-1})	52.13 ± 3.84^{b}	48.28 ± 6.09^{ab}	35.37 ± 2.82^{a}	41.16 ± 3.81^{ab}	38.20 ± 5.69^{ab}
ALT (U L^{-1})	13.43 ± 2.56	14.22 ± 1.91	14.62 ± 2.24	15.11 ± 1.40	15.34 ± 2.64
TC (mmol L^{-1})	5.56 ± 0.53^{b}	4.98 ± 0.75^{ab}	4.84 ± 0.42^{ab}	4.26 ± 0.37^{a}	3.99 ± 0.21^{a}
TG (mmol L^{-1})	2.87 ± 0.01^{c}	2.48 ± 0.23^{bc}	2.18 ± 0.17^{b}	1.74 ± 0.23^{a}	2.21 ± 0.13^{b}
HDL-C	0.39 ± 0.04^{a}	0.86 ± 0.07^{bc}	1.19 ± 0.18^{c}	0.94 ± 0.08^{bc}	0.70 ± 0.05^{ab}
LDL-C	2.38 ± 0.25^{b}	1.78 ± 0.19^{ab}	1.76 ± 0.18^{ab}	1.27 ± 0.21^{a}	1.81 ± 0.15^{ab}
BUN	6.44 ± 0.32	6.60 ± 0.19	6.34 ± 0.15	6.58 ± 0.34	6.33 ± 0.20

[1]Data are presented as the mean ± SD (n = 3 replicates).
Data in the same row with different superscripts show significant difference (P < 0.05).
AST, aspartate aminotransferase, ALT, alanine aminotransferase, TC, total cholesterol, TG, triglyceride, HDL-C, high density lipoprotein cholesterol, LDL-C, low density lipoprotein cholesterol, BUN, blood urea nitrogen.

TABLE 6 Effects of PQQ on antioxidant capacity in the serum of yellow catfish.

Groups[1]	Control	1.5 mg/kg	3.0 mg/kg	4.5 mg/kg	6.0 mg/kg
T-AOC (U mg^{-1} prot)	6.56 ± 0.56^{a}	7.75 ± 1.04^{a}	11.58 ± 1.74^{b}	11.22 ± 0.95^{b}	10.67 ± 0.92^{b}
SOD (U mg^{-1} prot)	1.82 ± 0.08^{a}	3.60 ± 0.28^{c}	2.38 ± 0.05^{b}	2.46 ± 0.34^{b}	2.72 ± 0.13^{b}
CAT (U mg^{-1} prot)	43.90 ± 4.43	42.98 ± 3.71	41.85 ± 5.90	40.36 ± 3.10	42.72 ± 4.26
GPX (U mg^{-1} prot)	315.80 ± 24.07	300.67 ± 16.79	324.41 ± 19.63	304.87 ± 28.29	307.19 ± 22.09
MDA (nmol mg^{-1} prot)	10.91 ± 1.23^{b}	11.52 ± 1.41^{b}	8.45 ± 1.02^{a}	7.44 ± 0.75^{a}	6.94 ± 0.86^{a}

[1]Data are presented as the mean ± SD (n = 3 replicates).
Data in the same row with different superscripts show significant difference (P < 0.05).
AOC, total antioxidant capacity, SOD, superoxide dismutase, GPX, glutathione peroxidase, CAT, catalase, MDA, malondialdehyde.

(Bauerly et al., 2006). PQQ was found to stimulate mitochondrial biogenesis through promoting the phosphorylation of cAMP response element-binding protein and increasing the expression of peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1α) (Chowanadisai et al., 2010). PQQ could also improve intestinal health to promote the growth of yellow catfish. Yin et al. (2019) revealed that PQQ can enhance intestinal morphology, promote intestinal barrier integrity, and improve the antioxidant status of the intestine. Wang et al. (2020) indicated that PQQ can alter the composition or metabolism of intestine microbiota, especially to increase the abundance of *Firmicutes* and decrease the levels of *Actinobacillus* and *Escherichia*, resulting in a more balanced bacterial structure. Moreover, the promotion of growth by PQQ supplementation could be also due to the increase of digestive enzyme activity. The digestive enzymes, such as protease, lipase, and amylase, etc., play a major role in food digestion and assimilation (Duan et al., 2017). An increase in the production of these enzymes is usually associated with an improvement in overall body metabolism (Midhun et al., 2019). In the present study, fish fed with 4.5 mg/kg and 6 mg/kg PQQ increased significantly the activities of protease. This observation indicated that PQQ in the diet might benefit for protein digestion and absorption in yellow catfish intestine, and thus improve growth performance in yellow catfish. The potential mechanism is that PQQ may have synergistic action along the intestinal tract and promote the development of intestinal tissue (Zheng et al., 2020), modulate intestinal microbial status (Wang et al., 2020), and ultimately stimulate enzyme expression.

Blood analysis plays a key role in the nutrition and physiology of fish, which can indirectly reflect the health status of fish (Hossain et al., 2016). AST and ALT are two of the most important aminotransferases in fish and are usually considered sensitive tools for indications of liver tissue damage. The decline of serum enzymes mentioned above in response to the

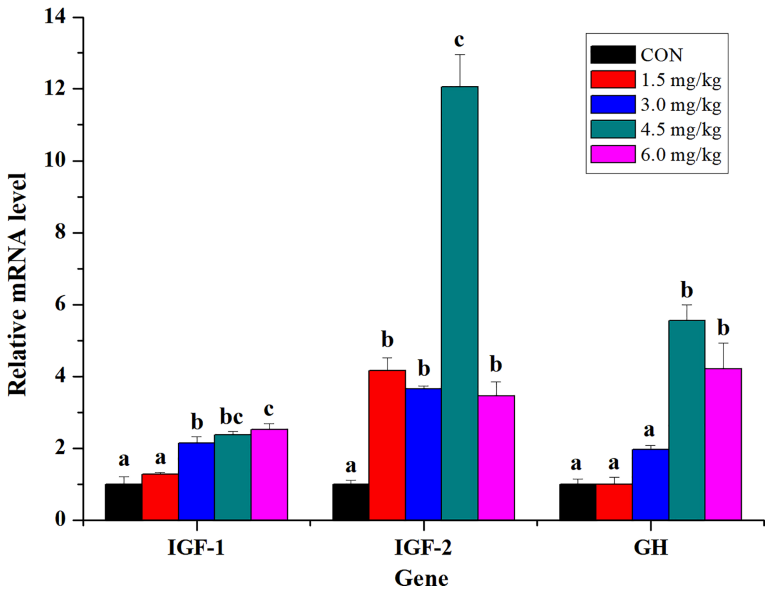

FIGURE 2
Effects of PQQ on the gene expression of growth-related genes *GH*, *IGF-1*, and *IGF-2* in the liver of yellow catfish. Bars with different letters in the same gene indicate significant difference between the corresponding treatment (P < 0.05) (n = 3).

nutritional agent is usually thought to improve liver function (Wang et al., 2016). In this study, the AST decreased significantly in fish fed with 3 mg/kg PQQ, suggesting that PQQ had a beneficial effect on liver health. Consistently, a previous study reported that PQQ showed a better hepatoprotective effect against oxidative stress by reducing the elevated AST activity in the serum caused by oxidized sunflower oil (Zhao et al., 2014). Serum TG and TC, are two important indicators of lipid levels in fish, which reflect the metabolism, and increase energy storage of lipids in fish, and high contents of TG and TC in the serum are believed to be involved in cardiovascular diseases (Castro et al., 2015). The present study revealed that PQQ could reduce the levels of serum TC and TG, suggesting that PQQ possessed a hypolipidemic effect in yellow catfish. Similar to our results, Zhao et al. (2014) demonstrated that PQQ can significantly inhibit the elevation of triglyceride and total cholesterol in the liver of laying hens induced by high-energy and low-protein diets. One possible mechanism that might explain these observations could be due to PQQ can protect the integrity of mitochondria in hepatocytes, promote β-oxidation of fatty acids, regulate the level of lipid metabolism in the body, increase the uptake and reduce the accumulation of TG in the liver tissues, and thus decrease serum and/or liver cholesterol levels (Chowanadisai et al., 2010; Bauerly et al., 2011). However, the exact mechanisms are still needed to be further investigated. Our results also revealed that PQQ significantly reduced serum LDL-C and increased serum HDL-C levels in yellow catfish. Similarly, Zhang et al. (2015) showed that PQQ can significantly increase HDL-C levels in the serum of broilers after 21 days of feeding. The decline of LDL-C is especially linked with HDL-C. Several researchers have reported that HDL plays antioxidant roles due to its antioxidant proteins and enzymes (Mackness and Mackness, 2012; Soran et al., 2015; Islam et al., 2018). Apolipoprotein-AI, the major structural protein of HDL, is considered the main antioxidant factor in HDL, and which is capable of removing LDL lipid hydroperoxides (Islam et al., 2018). The increased concentration of serum HDL in our study was accompanied by decreased levels of LDL and MDA.

The anti-oxidative enzymes CAT, SOD, and GPX are essential for the protection of important organelles and macromolecules in cells from oxidation-related damage by scavenging or neutralizing the pro-oxidants produced by normal animal metabolism (Rashidian et al., 2021). T-AOC directly reflected the antioxidant capacity of fish, which prevents reactive oxygen species' negative effects (Tan et al., 2017). MDA, an end-product of lipid peroxidation, indirectly reflected the extent of lipid peroxidation in tissue cells from free radicals attack (Cai et al., 2016). Usually, higher levels of SOD, CAT, and GPX activities revealed an increased antioxidant defense in fish. In this study, although PQQ supplementation had no impact on CAT and GPX activity, the activity of SOD and level of T-AOC in the serum were significantly increased by 3-6 mg/kg PQQ supplementation, while the MDA levels were markedly decreased. These data revealed that PQQ may improve antioxidant capacity and reduce lipid oxidation damage in yellow catfish. Similar results were also observed in the previous studies in laying hens (Wang et al., 2016), weaned pigs (Ming et al., 2021), and broilers (Samuel et al., 2015). PQQ was reported to be a potent non-enzymatic antioxidant, and its reduced form (pyrroloquinoline quinol, $PQQH_2$) can directly eliminate reactive oxygen species (superoxide anion, hydrogen peroxide, and lipid radicals), with $PQQH_2$ having a scavenging capacity 7.4 times higher than that of vitamin C, which is the most active water-soluble antioxidant (Ouchi et al., 2009). On the other hand, PQQ seems to enhance the antioxidant defense system by inducing antioxidant enzymes (Misra et al., 2004), consistent with our previous finding. A recent study had shown that PQQ could increase antioxidant enzyme activity by stimulating the PGC-1α and Nrf2-ARE signaling pathways of the peroxisome proliferator-activated receptor (Chowanadisai et al., 2010).

It is well known that the GH/IGF axis plays an important role in the regulation of fish growth (Picha et al., 2008). GH can bind to the growth hormone receptor (GHR) in targeted tissues, then promote IGFs production and release in the liver and in most peripheral tissues, which mediates many of the growth-promoting effects of GH (Tan et al., 2017). There are two principle IGFs referred to as IGF-1 and IGF-2 (Gabillard et al., 2006). In particular, IGF-1 promotes growth in large part depending on nutrient availability (Fox et al., 2010). IGF-2 is indicated to show a high structural homology with IGF-1 and extensively expressed in juvenile and adult fish (Terova et al., 2007). IGFs stimulate strongly growth, inducing an anabolic effect on protein and carbohydrate metabolism (Perez-Sanchez and Le Bail, 1999; Amin et al., 2019). Previous studies had showed that the increase in the number of mRNA copies of *GH* and *IGFs* expression levels likely reflects the improved growth performance of teleosts under the same nutrition conditions (Picha et al., 2008; Asaduzzaman et al., 2017; El-Kassas et al., 2020). In the current study, fish fed with 4.5 mg/kg and 6 mg/kg PQQ increased significantly the expressions of *GH*, *IGF-1*, and *IGF-2* in the liver of yellow catfish. This was in accordance with the result obtained in growth performance. We infer that PQQ could stimulate the growth of yellow catfish *via* its action on the GH/IGF axis. Currently, studies on the effects of dietary PQQ supplemented on the GH/IGF axis in fish are rarely reported. Hence, future studies are needed to better understand the underlying mechanisms of PQQ affecting GH/IGF axis. Moreover, the expressions of *IGF-1* and *IGF-2* in the liver were in parallel with that of *GH* in this study. This finding suggest that GH may directly promote the mitosis and differentiation of cells to indirectly trigger the production and release of IGF (Delgadin et al., 2015).

In conclusion, our study revealed that dietary PQQ supplementation had beneficial effects on growth performance,

serum biochemical parameters, and antioxidant status of juvenile yellow catfish, and the optimum supplemental level of PQQ is 4.92 mg/kg.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by The Institutional Animal Care and Use Committee of Neijiang Normal University, the Institutional Ethics Committee of the Chinese Institute of Chemical Biology guidelines. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

QS: Project administration, writing – original draft, Writing – review & editing. ZW: Review & editing. JW: Software, Formal analysis. PH and YZ: Investigation, Methodology. SW: Supervision. CQ: Review & editing, Funding acquisition. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Dietary sodium butyrate administration alleviates high soybean meal-induced growth retardation and enteritis of orange-spotted groupers (*Epinephelus coioides*)

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An 8-week feeding trial was conducted to investigate whether dietary sodium butyrate (SB) administration alleviates growth reduction and enteritis of orange-spotted grouper (*Epinephelus coioides*) caused by high soybean meal (SBM) feeding. The control diet (FM diet) was formulated to contain 48% protein and 11% fat. Soybean meal was used to replace 60% FM protein in FM diet to prepare a high SBM diet (HSBM diet). Sodium butyrate (SB) at 0.1%, 0.2%, and 0.3% were added to HSBM diets to prepare three diets. Triplicate groups of 30 groupers (initial weight: 33.0 $\pm$ 0.3 g) were fed one of the diets twice daily, to apparent satiety. HSBM diets had lowered growth rate and feed efficiency vs FM diets (P <0.05). Growth rate and feed efficiency were improved by dietary SB administration and were in a dose-dependent manner (P <0.05). A similar pattern to the growth rate was observed for plasma LDL-C and gut digestive activity of lipase, trypsin, and protease, but the opposite trend was observed for intestinal contents of D-lactic acid and endotoxin, in response to dietary SB inclusion levels (P >0.05). The muscular thickness in the middle and distal intestines in SB-treated diets were higher than that in HSBM diets (P <0.05). The mRNA levels of intestinal pro-inflammatory cytokines *IL-8*, *IL-1β*, *IL-12* and *TNF-α* had a decreasing trend, and the mRNA level of intestinal anti-inflammatory cytokine *TGF-β1* had the opposite trend, with increasing SB inclusion levels (P < 0.05). The above results indicate that dietary SB intervention could enhance growth and feed utilization of groupers with SBM-induced enteritis by promoting intestinal digestive enzyme activities, reducing mucosa permeability, maintaining the integrity of intestinal morphology and attenuating the intestinal inflammatory response.

KEYWORDS

sodium butyrate, growth performance, intestinal injury, *Epinephelus coioides*, inflammatory response

Introduction

Farmed marine fish generally require a high-quality protein feed, and fish meal (FM) is the major protein source that can fully meet the demand for the high-quality protein due to its high protein level, balanced amino acids profile, and less anti-nutritional factors in comparison with terrestrial animal and plant proteins (Zhao et al., 2021a; Mahamud et al., 2022). This determines the importance of FM as a protein source in marine aquaculture. However, over the past few decades, there has been a huge contrast between the rapid expansion of global aquaculture and the declining production of marine fishery catches (FAO, 2020). This causes a great deficit between FM supply and demand, making it a general consensus in the industry to find alternative protein sources of FM (Hardy, 2010). Soybean meal (SBM) is widely regarded as the most potential plant protein source to replace FM in marine fish feeds due to its relatively balanced amino acids profile, stable supply and low price. However, the majority of fish, especially carnivorous fish, show an obvious intolerance to SBM when given a diet containing a high percentage of SBM (Baeverfjord and Krogdahl, 1996; Zhu et al., 2021). Some anti-nutritional factors in SBM are the direct factors inducing enteritis, the so-called SBM induced enteritis (SBMIE) (Carmona, 2008), which leads to poor growth and other side effects, such as damage to the intestinal mucosal barrier, disturbance of the intestinal flora, an increased presence of inflammatory cells (Gatlin et al., 2007; Booman et al., 2018; Wang et al., 2020; Zhang et al., 2022). Therefore, how to prevent and control the widespread SBMIE is the key to maintaining normal daily fish culture and reducing disease risk. One of the effective ways to mitigate fish intestinal damage and enteritis caused by SBM-based feeds is the dietary use of functional feed additives through the nutrition regulation strategy (Peng et al., 2013; Ferrara et al., 2015; Rimoldi et al., 2016; Zhao et al., 2019).

Butyric acid is a metabolite of intestinal bacterium *Clostridium butyricum*, and as an important energy substance, provides energy for intestinal epithelial cell metabolism (Topping and Clifton, 2001; Hamer et al., 2007; Robles et al., 2013), promotes cellular function, and maintains intestinal health (Biagi et al., 2007). Butyric acid is a volatile fatty acid, and its stable form is sodium butyrate (SB). In animal feeding practice, SB, as a substitute for butyric acid, is often used as a functional additive in livestock and poultry feeds as well as aquafeeds (Bedford and Gong, 2018; Lin et al., 2020). The positive effects of dietary SB supplementation on growth performance have been achieved in fish such as turbot (*Scophthalmus maximus L.*) (Liu et al., 2019; Yu et al., 2021), yellow drum (*Nibea albiflora*) (Wu et al., 2020) and Pengze crucian carp (*Carassius auratus* Pengze) (Fang et al., 2021). The above study results showed that SB supplementation in high-SBM diets could alleviate SBMIE of fish by promoting digestion and absorption capacity and maintaining the integrity of intestinal morphology and structure, as well as intestinal microbial homeostasis (Liu et al., 2019). In addition, dietary SB was found to exert a regulatory role in the expression of related genes, such as intestinal inflammatory factor genes and growth-related factor genes (Wu et al., 2020; Abdel-Tawwab et al., 2021; Yu et al., 2021).

Grouper is a carnivorous marine fish that has been widely cultivated in Southeast Asia and China due to its fast growth and high quality of fish flesh (Dennis et al., 2020; Qin et al., 2022). The aquaculture output of grouper in China reached 192,045 tons in 2020 (China Fishery Statistics Yearbook, 2021). Great progress regarding its nutrition and feed research and development has been achieved (Ko et al., 2020; Bai et al., 2021; Kuo et al., 2021; Yang et al., 2021). However, there is still a lack of nutritional regulation research on the prevention of SBMIE in fish species. Our latest study also showed that a high SBM diet caused inflammatory reactions and reduced the growth performance of orange-spotted grouper (*Epinephelus coioides*) (Wang et al., 2017a; Zhao et al., 2021b). So far, the intervention effect of dietary SB on grouper SBMIE has not been reported yet. Therefore, the present study aimed to investigate the effects of SB supplementation (0.1%, 0.2%, and 0.3%) in high SBM diets on the growth performance, plasma components, and intestinal health of orange-spotted grouper. This study provided the basis for the prevention and control of SBMIE in the fish species.

Materials and methods

Experimental diets and rearing management

The ingredients and proximate composition of experimental diets are presented in Table 1. A basal diet (FM diet) was formulated to contain 48% crude protein and 11% crude lipid using fish meal (FM), gelatin and casein as the main protein sources and soybean and fish oil, and soy lecithin as the lipid sources. Soybean meal (SBM) was used to replace 60% FM protein in the FM diets to prepare a high SBM diet (HSBM diet). Sodium butyrate (SB) were added to the HSBM diet at 0.1%, 0.2%, and 0.3% to prepare three experimental diets (SB0.1 diet, SB0.2 diet, SB0.3 diet, respectively). Coarse dry feed ingredients were ground from a hammer mill (GH-20B, Jiangyin Kejia Machinery Manufacturing Co., Ltd., Jiangyin, Jiangsu, China), sieved through a 60-mesh sieve, then weighed and homogenized. Liquid ingredients (fish and soybean oil, soy lecithin and freshwater) were then incorporated into the dry feed ingredients and a mash was prepared. The dough was extruded into strands and made into 2.5 mm and 4 mm pellets through dies using the cold press extrusion method

TABLE 1 Formulations and nutrient level on experimental diets of grouper (on an as-fed basis, %).

Ingredients	Diets[1]				
	FM	HSBM	SB0.1	SB0.2	SB0.3
Fish meal	52	22	22	22	22
Casein	11.98	11.27	11.27	11.27	11.27
Gelatin	3	2.82	2.82	2.82	2.82
Soybean meal	–	47	47	47	47
Soybean oil	3.5	3.5	3.5	3.5	3.5
Fish oil	0.82	3.52	3.52	3.52	3.52
Soybean lecithin	2	2	2	2	2
Sodium butyrate	–	–	0.1	0.2	0.3
Corn starch	17.72	3.26	3.16	3.06	2.96
Sodium alginate	1	1	1	1	1
Ca(H2PO4)2	1.5	1.5	1.5	1.5	1.5
Choline chloride	0.4	0.4	0.4	0.4	0.4
Vitamin C	0.03	0.03	0.03	0.03	0.03
Vitamin mix	0.4	0.4	0.4	0.4	0.4
Mineral mix	0.5	0.5	0.5	0.5	0.5
Taurine	0.5	0.8	0.8	0.8	0.8
Microcrystalline cellulose	4.65	–	–	–	–
Total	100	100	100	100	100
Nutrient level (analyzed values)					
Dry matter (%)	95.06	95.78	95.11	95.19	95.57
Crude protein (%)	49.85	50.34	49.00	50.48	50.25
Crude lipid (%)	11.56	11.42	11.63	11.37	11.60
Energy MJ/kg	18.7	18.7	18.6	18.6	18.6

[1]FM, fish meal; HSBM, 60% FM protein replacement with SBM in FM diets without SB addition; SB0.1, SB0.2 and SB0.3 were added with 0.1%, 0.2%, and 0.3% SB in HSBM diets, respectively.
Fish meal (crude protein 70.34%, crude lipid 9.06%) and soybean (crude protein 46.60%, crude lipid 0.72%), and other feed ingredients except sodium butyrate and premix were provided by Jiakang Feed Co., Ltd., Xiamen, China.
Sodium butyrate (99% purity) was provided by Xinao Biotechnology Co., Ltd., China.
Vitamin and Mineral premix were obtained from Guangzhou Feixite Aquatic Technology Co., Ltd.

(CD4XITS, South China University of Technology, Guangzhou, Guangdong, China). The pellets were dried in a ventilated oven at 55°C for 24 h until the moisture was reduced to 10%, and then placed at room temperature for 24 h, before being sealed in plastic bags and stored at -20°C.

The juvenile groupers obtained from a commercial fish farm were transported to Dabeinong experimental station (Zhangzhou City, Fujian Province, China). Fish were stocked in a concrete pond and fed a commercial feed for 3-week acclimatization. At the beginning of the experiment, the fish (initial mean weight of 33.0 ± 0.3 g) were allocated to five groups each with triplicate tanks (500 L/tank), at a stocking density of 30 fish/tank in a water temperature-controlled recirculating culture system. Fish were fed one of the experimental diets twice daily (8:00, 17:00) to apparent satiety each meal under a natural photoperiod across a feeding period of 56-day. Excess feed was collected by siphoning 30 min after each meal, then dried at 65°C, and weighed to calculate feed intake (FI). Because daily sewage discharge will cause water loss in the aquaculture system, fresh seawater was refilled until the original water level of tanks was reached. During the feeding period, water temperature was kept at 28.5°C, the dissolved oxygen level was > 5.7 to 8.0 mg/L, and the ammonia nitrogen content was < 0.22 mg/L.

Sample collection

At the end of the growth trial, fish in each tank were caught and anesthetized with a dose of 100 mg/L solutions of MS-222 (tricaine methane sulphonate, Sigma-Aldrich Shanghai Trading Co., Ltd, Shanghai, China). Fish weight and number were then recorded for each tank to measure weight gain (WG), feed efficiency (FE), specific growth rate (SGR), and survival. Three fish from each tank were randomly sampled and pooled in plastic bags, and stored at -20°C for whole-body proximate composition determination. Nine fish per tank (27 fish each group) were weighed individually after an aesthesia with MS-222

(100 mg/L) to calculate the hepatosomatic index (HSI) and condition factor (CF). Blood was drawn from the caudal vein, using a 1-mL heparinized syringe, and centrifuged at 1027 × *g*, 4° C, 10 min. Plasma was then collected, pooled by tank and stored in 1.5-mL Eppendorf tubes at -80°C for the subsequent biochemical analysis. The intestine of nine fish per tank were aseptically removed and pooled into one tube by tank, stored at -80°C for the analysis of biochemical components, microbiota analysis, and gene expression.

Proximate composition analysis

Prior to component analysis, whole-fish samples were prepared according to the method described by Ye et al. (Ye et al., 2011). The proximate composition of diet and whole-body fish samples were determined according to standard methods (AOAC, 1995). Dry matter was determined by drying the samples in an oven at 105°C to a constant weight. Crude protein was determined by the Kjeldahl method ($N \times 6.25$) using Kjeltec TM 8400 Auto Sample Systems (Foss Teacher AB). The crude lipid content was determined by the Soxtec extraction method by using Soxtec Avanti 2050 (Foss Teacher AB). Ash was measured in the residues of samples burned in a muffle furnace at 550°C for 6 h.

Plasma component determination

The plasma contents of triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were determined using respective kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Gut digestive enzyme activity and mucosal function analysis

The intestinal activity for lipase and amylase was assayed using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) according to the protocols of the manufacturer. The activities of intestinal protease and trypsin were determined according to the method described by Hu et al. (2014). The contents of diamine oxidase (DAO), D-lactic acid (D-Lac), Endotoxin (ET) and Endothelin-1 (ET-1) in the intestine were determined using ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) according to the manufacturer's instructions.

Intestinal histological observation

To perform histological analysis, one fish was caught from each tank and dissected to obtain the whole gut, then divided into proximal, middle, and distal intestines (i. e. PI, MI, and DI, respectively), according to the method (Anguiano et al., 2013). All the segments were washed with normal saline, fixed in Bouin's solution for 24 h, rinsed with 70% (v/v) ethanol solution, and finally immersed in 70% (v/v) ethanol until histological processing was performed (Niu et al., 2021). The fixed gut segments were embedded in paraffin and 5-μm sections were cut by using a rotary microtome (KD-2258S, China). The serial histological sections were then mounted on glass slides, and stained with hematoxylin and eosin for morphometric analysis. Pictures were examined under a light microscope (Leica DM5500B, Germany), and digital images were taken and processed with a digital camera (Leica DFC450) equipped with the image program LAS AF (Version 4.3.0 Leica). Five slides were prepared for each gut segment sample and 30 measurements were made to determine the number of mucosal folds, muscle layer thickness, and length of the complete mucosal fold.

Intestinal microbiota analysis

Total DNA of the distal intestine (DI) of the fish was extracted using a DNA extraction kit (Omega Bio-teK, Norcross, GA, USA) according to the manufacturer's instructions. The integrity and quality, purity and quantity of DNA samples were assessed by electrophoresis on a 1% (w/v) agarose gel and spectrophotometer method (NanoDrop 2000, Wilmington, DE, U.S. 260nm/280nm optical density ratio), respectively. The V3-V4 region of the 16S rDNA gene of DI bacteria was amplified by polymerase chain reaction (PCR) using the forward primer 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and the reverse primer 806R (5'-GGACTACNNGGGTATCTAAT-3'). The PCR reaction system included pre-denaturation at 95°C for 5 min; denaturation at 95°C for 45 s, annealing at 55°C for 50 s, and extension at 72°C for 45 s, 32 cycles; extension at 72°C for 10 min. Subsequently, high-throughput sequencing was performed using Illumina Miseq PE300 at Beijing Allwegene Technology Co., Ltd (Beijing, China). The sequencing data of all samples were deposited into Sequence Read Archive (SRA) (Accession number: PRJNA875282). A library of small fragments was constructed using a paired-end for sequencing, and the data was passed through QIIME (v1.8.0) for removal of low-quality sequences and chimeras. Based on 97% sequence similarity, similar sequences were assigned to the same operational taxonomic units (OTU). Species classification information

corresponding to each OTU was obtained by comparing with the sliva database, and alpha diversity analysis (Shannon, Ace, and Chao1) was performed using Mothur software (version 1.31.2). Based on the weighted unifrac distance, the pheatmap of the R (v3.1.1) software package was used for clustering analysis. After the UniFrac algorithm, the information on system evolution was used to compare the difference in species communities among samples and beta diversity analysis was performed.

RNA extraction and gene expression analysis

The total RNA was extracted from the intestinal samples using TRIzol® reagent (Takara Co., Ltd, Japan) according to the manufacturer's instructions. Isolated RNA was quantified using the NanoDrop ND-2000 Spectrophotometer, and its integrity was confirmed by agarose gel electrophoresis. The cDNA was generated from 1 μg DNase-treated RNA and synthesized by a PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara Co., Ltd, Japan). Real-time PCR was employed to determine mRNA levels based on the TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) (Takara Co., Ltd, Japan) using a QuantStudio™ Real-Time PCR System (ABI) quantitative thermal cycler. The fluorescent quantitative PCR solution consisted of 10 μL TB Green Premix Ex Taq™ II (Tli RNaseH Plus) (2×), 0.8 μL PCR forward primer (10 μM), 0.8 μL PCR reverse primer (10 μM), 2.0 μL RT reaction (cDNA solution), and 6 μL dH_2O. The thermal program included 30 s at 95°C, 40 cycles at 95°C for 5 s, and 60°C for 30 s. The sequences of primers are shown in Table 2. All amplicons were initially separated by agarose gel electrophoresis to ensure that they were of the correct size. *β*-actin served as the internal reference gene to normalize cDNA loading. The gene expression levels of the target genes were analyzed by the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008) after verifying that the primers were amplified with an efficiency of approximately 100% (Hanaki et al., 2014), and the data for all treatment groups were compared with the data for the control group.

Statistical analysis

All data were presented as mean and standard error of the mean (SEM). The data were analyzed using a one-way analysis of variance (ANOVA) to test for differences between treatments and then the Student-Neuman-Keuls multiple comparison test was performed after confirming the normality and homogeneity of variance using the Kolmogorov-Smirnov test and Levene's test in SPSS Statistics 25.0 (SPSS, Michigan Avenue, Chicago, IL, USA). The data expressed as percentages or ratios were subjected to data conversion prior to statistical analysis. *P*-values <.05 was deemed as significant difference.

Results

Growth performance and proximate composition

The results of growth performance and proximate composition of groupers are presented in Table 3. The fish fed HSBM diet had significantly lower WG and SGR compared with those fed FM diet ($P < 0.05$). The WG and SGR in fish fed diets SB0.1-SB0.3 were higher than that in fish fed diet HSBM, and reached the level of diet FM ($P > 0.05$). The WG and FE were in a dose-dependent manner with the dietary SB inclusion levels (Figure 1). The maximum values for WG and FE were observed for diet SB0.2 and diet SB0.3 respectively. The FE, HSI, CF and whole-body proximate composition were not affected by dietary treatments ($P > 0.05$).

Plasma components

As shown in Table 4, plasma LDL-C concentration in HSBM group was significantly lower ($P < 0.05$). However, plasma LDL-C concentration in dietary SB-treated groups was not different from those of SB0.1 group and SB0.2 groups ($P > 0.05$), and lower than that in SB0.3 group ($P < 0.05$). The plasma HDL-C,

TABLE 2 Primer sequences for real-time PCR assay.

Genes	Forward (5'-3')	Reverse (5'-3')	Efficiency (%)	Accession number
IL-8	AAGTTTGCCTTGACCCCGAA	TGAAGCAGATCTCTCCCGGT	94	FJ913064.1
IL-1β	GCAACTCCACCGACTGATGA	ACCAGGCTGTTATTGACCCG	116	EF582837.1
IL-10	GTCCACCAGCATGACTCCTC	AGGGAAACCCTCCACGAATC	99	KJ741852.1
TGF-β1	GCTTACGTGGGTGCAAACAG	ACCATCTCTAGGTCCAGCGT	102	GQ503351.1
IL-12	CCAGATTGCACAGCTCAGGA	CCGGACACAGATGGCCTTAG	115	KC662465.1
TNF-a	GGATCTGGCGCTACTCAGAC	CGCCCAGATAAATGGCGTTG	91	FJ009049.1
β-actin	TGCTGTCCCTGTATGCCTCT	CCTTGATGTCACGCACGAT	104	AY510710.2

IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor.

TABLE 3 Effects of sodium butyrate (SB) addition in high soybean meal (SBM) diets on growth performance and proximate composition of groupers in a 56-d feeding period.

Item	Diets[1]				
	FM	HSBM	SB0.1	SB0.2	SB0.3
Growth performance					
IBW[2] (g/fish)	33.82 ± 0.10	33.76 ± 0.06	33.80 ± 0.07	33.78 ± 0.06	33.83 ± 0.02
FBW[2] (g/fish)	113.24 ± 0.66^{b}	95.47 ± 2.59^{a}	101.32 ± 4.45^{ab}	115.43 ± 7.32^{b}	111.83 ± 1.22^{b}
WG[2] (%)	234.81 ± 2.53^{ab}	182.81 ± 7.24^{a}	199.83 ± 13.74^{ab}	241.80 ± 22.18^{b}	230.53 ± 3.72^{ab}
SGR[2] (%/d)	2.16 ± 0.01^{b}	1.86 ± 0.04^{a}	1.96 ± 0.09^{ab}	2.18 ± 0.11^{b}	2.14 ± 0.02^{b}
FE[2]	0.93 ± 0.02	0.72 ± 0.03	0.89 ± 0.01	1.01 ± 0.10	1.03 ± 0.03
HSI[3] (%)	1.31 ± 0.09	1.24 ± 0.04	1.22 ± 0.03	1.15 ± 0.05	1.11 ± 0.01
CF[3]	3.16 ± 0.08	3.05 ± 0.11	3.17 ± 0.04	3.19 ± 0.04	3.08 ± 0.08
Whole-body composition (%)					
Moisture[2]	67.05 ± 0.21	67.27 ± 0.22	67.67 ± 0.33	67.54 ± 0.36	67.13 ± 0.17
Crude protein[2]	18.01 ± 0.49	17.95 ± 0.27	17.34 ± 0.33	18.92 ± 1.26	18.53 ± 0.26
Crude lipid[2]	8.25 ± 0.17	7.90 ± 0.29	8.04 ± 0.16	8.19 ± 0.26	8.10 ± 0.23
Ash[2]	5.00 ± 0.15	4.96 ± 0.07	4.82 ± 0.08	4.75 ± 0.01	5.05 ± 0.06

[1]FM, fish meal; HSBM, 60% FM protein replacement by SBM in FM diets without SB addition; SB0.1, SB0.2 and SB0.3, 0.1%, 0.2%, and 0.3% SB were added in the HSBM diets, respectively.
[2]Data are presented as the means of per dietary treatment (n = 3 tanks).[3] Data are presented as the means of per dietary treatment (n = 27 fish). Values in the same row with different superscripts indicate significant differences (P < 0.05), while that with the same letter or no letter superscripts indicate no significant difference (P > 0.05).
WG (weight gain, %) = 100×(FBW - IBW)/IBW; SGR (specific growth rate, %/d) = 100×(ln FBW - ln IBW)/d; FE (feed efficiency) = (FBW - IBW)/FI; HSI (hepatosomatic index, %) = 100×(liver weight (g)/body weight (g)); CF (condition factor) = 100×body weight (g)/(body length (cm))^{3}. IBW, initial body weight (g); FBW, final body weight (g); FI, feed intake (g).

TC and TG concentrations were not affected by dietary treatments (P>0.05).

Gut digestive enzymes activity

There was a general reduction in lipase, trypsin, and protease activity for HSBM diet vs for FM diet (Table 5). A general enhancement in lipase, trypsin, and protease activity by dietary SB addition, and showed positive quadratic (P < 0.05) responses to increasing dietary SB inclusion levels, and the maximum value both were observed for diet SB0.1 (Figure 2). Dietary treatment did not affect intestinal amylase activity (P >0.05).

Intestinal permeability

As shown in Table 6, intestinal D-Lac and ET concentrations were higher in HSBM group than that in FM group (P < 0.05). However, dietary SB addition lowered intestinal DAO activity,

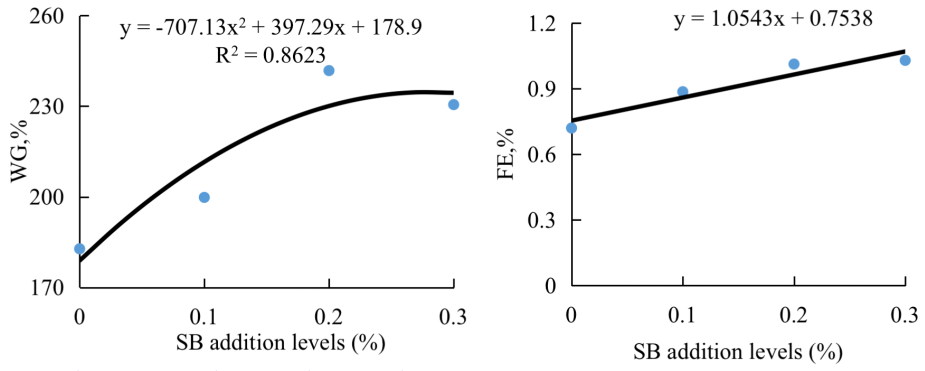

FIGURE 1
The relationship between weight gain (WG) or feed efficiency (FE) of groupers and sodium butyrate (SB) inclusion levels in HSBM diets in a 56-d feeding period. Data are presented as the means of per dietary treatment (n = 3 tanks). HSBM, 60% FM protein replacement with soybean meal (SBM) in FM diet and without sodium butyrate (SB) addition; SB0.1, SB0.2 and SB0.3, 0.1%, 0.2%, and 0.3% SB were added in HSBM diets,respectively.

TABLE 4 Effect of sodium butyrate (SB) addition in high soybean meal (SBM) diets on plasma components of groupers in a 56-d feed period.

Parameters	Diets[1]				
	FM	HSBM	SB0.1	SB0.2	SB0.3
HDL-C (mmol/L)	1.06 ± 0.05	1.00 ± 0.03	0.98 ± 0.11	0.85 ± 0.11	1.05 ± 0.07
LDL-C (mmol/L)	0.28 ± 0.01^{c}	0.19 ± 0.01^{b}	0.18 ± 0.01^{ab}	0.18 ± 0.02^{ab}	0.14 ± 0.01^{a}
TC (mmol/L)	3.77 ± 0.21	3.49 ± 0.23	3.38 ± 0.35	3.20 ± 0.29	2.76 ± 0.14
TG (mmol/L)	1.61 ± 0.17	1.36 ± 0.08	1.40 ± 0.08	1.43 ± 0.18	1.43 ± 0.12

[1]FM, fish meal; HSBM, 60% FM protein replacement with SBM in FM diets without SB addition; SB0.1, SB0.2 and SB0.3, 0.1%, 0.2%, and 0.3% SB were added in the HSBM diets, respectively. Data are presented as the means of per dietary treatment (n = 3 tanks). Values in the same row with different superscripts indicate significant differences (P < 0.05), while that with the same letter or no letter superscripts indicate no significant difference (P > 0.05). HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; TC, total cholesterol.

D-Lac and ET concentrations, and intestinal D-Lac concentration showed a declining trend with dietary SB inclusion levels. Furthermore, intestinal D-Lac and ET concentrations were reduced to the level of FM group (P >0.05). The intestinal ET-1 content was not affected by dietary treatments (P >0.05).

Intestinal morphology

As shown in Figure 3, the sizes of intestinal lumen and MFN were ordered as PI >MI >DI. The thickness of lamina propria and submucosa of MI in the HSBM group was increased compared to others. There was a reduction in the number of goblet cells with lymphocyte infiltration of DI for HSBM diet vs for FM diet, but an increase with increasing SB inclusion levels.

Table 7 shows the results of intestinal morphometrical parameters (MFH, mucosal fold height; MT, muscular thickness; MFN, mucosal fold number) in three intestinal segments (PI, MI, and DI) of groupers. There was a significant reduction (P < 0.05) in MT of MI and DI in fish fed the HSBM diet compared with those fed with FM diet. The MT of MI was increased with increasing SB inclusion levels in HSBM diets, the maximum value was observed for SB0.3 diet (P < 0.05), and MT value for SB0.2 diet returned to that of FM diet (P > 0.05). The MT of DI was also promoted by dietary SB inclusion levels, but the values in SB diets were lower than that in the FM diet (P < 0.05). The MFH of PI, MI, and DI, the MT of PI, as well as MFN of PI, MI, and DI remained unaffected across dietary treatments (P > 0.05).

Intestinal microbial abundance

The species abundance and diversity of DI samples are shown in Table 8. Although OTU and Ace showed a declining trend and Simpson and Shannon showed an upward trend with increasing dietary SB levels, the species abundance and diversity remained unaffected across dietary treatments (P > 0.05). At the phylum level, the average bacteria in the DI flora map of grouper were dominated by Firmicutes (relative abundance of 43.13%), Bacteroidetes (26.82%) and Proteobacteria (22.53%). Spirochaetae (1.53%) and Fusobacteria (1.08%) were subdominants (Figure 4A). However, these phyla bacterial abundance among all groups did not show significant differences (Figure 4B, P > 0.05). At the genus level, the bacteria in the DI flora map of grouper mainly included *Photobacterium* (FM: 18.9%; HSBM: 11.25%; SB0.1: 5.43%; SB0.2: 25.47%; SB0.3: 0.42%), *Selenomonas_1* (FM: 13.70%; HSBM: 13.04%; SB0.1: 11.76%; SB0.2: 7.90%; SB0.3: 12.64%), *Prevotella_1* (FM: 8.34%; HSBM: 7.88%; SB0.1: 7.61%; SB0.2: 6.08%; SB0.3: 9.16%), *Rikenellaceae_RC9_gut_group* (FM:

TABLE 5 Effects of sodium butyrate (SB) addition in high soybean meal (SBM) diets on activities of gut digestive enzymes of groupers in a 56-d feed period.

Parameters	Diets[1]				
	FM	HSBM	SB0.1	SB0.2	SB0.3
Lipase (U/mg prot)	0.80 ± 0.02^{a}	0.74 ± 0.06^{ab}	0.88 ± 0.01^{b}	0.77 ± 0.65^{ab}	0.73 ± 0.03^{ab}
Amylase (U/mg prot)	0.76 ± 0.06	0.73 ± 0.11	0.88 ± 0.06	0.91 ± 0.10	0.85 ± 0.09
Trypsin (U/g prot)	256.07 ± 17.23^{b}	175.55 ± 17.55^{a}	288.35 ± 10.66^{b}	282.25 ± 21.29^{b}	265.65 ± 5.36^{b}
Protease (U/mg prot)	20.54 ± 0.87^{b}	15.91 ± 2.04^{a}	24.03 ± 1.40^{b}	22.25 ± 1.75^{b}	22.15 ± 0.79^{b}

[1] FM, fish meal; HSBM, 60% FM protein replacement with SBM in FM diets without SB addition; SB0.1, SB0.2 and SB0.3, 0.1%, 0.2%, and 0.3% SB were added in the HSBM diets, respectively. Data are presented as the means of per dietary treatment (n = 3 tanks). Values in the same row with different superscripts indicate significant differences (P < 0.05), while that with the same letter or no letter superscripts indicate no significant difference (P > 0.05).

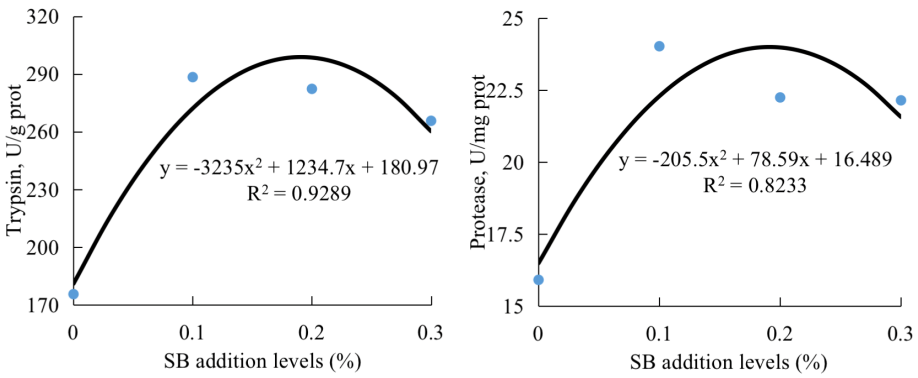

FIGURE 2
The relationship between intestinal activities of trypsin or protease of groupers and sodium butyrate (SB) inclusion levels in HSBM diets in a 56-d feeding period. Data are presented as the means of per dietary treatment. (n = 3 tanks). HSBM, 60% FM protein replacement with soybean meal (SBM) in FM diet and without SB addition; SB0.1, SB0.2 and SB0.3, 0.1%, 0.2%, and 0.3% SB were added in HSBM diets, respectively.

3.24%; HSBM: 3.81%; SB0.1: 4.88%; SB0.2: 5.75%; SB0.3: 6.37%) and *Curvibacter* (FM: 2.97%; HSBM: 2.81%; SB0.1: 2.76%; SB0.2: 1.50%; SB0.3: 2.08%) (Figure 5A) and marked difference in the abundance of these genus bacteria between groups was not observed (Figure 5B, $P > 0.05$). There was no variation in the relative abundance of *Anaerovibrio* among dietary treatments except for the SB0.3 diet, whose value was significantly higher than in FM and SB0.2 diets ($P < 0.05$). The relative abundance of *Vibrio* in SB diets was generally lower ($P > 0.05$) than that in HSBM diet and was similar to that of FM diet.

Expression of intestinal inflammatory factor genes

The relative mRNA levels of intestinal inflammatory factor genes are presented in Figure 6. HSBM group had higher mRNA levels for *IL-8*, *IL-1β*, *IL-12* and *TNF-α* genes vs FM group ($P < 0.05$), but the opposite was true for *IL-10* gene. The mRNA level of *TGF-β1* for HSBM diet was not different from that for FM diet ($P > 0.05$). The mRNA levels of *IL-8, IL-1β, IL-12 and TNF-α* showed a decreasing trend with increasing SB inclusion levels in HSBM diets, and the values of these parameters reduced to the similar level of FM diet, or even lower ($P > 0.05$). The mRNA level of *TGF-β1* in SB0.3 group was the highest among dietary treatments and higher than any other group ($P < 0.05$). The mRNA level of *IL-10* was not affected by dietary treatments ($P > 0.05$).

Discussion

Growth performance and proximate composition

Results of the present study showed that in HSBM diet decreased significantly WG and SGR vs FM diet, which was observed in previous studies on FM replacement with high SBM (Wang et al., 2017b; Zhu et al., 2021; Zhang et al., 2022). The growth-limiting effect is attributed to the presence of antinutritional factors (ANFs), poor palatability, and lack of some nutrients (Gatlin et al., 2007), of which the most typical

TABLE 6 Effects of sodium butyrate (SB) addition in high soybean meal (SBM) diets on intestinal permeability of groupers in a 56-d feeding period.

Parameters	Diets[1]				
	FM	HSBM	SB0.1	SB0.2	SB0.3
DAO (U/L)	19.75 ± 1.39	20.59 ± 1.05	15.27 ± 1.72	16.24 ± 2.10	15.18 ± 1.89
D-Lac (nmol/mL)	2.03 ± 0.20a	4.05 ± 0.23b	2.25 ± 0.15a	2.03 ± 0.12a	1.90 ± 0.04a
ET-1 (ng/L)	1.91 ± 0.07	2.12 ± 0.09	2.24 ± 0.17	1.93 ± 0.12	1.90 ± 0.12
ET (EU/L)	1.51 ± 0.03a	1.70 ± 0.10b	1.46 ± 0.01a	1.38 ± 0.03a	1.46 ± 0.02a

[1]FM, fish meal; HSBM, 60% FM protein replacement with SBM in FM diets without SB addition; SB0.1, SB0.2 and SB0.3, 0.1%, 0.2%, and 0.3% SB were added in the HSBM diets, respectively. Data are presented as the means of per dietary treatment (n = 3 tanks). Values in the same row with different superscripts indicate significant differences ($P < 0.05$), while that with the same letter or no letter superscripts indicate no significant difference ($P > 0.05$). DAO, Diamine oxidase; D-lac, D-lactic acid; ET, Endotoxin; ET-1, Endothelin-1.

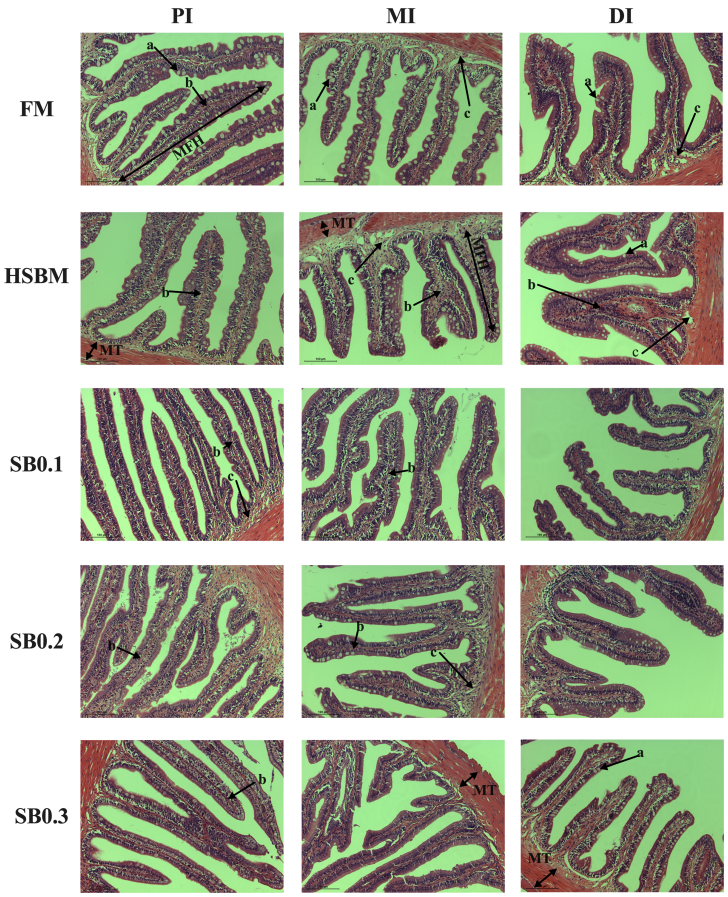

FIGURE 3
Intestinal histological examinations of sodium butyrate (SB) addition in high soybean meal (SBM) diets on the proximal intestine (PI), mid intestine (MI), and distal intestine (DI) in groupers in a 56-d feeding period (magnification 200 X, scale bar = 100 mm). FM, fish meal; HSBM, 60% FM protein replacement with SBM in FM diets without SB addition; SB0.1, SB0.2 and SB0.3, 0.1%, 0.2%, and 0.3% SB were added in the HSBM diets, respectively. MFH, mucosal fold height; MT, muscular thickness; a, goblet cell; b, lamina propria; c, submucosa.

TABLE 7 Effects of sodium butyrate (SB) addition in high soybean meal (SBM) diets on the intestinal morphology of groupers in a 56-d feeding period.

Parameters		Diets[1]				
		FM	HSBM	SB0.1	SB0.2	SB0.3
PI	MFH (μm)	577.30 ± 87.68	489.10 ± 54.31	559.30 ± 35.39	495.92 ± 50.19	572.25 ± 40.22
	MT (μm)	63.24 ± 6.74	64.56 ± 8.11	56.70 ± 8.72	65.78 ± 10.6	78.59 ± 3.02
	MFN (unit)	42.50 ± 4.25	45.83 ± 3.09	40.67 ± 2.20	45.67 ± 4.15	54.33 ± 3.94
MI	MFH (μm)	465.12 ± 50.20	356.66 ± 9.37	403.74 ± 26.77	425.36 ± 36.05	516.26 ± 81.26
	MT (μm)	53.53 ± 2.44^{ab}	44.96 ± 4.06^{a}	55.06 ± 1.54^{ab}	57.65 ± 5.30^{ab}	72.29 ± 9.48^{b}
	MFN (unit)	34.33 ± 2.20	31.67 ± 1.01	33.00 ± 3.62	41.50 ± 1.04	42.50 ± 5.41
DI	MFH (μm)	417.87 ± 63.72	337.13 ± 44.48	373.43 ± 25.76	361.66 ± 49.92	458.86 ± 91.13
	MT (μm)	87.58 ± 7.61^{c}	51.53 ± 1.48^{a}	69.02 ± 5.05^{ab}	63.72 ± 7.81^{ab}	68.32 ± 9.18^{ab}
	MFN (unit)	32.00 ± 5.20	37.00 ± 4.00	35.83 ± 0.17	34.67 ± 0.88	39.67 ± 6.69

[1]FM, fish meal; HSBM, 60% FM protein replacement with SBM in FM diets without SB addition; SB0.1, SB0.2 and SB0.3, 0.1%, 0.2%, and 0.3% SB were added in the HSBM diets, respectively. Data are presented as the means of per dietary treatment (n = 3 tanks). Values in the same row with different superscripts indicate significant differences ($P < 0.05$), while that with the same letter or no letter superscripts indicate no significant difference ($P > 0.05$). PI, proximal intestine; MI, middle intestine; DI, distal intestine; MFH, mucosal fold height; MT, muscular thickness; MFN, mucosal fold number.

TABLE 8 Richness and diversity index of bacterial community for different samples in distal intestine of groupers.

Parameters	Diets[1]				
	FM	HSBM	SB0.1	SB0.2	SB0.3
OTU	343.27 ± 38.99	415.33 ± 8.19	423.33 ± 12.14	409.33 ± 9.94	384.63 ± 13.89
Ace	578.20 ± 66.61	719.44 ± 31.36	701.05 ± 57.57	693.61 ± 9.90	619.92 ± 1.56
Chao1	533.97 ± 56.09	619.46 ± 5.32	671.71 ± 2.65	632.32 ± 21.86	631.90 ± 45.21
Simpson	0.93 ± 0.02	0.65 ± 0.22	0.91 ± 0.06	0.96 ± 0.01	0.98 ± 0.00
Shannon	5.31 ± 0.29	3.68 ± 1.44	5.54 ± 0.57	5.85 ± 0.15	6.12 ± 0.04

[1]FM, fish meal; HSBM, 60% FM protein replacement with SBM in FM diets without SB addition; SB0.1, SB0.2 and SB0.3, 0.1%, 0.2%, and 0.3% SB were added in the HSBM diets, respectively. Data are presented as the means of per dietary treatment (n = 3 tanks). OTU, operational taxonomic units; Ace, abundance-based coverage estimator.

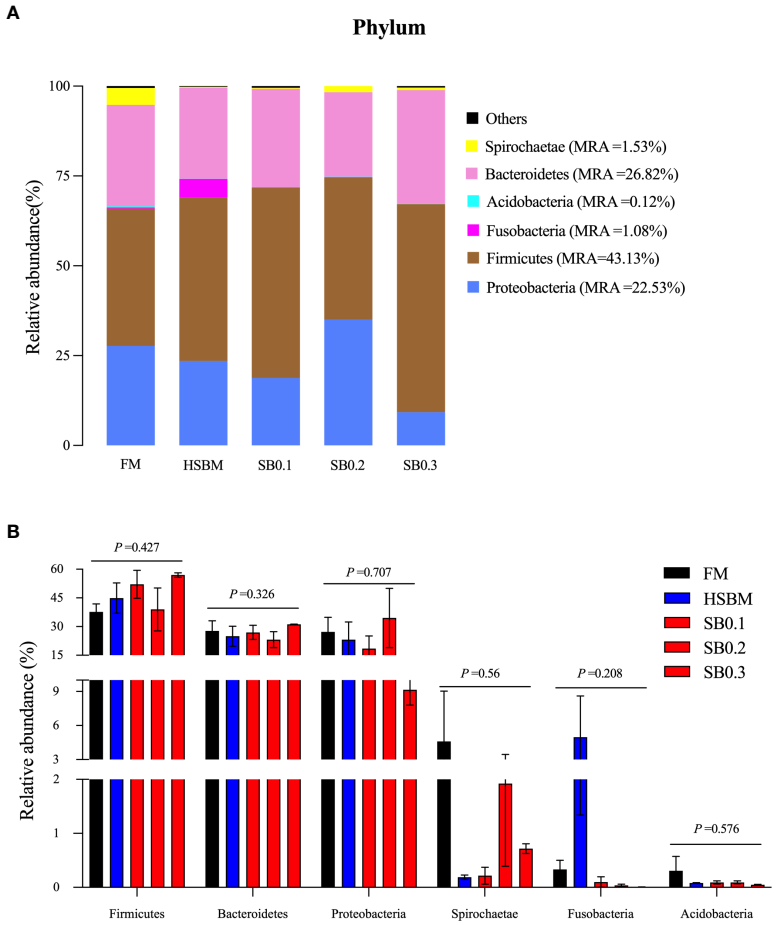

FIGURE 4
Relative abundance of the dominant bacterial phylum in different samples in the distal intestine of *E. coioides* **(A)** Intestine microbial composition at phylum level, **(B)** Differential analysis at phylum level. Bars bearing the different letters indicate significant differences ($P < 0.05$), while that with the same letters indicate no significant difference ($P > 0.05$). Data are presented as means ± SEM (n = 3 tanks). Statistical analysis was performed by one-way ANOVA, followed by S-N-K test. FM, fish meal; HSBM, 60% FM protein replacement with SBM in FM diets without SB addition; SB0.1, SB0.2 and SB0.3, 0.1%, 0.2%, and 0.3% SB were added in the HSBM diets, respectively. MRA, mean relative abundance.

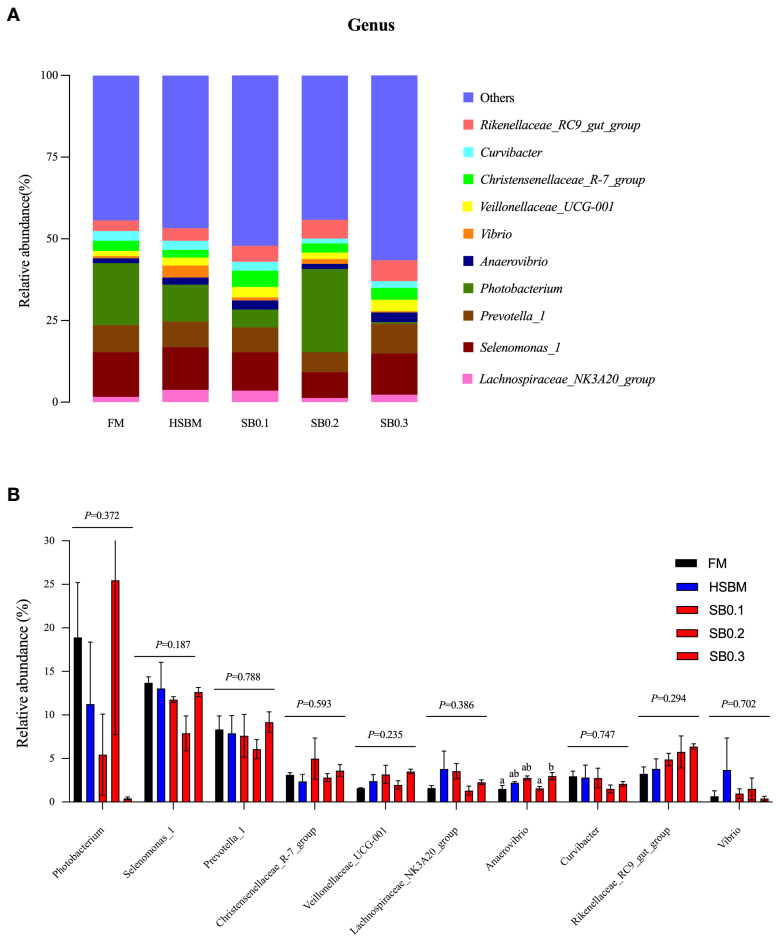

FIGURE 5
Relative abundance of the dominant bacterial genus in different samples in distal intestine of *E. coioides*. **(A)** Intestine microbial composition at genus level, **(B)** Differential analysis at genus level. Bars bearing the different letters indicate significant differences ($P < 0.05$), while that with the same letters indicate no significant difference ($P > 0.05$). Data are presented as the means of per dietary treatment (n = 3 tanks). FM, fish meal; HSBM, 60% FM protein replacement with SBM in FM diets without SB addition; SB0.1, SB0.2 and SB0.3, 0.1%, 0.2%, and 0.3% SB were added in the HSBM diets, respectively.

side effect is considered as SBMIE (Sahlmann et al., 2013). The SB supplementation in HSBM diets significantly promoted growth performance in a dose-dependent manner and had results comparable to the FM diet in this study. Our current study supported what has been reported in sea bream (Robles et al., 2013), and common carp (Liu et al., 2014a), turbot (Liu et al., 2019), yellow drum (Wu et al., 2020), and Nile tilapia (Abdel-Tawwab et al., 2021).

The whole-body composition was not affected by dietary treatments in this study. This finding was supported by the results observed in similar experiments on grass carp (Liu et al., 2017), black seabream (Ullah et al., 2020), and yellow drum (Wu et al., 2020). In contrast, several studies reported an inconsistent result that dietary supplementation of SB significantly promoted whole-body crude protein contents of Nile tilapia (Ahmed and Sadek, 2015) and European seabass (Abdel-Mohsen et al., 2018), as a result of the promotion of proliferation and differentiation of intestinal epithelial cells (Canani et al., 2012) and upregulated expression of the intestinal small peptide transporter PepT1 (Liu et al., 2014b) in the case of dietary SB administration.

Plasma components

Plasma biochemical indicators reflect the metabolic function and nutritional status of fish (Ren et al., 2021). Plasma HDL-C and LDL-C are a family of lipoproteins involved in cholesterol transport. HDL-C is mainly responsible for TG clearance and TC removal from peripheral tissues, while LDL-C transports cholesterol from the liver to peripheral tissues (Eisenberg, 1984; Jiang et al., 2015). In the current study, except for LDL-C, plasma HDL-C, TC and TG contents were not affected by

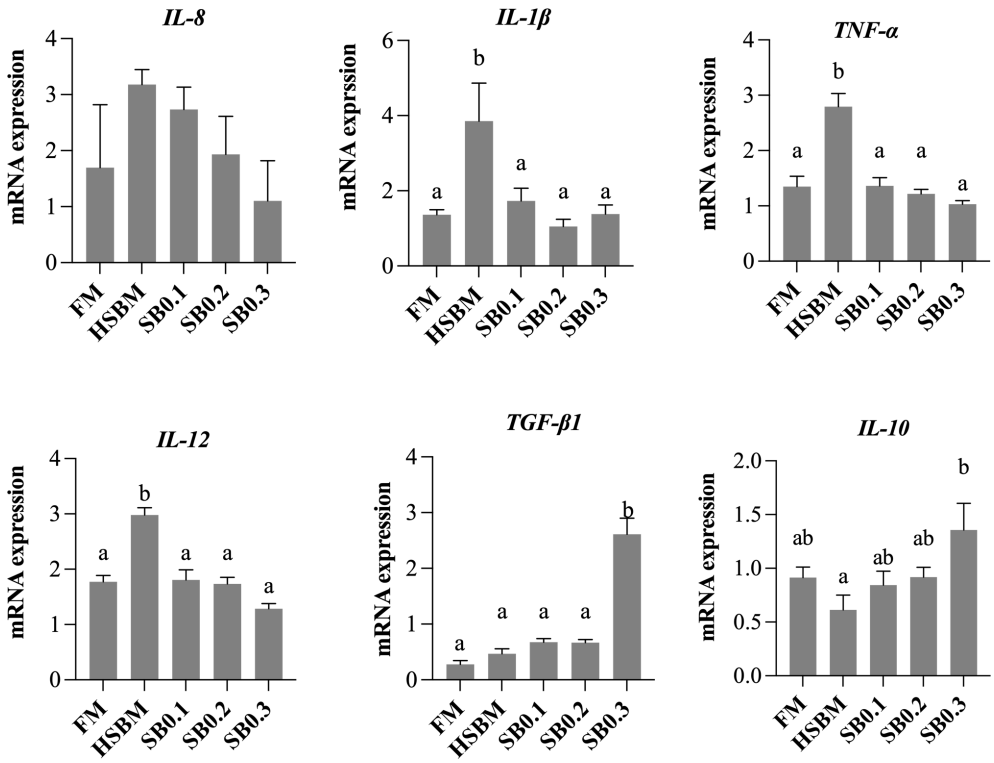

FIGURE 6
Effects of sodium butyrate (SB) addition in high soybean meal (SBM) diets on mRNA levels of intestinal inflammatory factor genes of groupers in a 56-d feeding period. Bars bearing the different letters indicate significant differences ($P < 0.05$), while that with the same letters indicate no significant difference ($P > 0.05$). Data are presented as means ± SEM (n = 3 tanks). Statistical analysis was performed by one-way ANOVA, followed by S-N-K test. FM, fish meal; HSBM, 60% FM protein replacement with SBM in FM diets without SB addition; SB0.1, SB0.2 and SB0.3, 0.1%, 0.2%, and 0.3% SB were added in the HSBM diets, respectively. *IL-8*, interleukin-8; *IL-1β*, interleukin-1β; *TNF-α*, tumor necrosis factor-alpha; *IL-12*, interleukin-12; TGF-β1, transforming growth factor-beta 1; IL-10, interleukin-10.

dietary treatments. The decreased plasma LDL-C content caused by dietary high SBM was also observed in previous studies on FM replacement with SBM in groupers (Ye et al., 2019; Zhao et al., 2021b). As observed in our current study, high SBM diets could decrease plasma TC, TG, LDL-C, and/or HDL-C contents in several previous studies with other fishes (Yamamoto et al., 2010; Dossou et al., 2018; Rahimnejad et al., 2021). However, the values of the index were not improved after dietary SB administration in HSBM diets in this study. Therefore, it seems that groupers were still in a certain degree of malnutrition caused by HSBM diets, though growth improvement by the dietary intervention of SB.

Gut digestive enzymes activity

The activity of digestive enzymes is directly associated with the digestion of nutrients (Jesus et al., 2019). In this study, the high SBM diet resulted in reduced trypsin and protease activities vs FM diet, which was consistent with other studies with other fishes such as hybrid tilapia (Lin and Luo, 2011), Japanese seabass (Zhang et al., 2018), *Totoaba macdonaldi* (Fuentes-Quesada et al., 2018). The reduction of intestinal trypsin and the protease activities were attributed to the presence of trypsin inhibitors or other ANFs in SBM, resulting in poor growth and feed utilization in fish (Dias et al., 2005; Santigosa et al., 2008; Lin and Luo, 2011; Yaghoubi et al., 2016). After dietary SB intervention, the intestinal activities of trypsin and protease were enhanced in comparison with HSBM group and showed an increasing trend with increasing dietary SB inclusion levels. As evidenced by previous studies of SB intervention on poor growth caused by high SBM feeding, on account of the ability of SB to activate digestive enzyme activity (Tian et al., 2017; Fang et al., 2021; Xie et al., 2021).

Intestinal mucosal barrier

The intestine is not only the site of nutrient digestion and absorption, but also provides an important barrier against

exogenous pathogens (Tang et al., 2022). Butyric acid is generally considered as an important substance for the energy supply and proliferation of intestinal epithelial cells (Biagi et al., 2007). Many studies showed that the increased intestinal DAO activity, D-Lac and ET concentrations reflect impaired mucosal permeability, indicating the intestinal mucosal capillary endothelial damage and intestinal flora disorder (Fukudome et al., 2014; Long et al., 2022). Feeding high-SBM diets resulted in increased intestinal D-Lac and ET concentrations vs feeding low-SBM diets or FM diets without SBM (Zhang et al., 2018; Rahimnejad et al., 2021) and in our current study. In the present study, we observed reduced intestinal DAO activity, D-Lac and ET concentrations in fish administrated with dietary SB vs those of fish fed HSBM diets, and reduced to the level of FM diets. Similar results were observed in broilers (Zou et al., 2019) and weaning piglets (Lin et al., 2020) with dietary SB administration. This indicated that dietary SB administration could reduce the intestinal mucosal permeability of farmed animals including fish.

The intestinal histomorphology also acts as a physical barrier to intestinal mucosa (Escaffre et al., 2007). It is clear that the typical signs of SBMIE can cause thickening of the lamina propria and submucosa, and disappearance of supranuclear vacuoles in the enterocytes of DI, accompanied by an infiltration of inflammatory cells (Baeverfjord and Krogdahl, 1996; Urán et al., 2009). Consistent with the previously reported results, we observed a reduction in the number of goblet cells with lymphocyte infiltration, and an increase in the thickness of lamina propria and submucosa caused by high SBM in this study. Higher intestinal MT means a larger surface area for absorbing nutrients (Caspary, 1992). The intestinal MT of fish fed high-SBM diets was lower than that of fish fed FM diets in this study and previous studies with Japanese seabass (Zhang et al., 2018), hybrid grouper (Zhou et al., 2020), and spotted seabass (Rahimnejad et al., 2021). After dietary SB intervention, the intestinal MT was increased with increasing SB inclusion levels, which supported the similar results on European Seabass (Abdel-Mohsen et al., 2018), yellow drum (Wu et al., 2020), and yellow catfish (Zhao et al., 2021a). The number of goblet cells of DI was increased with increasing SB inclusion levels in this study, facilitating the expulsion of pathogens and maintaining the integrity of mucus protective layers (Kim and Ho, 2010). As a result, the intervention effect of dietary SB administration on the SBMIE of groupers can also be achieved by improving histomorphology.

Intestinal microbiota

Dysbacteriosis of the intestinal flora increases the susceptibility to intestinal pathogens, and in severe cases, it will further develop into intestinal infection and reduce immune function (Fu et al., 2021; Vargas-Albores et al., 2021). In the current study, no alterations in intestinal microbial abundance and diversity were observed at both phylum and genus levels among dietary treatments. Previous research reveals that Proteobacteria, Firmicutes, and Bacteroidetes are the dominant phyla of intestine of marine carnivorous fish (Nayak, 2010; Wang et al., 2018), which was consistent with our current results. The change of intestinal bacterial abundance of fish fed high-SBM diets vs FM diets conflicted with what has been reported in phyla and genus abundance of turbot (Li et al., 2020), and in genus abundance of large yellow croaker (Wang et al., 2019) when fed a fermented SBM diet. This inconsistency may be that the latter used fermented SBM instead of SBM as an alternative protein source for FM. Although the changes in phylum and genus intestinal bacterial abundance in fish fed the diets administrated with SB were consistent with a study with common carp (Liu et al., 2014a), our results still deviated from many other experimental results on gilthead sea bream (Piazzon et al., 2017) and turbot (Liu et al., 2019), and in genus abundance changes of young grass carp (Tian et al., 2017) and European seabass (Abdel-Mohsen et al., 2018). The inconsistency regarding the effect of dietary SB on the intestinal flora of fish may be due to complex rearing environmental factors such as fish species, growth stage, and feed composition. Further study is required in this regard.

Expression of intestinal inflammatory factor genes

The presence of an inflammatory response is a complex pathophysiological process, which is mediated by the activation of a variety of cytokines and complement factors secreted by macrophages and leukocytes (Ruhee et al., 2019). Previous studies showed that the SBMIE mediated the expression of up-regulated pro-inflammatory cytokine genes (*IL-8*, *IL-1β*, *TNF-α* and *IL-12*) and the expression of down-regulated anti-inflammatory factor genes (*TGF-β1* and *IL-10*) in fish (Urán et al., 2008; Wang and Secombes, 2013). Similarly, we observed an up-regulation expression of intestinal *IL-8*, *IL-1β*, *TNF-α* and *IL-12* and the down-regulation of intestinal *IL-10* in SBM-fed fish, which was in accordance with previous observations (Wang et al., 2017a; Li et al., 2020; Zhao et al., 2021b). Intestinal *TGF-β1* is involved in the inactivation of the NF-κB signaling pathway, playing a role in the regulation of the inflammatory response and controlling the expression of genes encoding pro-inflammatory cytokines (Inan et al., 2000; Pedersen et al., 2022). Butyrate and SB are shown to function as an energy source for intestinal epithelial cells to exhibit anti-inflammatory as well as immune modulatory effects on mammals (Nancey et al., 2002; Weber and Kerr, 2006; Meijer et al., 2010; Ali et al., 2022). In this study, the SB intervention achieved a reduction in the expression of intestinal *IL-8*, *IL-1β*, *TNF-α* and *IL-12* and an increase in the expression of intestinal *TGF-β1*. This finding indicates dietary SB intervention could attenuate

SBMIE of fish by promoting the production of anti-inflammatory factors and preventing the production of pro-inflammatory factors (Tian et al., 2017; Liu et al., 2019; Yu et al., 2021).

Conclusions

The present study shows that supplementation of SB in high-SBM diets not only improves growth and feed utilization, but also reduces the permeability of intestinal mucosal cells and attenuates the intestinal inflammatory response in juvenile orange-spotted groupers. The optimal inclusion level was 0.2% SB in a high-SBM diet according to the regression analysis of percent weight gain against dietary SB inclusion levels. This is the first report on the intervention effect of dietary SB on grouper enteritis induced by high-SBM diet feeding. Our current study will provide a basis for dietary SB use as a functional feed additive to alleviate SBMIE of fish.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: BioProject, accession number PRJNA875282.

Ethics statement

The animal study was reviewed and approved by Jimei University, Xiamen.

Author contributions

LK: Investigation, Methodology, and Writing-original draft. YQ: Data curation, Formal analysis, and Investigation. TS: Investigation and data curation. KW: Project Administration and Supervision. JY: Conceptualization, Supervision, and Writing-review. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Effects of three feed attractants on the growth performance and meat quality of the largemouth bass (*Micropterus salmoides*)

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The trial was conducted to investigate the effects of limonene, allicin and betaine supplementation in low fish meal (FM) diet on growth performance, antioxidant capacity, meat quality and intestinal health in largemouth bass (*M. salmoides*). The biting-balls test and feeding trial were successively conducted. For the one, the results of the biting-ball test showed that with the increase of the concentration of the three attractants, the attracting effect firstly increased, then decreased, and the effect reached maximum at 0.2% concentration. ($P < 0.05$). Further, a 9-week feeding trial was conducted using five diets, including a basal diet with 30% and 40% fish meal without attractant, 30% fish meal supplemented with 0.2% limonene, 0.2% allicin or 0.2% betaine (the diets were named FM30, FM40, FM30 + L, FM30 + A, FM30 + B, respectively). The results demonstrated that adding limonene, allicin and betaine at concentration of 0.2% to the low fish meal feed could improve final body weight, weight gain rate, and specific growth rate of *M. salmoides* but only in 4 weeks ($P > 0.05$). Besides, dietary supplementation with attractants could significantly reduce the content of MDA in serum and liver, and increase the activity of GSH in liver ($P < 0.05$). Compared with FM30 group, the supplementation with limonene, allicin or betaine diet had higher pH, redness (a^*), yellowness (b^*) ($P > 0.05$), and lower refrigeration loss, cooking loss values ($P < 0.05$). Furthermore, supplementation with attractants groups had higher values for villus height, lamina propria, crypt depth, submucous layer, and serous layer ($P < 0.05$). Taken together, these results indicated that limonene, allicin and betaine had a time effect on the growth performance, and could improve antioxidant capacity, meat quality and intestinal health of *M. salmoide*.

KEYWORDS

feed attractants, largemouth bass, meat quality, physiological biochemistry, intestinal health

Introduction

Fish meal is the preferred protein source for manufacturing aquafeed due to its nutritional contents, such as protein, fatty acids, and amino acid profile, as well as its excellent digestibility and palatability (Niu et al., 2020). However, resource depletion and rising prices seriously limited the use of fish meal in aquaculture (Li X. et al., 2021). Earlier, a number of studies conducted on various fish species demonstrated that low-fishmeal (LFM) diets can lead to poor feed palatability, decrease food intake and reduce the growth performance. For instance, olive flounder (*Paralichthys olivaceus*) (Niu et al., 2019), rainbow trout (*Oncorhynchus mykiss*) (Lazzarotto et al., 2018), Nile Tilapia (*Oreochromis niloticus*) (Wattanakul et al., 2019), Japanese seabass (*Lateolabrax japonicu*) (Rahimnejad et al., 2019). While, the attractants such as L-amino acids, taurine, betaine, glycine, fish meal, earthworms, Chinese herbs, and herbal extracts (Lunger et al., 2007; Shamushaki et al., 2007; Pu et al., 2017; Rufchaei et al., 2019; Xu et al., 2020) supplementation in LFM diets were considered as one of the most effective and reliable ways to improve the feed palatability (Hirt-Chabbert et al., 2012; Dar et al., 2019). But, it was also found that such odorants supplementation in fish feeds could affect the foraging behaviors of some species (Schmachtenberg, 2015). Therefore, the formulation of fish feeds using plants with distinct smells merits investigation to discover beneficial effects on feeding attractant activity.

Limonene is an aromatic compound in essential oils, commonly used food additive obtained from oranges, grapefruits, and lemons (Cicero et al., 2015; Giarratana et al., 2016; Ravichandran et al., 2018). It has been reported that limonene has with a variety of beneficial impact including growth improvement (Kesbiç et al., 2019), nutrient absorption (Aanyu et al., 2018), antioxidant enzymatic activity (Djenane, 2015), and can also improve the specific immunity (de Souza et al., 2019; Han et al., 2019). Similarly, allicin is an important biologically active sulfur containing organic compound extracted from the bulbs of garlic (Huang et al., 2020). Currently, various studies have shown that allicin could improve the growth performance (Lee et al., 2014; Ajiboye et al., 2016), reduce oxidative stress (Abdel-Daim et al., 2015), strengthen immunity (Hamed et al., 2021) as well as improve meat quality (Kaswinarni, 2015) of fish. And it has been found that allicin could promote the daily feed intake of many fish such as *Litopenaeus vannamei* (Samadi et al., 2016), common carp (*Cyprinus carpio L*) (Mohammad, 2020), Nile Tilapia (*Oreochromis niloticus*) (Soltan and Amal Elfeky, 2016), benni fish (*Mesopotamichthys sharpeyi*) (Milad Maniat et al., 2014) and African catfish (*Clarias gariepinus*) (Gabriel et al., 2019). In addition, diet replenished with allicin improved the survival and growth of large yellow croaker (*Larimichthys crocea*) larvae probably by promoting the intestinal development, alleviating inflammation and enhancing appetite (Huang et al., 2020). Betaine, a stable and non-toxic natural substance, is mainly extracted from the processing of sugar beet (Zhao et al., 2018) and was observed to improve growth performance, health status, feed digestibility, as well as flesh quality and the immune status of fish (Hirt-Chabbert et al., 2012; Pinedo-Gil et al., 2017; Ismail et al., 2020; Sun et al., 2020). It has been proven that betaine could act as a feed attractant and appetizer through stimulating the olfactory bulb, leading to increase the feed intake, which minimize the feed wastage and water pollution (Danaceau and Lucero, 2000).

In China, largemouth bass (*Micropterus salmoides*) typically a freshwater carnivore fish traditionally been cultured due to high commercial values and over the past decade its production has expanded over 600,000 tons because of its suitability for aquaculture, marketability, and high nutritional value (China Fishery Statistics Yearbook 2020). So far, there are no comprehensive studies have been reported though using betaine, limonene and allicin as a natural attractant in largemouth bass fed low fishmeal diets. Thus, the current study aimed to evaluate the effects of three herbal extracts on feed intake, growth performance, antioxidant capability, meat quality and intestinal health for largemouth bass supplemented low fishmeal diets.

Materials and methods

The biting-balls test

A biting-ball test device was prepared as reported previously and the schematics was shown in Figure 1 (Yu et al., 2021). A total of 150 fishes were placed into 3 tanks evenly [(150 × 150 × 60 cm) (height × width × length)], supplied with dechlorinated water. The water depth was maintained at 40 cm during the experiment and the experiment was carried out twice a day at 8:30 and 17:00 for three days. Five different concentrations (0.0%, 0.1%, 0.2%, 0.6% and 1.0%) solution of limonene, allicin and betaine were prepared and stored at 4°C, then injected into a cotton ball and wrapped with gauze, respectively. The biting-ball was fixed with iron wire and submerged 5 cm under the water's surface to allow the fish to touch or bite it. In addition, a 10 cm diameter circle was drawn at the tank's bottom as an effective region based on the center of the biting ball. The mobile phone recorded the number of bites of each bait ball and entries into the effective region within 10 minutes in order to determine the proper concentration of limonene, allicin, and betaine.

Experimental design and diet preparation

Five experimental diets were formulated and the formulation, and proximate composition of the experimental

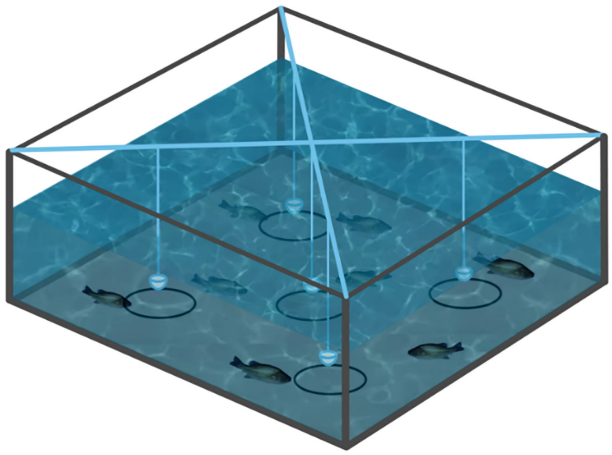

FIGURE 1
Schematic diagram of biting-balls test device.

diets are presented in Table 1. The basal diet was prepared with fish meal, soybean meal and peanut meal as the main protein source, and fish oil, and wheat flour as the main lipid and carbohydrate source respectively. According to the results in the biting-balls test, we selected the same concentration (0.2%) of allicin, betaine, limonene for further experiments. All the five test diets were designed as follows: (1) the normal fishmeal group (FM 40); (2) the low fishmeal group (FM 30); (3) the low fishmeal diet supplemented with 0.2% limonene (FM30 + L); (4) the low fishmeal supplemented with 0.2% allicin (FM30 + A); (5) the low fish meal supplemented with 0.2% betaine (FM30 + B) as presented Table 1. Crystalline amino acids (lysine, methionine) also were added to the diet to balance the dietary amino acid requirements in low fish meal diets. All dry ingredients were mixed thoroughly, and then oil and water were added. The mixture was extruded as an expanded particle diet (diameter of 1.5 mm) using a DS32-II type two-screw extruder (Guangzhou Vilavi Mechanical Equipment Co., Ltd.) after water addition, then air-dried and stored at -20°C until use.

Feeding trial and experimental conditions

M. salmoides were obtained from Guangdong Ho's Aquatic Products Co., Ltd. (Guangdong, China) and cultured in recirculating water system in Foshan University. Throughout the experiment, water temperature, pH, NH_{4+}, nitrite, nitrate and dissolved O_2 in water were maintained at 24-30°C, 6.5-7.5, < 1 mg/L, < 1 mg/L, < 20 ppm, and > 6 mg/L, respectively. After acclimation for 2 weeks, a total of 600 fish with similar body weight (mean initial weight 6.26 ± 0.01 g) were randomly assigned into 20 tanks. Each group contained four replicate tanks (30 fish/tank). All groups were fed two times per day at 8:30 and 17:00. The weight of the fish in each tank was recorded at fourth and sixth week.

Sample collection

After fasting for 24 h, fishes were anaesthetized with buffered MS-222, and the fishes in each tank were weighed to evaluate the growth performance parameters. Three whole fishes from each tank were sampled and stored at -20°C for subsequent proximate composition analysis. Blood was collected from the caudal vein of eleven fishes of each tank and blood samples were centrifuged (3000 r/min, 15 min) at 4°C, and the supernatant (serum) was stored at -80°C for further analysis. The livers and intestines of five fish per tank were collected and used for histopathological and enzyme activity analyses. Similarly, the dorsal muscles of six fish/tank were collected for flesh quality parameters analysis.

Enzyme assays

The collected livers were centrifuged for 10 min (2000 r/min, 4°C) before collecting the supernatant and then kept at -80°C. The supernatant of livers and serum were used to determine the superoxide dismutase (SOD) (determined by AST-1 method), malondialdehyde (MDA) (determined by thiobarbituric acid (TBA) test method), catalase (CAT) (determined by ammonium molybdenum acid method), glutathione (GSH) (determined by microplate method) and total protein (TP) (determined by coomassie blue staining) using the kits purchased from Nanjing Jiancheng Bioengineering Institute, China. All the analyses were performed according to the instructions of the manufacturer.

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TABLE 1 Composition and nutrient levels of experimental diets for *M. salmoides* (dry-weight basis).

Ingredients (%)	FM30	FM40	FM30 + L	FM30 + A	FM30 + B
Fish meal	30	40	30	30	30
Soybean meal	22	22	22	22	22
Peanut meal	21	10	21	21	21
Wheat flour	8.6	11	8.4	8.4	8.4
Vital wheat gluten	6	6	6	6	6
Beer yeast	3	3	3	3	3
Soybean lecithin	1	1	1	1	1
Fish oil	3	3	3	3	3
Choline chloride	0.5	0.5	0.5	0.5	0.5
Calcium dihydrogen phosphate	1.5	1.5	1.5	1.5	1.5
Compound premix[a]	3	3	3	3	3
Crystalline lysine	0.29	0	0.29	0.29	0.29
Crystalline methionine	0.11	0	0.11	0.11	0.11
Limonene[b]	0	0	0.2	0	0
Allicin[c]	0	0	0	0.2	0
Betaine[d]	0	0	0	0	0.2
Totel	100	100	100	100	100
Proximate composition					
Crude protein	44.73	47.88	46.08	45.92	46.54
Crude lipid	13.93	14.02	14.16	13.96	14.39
Crude ash	11.65	12.52	11.72	11.82	11.98

[a]Compound premix: (kg^{-1} of diet): vitamin A, 250,000 IU; riboflavin, 750 mg; pyridoxine HCL, 400 mg; cyanocobalamin, 1 mg; thiamin, 250 mg; menadione, 250 mg; folic acid,125 mg; biotin, 10 mg;α-tocopherol, 2.5 g; myo-inositol, 8000 mg; calcium pantothenate,1250 mg; nicotinic acid, 2000 mg; choline chloride, 8000 mg; vitamin D3,45,000 IU; vitamin C, 7000 mg; $ZnSO4{\cdot}7H_2O$, 0.04 g; CaCO3, 37.9 g; KCl, 5.3 g; KI, 0.04 g; NaCl, 2.6 g; $CuSO4{\cdot}5H_2O$, 0.02 g; $CoSO4{\cdot}7H_2O$, 0.02 g; $FeSO4{\cdot}7H_2O$, 0.9 g; $MnSO4{\cdot}H_2O$, 0.03 g; $MgSO4{\cdot}7H_2O$, 3.5 g; $Ca(HPO_4)_2{\cdot}2H_2O$, 9.8 g.
[b]Purchased from Xi'an Victory Biochemical Technology Co., Ltd (Victorybio).
[cd]Purchased from Shanghai Aladdin Biochemical Technology Co., Ltd.

Muscle quality measurement

The muscle quality related parameters including the pH, lightness (L^*), redness (a^*), yellowness (b^*) and water holding capacity (included thawing loss, refrigeration loss, centrifugal loss, cooking loss, drop loss and pressure loss) of dorsal muscle were measured as earlier been reported by Caimi et al. (Caimi et al., 2021). The L^*, a^* and b^* of muscle were analyses using colorimeter (SCQ-1A Tenovo International Co., Limited) while, muscle pH was measured with a direct pH meter (accurate to 0.01, pH star, Mets, Germany).

Intestinal morphology analysis

The whole intestines were fixed in 4% paraformaldehyde, dehydrated in a graded alcohol series, cleared in xylol, embedded in paraffin, sectioned at 5 μm thickness, and hematoxylin and eosin (H&E) staining were performed. Lastly, the stained sections were observed under the microscope camera NLCD 500 (Nanjing China). Image J software (W. Rasband, NIH, USA) was used to measure the villi height (VH), villi width (VW), muscle thickness (ML), lamina propria (LP), crypt depth (CD), submucous layer (SML) and serous layer (SL).

Calculation and statistical method

Growth performance of *M. salmoides* was calculated as follows:

Final body weight (FBW) = the weight of fish in the tank/the number of fish in the tank;

Weight gain rate (WGR, %) = 100 × (final body weight-initial body weight)/initial body weight;

Daily feed intake (DFI, g/fish) = (amount of feed consumed by all fish in a tank/(days of the experiment × (IBW +FBW)/2) ×100%);

Specific growth rate (SGR, %/d) = 100 × (Ln final body weight-Ln initial body weight)/days of the experiment;

Feed conversion ratio (FCR) = feed intake/body weight gain;

Survival rate (SR, %) = 100 × (final number of fish)/(initial number of fish);

Condition factor (CF, g/cm^3) = 100 × body wet weight (g)/ body length (cm)^3;

Hepatosomatic index (HSI, %) = 100 × (liver weight/whole body weight);

Viscerosomatic index (VSI, %) = 100 × (viscera weight/ whole body weight);

Intestinal index (ISI, %) = 100 × (intestine weight/whole body weight);

Intestinal length index (ILI, %) = 100 × (intestine length/ body length).

All the data were statistically analyzed by using SPSS 26.0 (SPSS Inc., Michigan Avenue, Chicago, IL, USA). One-way ANOVA followed by Duncan's multiple range tests was used and all the results were presented as means ± S.E.M (standard error of the mean). Whereas, the values of $P \leq 0.05$ were considered as level of significance.

Results

The biting-balls test

It has been observed that at 8:00, only the effect of 0.2% allicin and betaine as food attractants was significantly higher than that of the 0.0% group ($P < 0.05$). While at 17:00, all the three food attractants (limonene, allicin, and betaine) with 0.2% concentration have a substantially higher effect than that of the 0.0% group ($P < 0.05$). Furthermore, limonene, allicin, and betaine as a food attractant with different concentrations 0.0%, 0.1%, 0.2%, 0.6%, and 1.0% are given in Table 2.

Growth performance

Similarly, the growth performance, feed utilization and biometric indices were also evaluated and are presented in Table 3. At 4th week the group fed with FM40 diet presented significantly higher FBW, WGR, and SGR than the group fed with FM30 diet ($P < 0.05$), meanwhile, no difference was observed for FBW, WGR, or SGR among all the attractant groups ($P > 0.05$). Although, both the FM30 and FM40 groups at 6th week exhibited an insignificant ($P > 0.05$) differences for DFI and FCR whereas, at 6th week the DFI in FM30 + A group was significantly higher than that of the FM30 group ($P < 0.05$). Additionally, no significant difference was observed for FBW, WGR, and SGR among the experimental groups ($P > 0.05$) at 9th week.

Furthermore, the CF, ISI and ILI was not changed among the experimental groups after 9 weeks ($P > 0.05$). Besides, the FM30 + L diet group had significantly higher levels of HSI than the FM30 diet group ($P < 0.05$) after 9 weeks, but there was no significant difference in HSI between the FM40 and the supplementation with limonene, allicin or betaine groups ($P > 0.05$).

Whole-body and muscle chemical composition

All the dietary treatments had an insignificant ($P > 0.05$) effect on the contents of the crude protein, crude lipid, and moisture levels of the whole body and muscle mass. However, the contents of the crude ash in FM30, FM30 + L, FM30 + A and FM30 + B groups were lower than that in FM40 group to varying degrees, and the FM30 + B group was significantly lower than that in FM40 group. The results of the whole body and muscle composition analysis are depicted in Tables 4, 5.

Liver antioxidant capability

A significantly higher contents of the MDA were detected in FM30 diet group ($P < 0.05$) as compared to the FM30 + L and FM30 + B groups, while no change in MDA contents were observed between the FM40 group and the supplementation with limonene, allicin or betaine groups ($P > 0.05$) as shown in Figure 2A. Similarly, the GSH contents in FM30 + A and FM30 + B diet groups were significantly higher than that of the FM30 diet group ($P < 0.05$), but insignificant difference was perceived between the FM40 group and

TABLE 2 The effects of different concentrations of limonene, allicin and betaine on attracting *of M. salmoides* at 8:00 and 17:00.

Items	0	0.1%	0.2%	0.6%	1%
8:00					
Limonene	17.67 ± 1.58	19.44 ± 0.62	24.22 ± 3.12	21.67 ± 6.77	14.67 ± 4.51
Allicin	11.22 ± 2.31^{ab}	19.44 ± 2.78^{bc}	23.56 ± 4.33^{c}	6.00 ± 3.18^{a}	9.78 ± 3.36^{ab}
Betaine	12.33 ± 1.33^{a}	20.44 ± 1.25^{b}	25.67 ± 2.07^{b}	14.00 ± 2.80^{a}	8.89 ± 1.11^{a}
17:00					
Limonene	14.56 ± 0.78^{a}	19.00 ± 2.71^{ab}	25.22 ± 1.50^{b}	22.00 ± 2.34^{b}	14.67 ± 2.85^{a}
Allicin	10.89 ± 4.05^{a}	20.33 ± 0.33^{ab}	27.78 ± 2.31^{b}	11.00 ± 5.50^{a}	10.78 ± 5.31^{a}
Betaine	16.78 ± 2.35^{a}	27.22 ± 2.74^{ab}	34.78 ± 1.83^{b}	26.78 ± 4.44^{ab}	21.11 ± 4.29^{a}

Values marked with different letters are significantly different (P < 0.05) between treatments.

TABLE 3 Effects of limonene, allicin and betaine on growth performance of *M. salmoides* for 4, 6 and 9 weeks.

Items	FM30	FM40	FM30 + L	FM30 + A	FM30 + B
4 weeks					
IBW (g)	6.26 ± 0.00	6.25 ± 0.00	6.25 ± 0.01	6.26 ± 0.01	6.26 ± 0.01
FBW (g)	18.99 ± 0.51^{a}	22.58 ± 0.70 ^{b}	20.94 ± 0.20^{ab}	19.89 ± 0.54^{a}	20.96 ± 1.14^{ab}
WGR (%)	203.41 ± 8.20^{a}	261.02 ± 11.09^{b}	234.81 ± 3.15^{ab}	217.98 ± 9.15^{a}	230.34 ± 6.65^{ab}
SGR (%/d)	3.96 ± 0.09^{a}	4.55 ± 0.11^{b}	4.31 ± 0.04^{ab}	4.12 ± 0.10^{ab}	4.25 ± 0.24^{ab}
DFI (%/d)	3.62 ± 0.17^{ab}	3.38 ± 0.05^{ab}	3.60 ± 0.08^{ab}	3.76 ± 0.02^{b}	3.49 ± 0.04^{ab}
FCR	1.01 ± 0.06^{b}	0.84 ± 0.02^{a}	0.93 ± 0.01^{ab}	1.01 ± 0.03^{b}	0.91 ± 0.03^{ab}
6 weeks					
FBW (g)	29.28 ± 0.54	32.19 ± 0.40	30.23 ± 0.26	29.40 ± 1.14	30.41 ± 1.14
WGR (%)	367.92 ± 8.62	414.74 ± 3.51	383.36 ± 4.14	370.14 ± 18.50	385.72 ± 17.88
SGR (%/d)	3.67 ± 0.04	3.90 ± 0.02	3.75 ± 0.02	3.68 ± 0.09	3.76 ± 0.09
DFI (%/d)	2.79 ± 0.03^{ab}	2.74 ± 0.06^{a}	2.92 ± 0.06^{bc}	2.96 ± 0.06^{c}	2.78 ± 0.05^{ab}
FCR	0.91 ± 0.01^{ab}	0.85 ± 0.01^{a}	0.93 ± 0.02^{b}	0.95 ± 0.03^{b}	0.88 ± 0.02^{ab}
9 weeks					
FBW (g)	48.05 ± 1.53	49.05 ± 0.59	47.92 ± 1.87	46.78 ± 0.62	48.85 ± 1.24
WGR (%)	667.90 ± 24.42	684.23 ± 8.87	666.38 ± 30.73	648.14 ± 10.3	680.29 ± 19.49
SGR (%/d)	3.23 ± 0.05	3.27 ± 0.02	3.23 ± 0.06	3.19 ± 0.02	3.26 ± 0.04
DFI (%/d)	2.30 ± 0.03^{ab}	2.22 ± 0.01^{a}	2.34 ± 0.05^{ab}	2.41 ± 0.05^{b}	2.27 ± 0.03^{a}
FCR	0.91 ± 0.01^{ab}	0.86 ± 0.01^{a}	0.91 ± 0.03^{ab}	0.94 ± 0.02^{b}	0.88 ± 0.01^{ab}
SR (%)	99.17 ± 0.83	100.00 ± 0.00	100.00 ± 0.00	98.33 ± 0.96	98.33 ± 0.96
CF (g/cm^{3})	1.25 ± 0.03	1.29 ± 0.03	1.29 ± 0.02	1.28 ± 0.02	1.28 ± 0.02
HSI (%)	2.58 ± 0.19^{a}	3.14 ± 0.15^{b}	3.22 ± 0.17^{b}	2.85 ± 0.12^{ab}	2.86 ± 0.16^{ab}
VSI (%)	7.51 ± 0.18^{abc}	7.73 ± 0.21^{bc}	7.90 ± 0.21^{c}	7.3 ± 0.14^{ab}	7.16 ± 0.14^{a}
ISI (%)	0.79 ± 0.03	0.71 ± 0.02	0.73 ± 0.05	0.71 ± 0.03	0.78 ± 0.02

Values marked with different letters are significantly different (P < 0.05) between treatments.

the supplementation with limonene, allicin or betaine group (P > 0.05) (Figure 2B). In addition, among all the groups (P > 0.05) the activity of SOD was not differ (Figure 2C). Moreover, the CAT activity of the FM30 + L, FM30 + A and the FM30 + B group were significantly lower than that of the FM30 and the FM40 groups (P > 0.05) as presented in Figure 2D.

Serum antioxidant capacity

The contents of MDA in FM30 + A group was significantly lower than that of the FM30 group (P < 0.05), while was not differ between the FM40 group and supplementation with limonene, allicin or betaine group (P > 0.05) (Figure 3A). Similarly, the GSH contents in FM30 + A and FM30 + B groups were markedly lower than the FM40 group (P < 0.05) (Figure 3B). Moreover, the FM30 + A group had lower SOD activity compared to other groups (P < 0.05) (Figure 3C), however, no difference has been observed for CAT activity among all the groups (P > 0.05) (Figure 3D).

Meat quality

As shown in Table 6, the pH, L^{*}, thawing loss, centrifugal loss, drop loss and pressure loss were not differ among all the experimental groups (P > 0.05), while, the a^{*} and b^{*} of FM40 group was significantly higher than that of the FM30 group (P <

TABLE 4 Effects of limonene, allicin and betaine on whole-body composition (dry-weight basis) of *M. salmoides* for 9 weeks.

Items	FM30	FM40	FM30 + L	FM30 + A	FM30 + B
Crude protein (%)	59.52 ± 0.42	62.81 ± 1.46	62.23 ± 1.76	60.93 ± 0.71	61.20 ± 0.19
Crude lipid (%)	23.18 ± 0.37	22.82 ± 0.29	23.87 ± 0.33	23.49 ± 0.33	23.47 ± 0.47
Crude ash (%)	13.52 ± 0.19^{b}	13.42 ± 0.10^{ab}	13.29 ± 0.13^{ab}	13.24 ± 0.09^{ab}	13.00 ± 0.19^{a}
Moisture (%)	2.70 ± 0.08	2.28 ± 0.10	2.54 ± 0.14	2.58 ± 0.14	2.45 ± 0.11

Values marked with different letters are significantly different (P < 0.05) between treatments.

TABLE 5 Effects of limonene, allicin and betaine on muscle composition (dry-weight basis) of *M. salmoides* for 9 weeks.

Items	FM30	FM40	FM30 + L	FM30 + A	FM30 + B
Crude protein (%)	89.54 ± 0.11	89.54 ± 0.20	90.37 ± 0.51	90.39 ± 0.32	90.43 ± 0.48
Crude lipid (%)	11.57 ± 0.48	11.46 ± 0.55	10.80 ± 0.33	10.72 ± 0.72	11.01 ± 0.24
Crude ash (%)	5.88 ± 0.25	6.08 ± 0.10	5.87 ± 0.05	6.05 ± 0.06	6.00 ± 0.11
Moisture (%)	2.18 ± 0.21	1.97 ± 0.11	2.05 ± 0.04	2.03 ± 0.12	1.94 ± 0.20

Values marked with different letters are significantly different (P < 0.05) between treatment.

0.05), but no difference was there between the FM40 group and the supplementation with limonene, allicin or betaine groups ($P > 0.05$). The refrigeration loss of the FM30 + A and FM30 + B diets were significantly lower than that of the FM30 and FM40 groups ($P < 0.05$). The cooking loss of the FM30 + L group was also lower than that of the FM30 group ($P < 0.05$).

Intestinal morphology

The intestinal morphology showed that the villi of FM30 group were injured and broken, and the thickness of the small intestinal wall was heterogeneous as depicted in Figure 4A. The FM30 + B group had significantly higher number of villi compared with the FM40 group ($P < 0.05$) (Figure 4B; Table 7) But the width and muscular layer of fishes'villus were not differ in all groups ($P > 0.05$). The lamina propria of FM30 + A and FM30 + B groups were significantly higher compared to the FM30 group ($P < 0.05$) (Table 7). Meanwhile, FM40, FM30 + L, FM30 + A and FM30 +B groups had significantly deeper crypt depth compared with FM30 group ($P < 0.05$) (Figures 4C–E). The submucous layer value of FM30 + L and FM30 + A group were significantly higher than FM30 group ($P < 0.05$). while, the serous layer of FM30 + L group was significantly thicker than

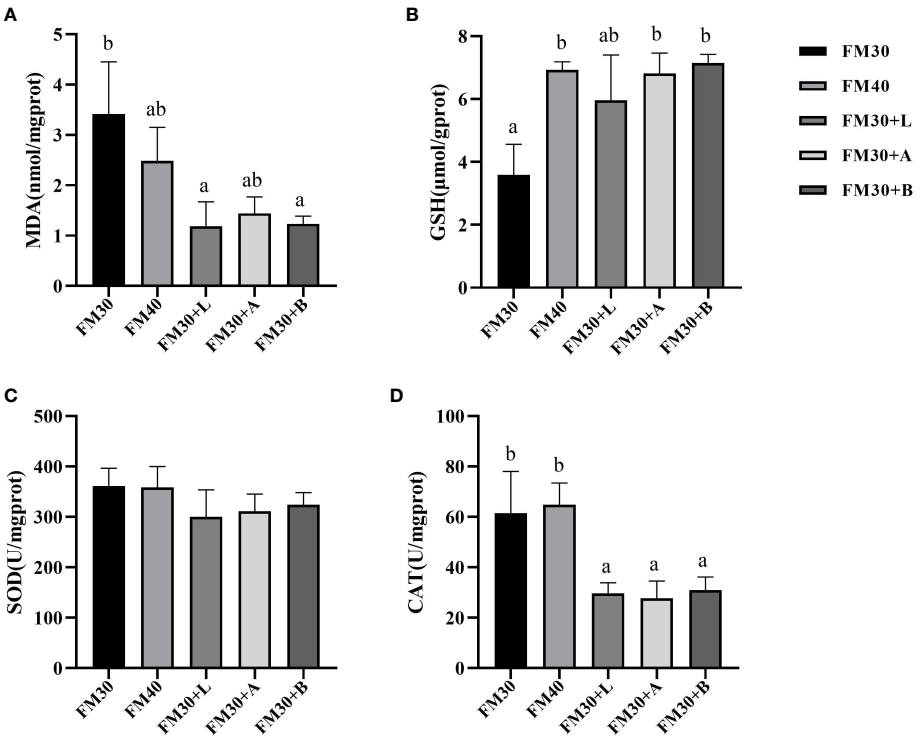

FIGURE 2
Effects of limonene, allicin and betaine on liver antioxidant capability of *M. salmoides* for 9 weeks. **(A)** Malondialdehyde (MDA); **(B)** glutathione (GSH); **(C)** superoxide dismutase (SOD); **(D)** catalase (CAT). Values (mean ± standard error of the mean, SEM) in bars that have the same letter are not significantly different (P > 0.05) between treatments.

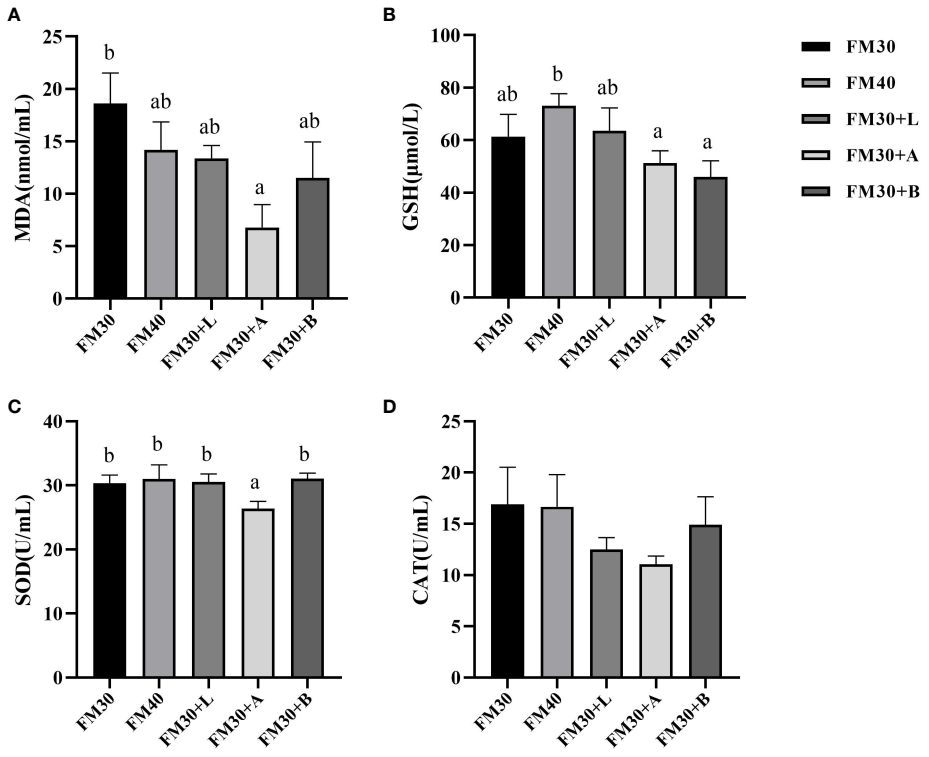

FIGURE 3
Effects of limonene, allicin and betaine on serum antioxidant capability of *M. salmoides* for 9 weeks. **(A)** Malondialdehyde (MDA); **(B)** glutathione (GSH); **(C)** superoxide dismutase (SOD); **(D)** catalase (CAT). Values (mean ± standard error of the mean, SEM) in bars that have the same letter are not significantly different (P > 0.05) between treatments.

FM30, FM40 and FM30 + B groups ($P < 0.05$). The villus height, villus width, muscular layer, lamina propria, crypt depth, and submucous layer values of fishes was not varied in limonene-, allicin- or betaine-supplementation groups ($P > 0.05$).

Discussion

In aquaculture, the commercial bait is composed of food-based basic materials and attractants, among which the attractants play a decisive role in the entire bait due to their characteristic flavour (Yu et al., 2021). Simultaneously, fish predominantly rely on their olfaction for a variety of fundamental behaviors such as foraging (Volz et al., 2020), and food attractants that could stimulate the olfactory receptors (Wang et al., 2021). Limonene is a translucent liquid with pleasant lemon-like odor (Ibáñez et al., 2020), allicin is the compound responsible for garlic's pungent odor (Borlinghaus et al., 2014), and betaine is a flavor enhancer used to reduce bitterness and imparting optimal sweetness and umami to food (Tu et al., 2020). Based on these theories, we designed this biting-balls test, it showed that these three compounds limonene, allicin and betaine do have positive impact on food consumption. And it is consistent with the results of our biting-balls test, indicating that limonene, allicin and betaine could stimulate the smell or taste receptors of *M. salmoides* and had a strong attraction effect (Reyes-Camacho et al., 2021). In addition, the attraction effects of different food attractants varied, which could be attributed to differences in the number of olfactory receptor genes responsible for detecting different odor molecules (Liu et al., 2021), resulting in different sensations or recognition capacities of olfactory and taste receptors to attractants in *M. salmoides*. In this study, when the concentrations of limonene, allicin and betaine were higher than 0.2%, the attraction effects on *M. salmoides* was gradually weakened, even lower than that of the control group. It might be because high concentrations of limonene, allicin and betaine were beyond the tolerance range of *M. salmoides*.

Interestingly, a gradual decrease in specific growth rate and daily feed intake was observed from 4th to 9th weeks, and the variations in growth performance progressively became inconspicuous among all the experimental groups. We speculate that *M. salmoides* might adapt to the taste and texture of the different diets with the extension of the feeding time, similar phenomenon was also reflected in previous researches (Tian et al., 2016; Lazado et al., 2019; Le et al., 2020; Martchenko et al., 2021). In this study, the crude

TABLE 6 Effects of limonene, allicin and betaine on meat quality of *M. salmoides* for 9 weeks.

Items	FM30	FM40	FM30 + L	FM30 + A	FM30 + B
H	6.16 ± 0.27	6.40 ± 0.03	6.42 ± 0.04	6.38 ± 0.02	6.33 ± 0.06
L^*	52.76 ± 2.07	55.37 ± 0.89	54.06 ± 1.75	52.08 ± 1.87	55.22 ± 1.34
a^*	1.98 ± 0.32^{a}	3.30 ± 0.49^{b}	2.25 ± 0.24^{ab}	3.15 ± 0.43^{ab}	2.40 ± 0.31^{ab}
b^*	4.64 ± 0.56^{a}	6.88 ± 0.61^{b}	5.33 ± 0.66^{ab}	5.97 ± 0.53^{ab}	6.36 ± 0.47^{ab}
Thawing loss (%)	2.01 ± 0.28	2.54 ± 0.37	2.17 ± 0.17	1.80 ± 0.16	2.10 ± 0.18
Refrigeration loss (%)	1.64 ± 0.13^{b}	1.75 ± 0.17^{b}	1.49 ± 0.13^{ab}	1.17 ± 0.15^{a}	1.16 ± 0.09^{a}
Centrifugal loss (%)	9.93 ± 0.80	8.83 ± 0.59	9.23 ± 0.71	9.43 ± 0.49	9.27 ± 0.69
Cooking loss (%)	22.04 ± 1.37^{b}	20.64 ± 0.36^{ab}	18.90 ± 0.5^{a}	20.19 ± 0.69^{ab}	21.00 ± 0.79^{ab}
Drop loss (%)	3.69 ± 0.33	3.01 ± 0.24	3.58 ± 0.29	3.04 ± 0.18	3.13 ± 0.21
Pressure loss (%)	5.26 ± 0.39	5.00 ± 0.26	4.61 ± 0.21	4.61 ± 0.15	5.07 ± 0.52

Values marked with different letters are significantly different ($P < 0.05$) between treatment.

protein, crude lipid and moisture of whole body and muscle did not differ among all the dietary treatments. The content of crude protein and moisture in whole fish and muscle of *M. salmoides* in the FM30 + L, FM30 + A and FM30 + B groups were closer to those of the FM40 group. Various factors have contributed to the nutritional composition of fish, such as genetic factors (Cai et al., 2021), water environment (Mohanty et al., 2019; Byrd et al., 2020), and season (Duarte et al., 2022), while the most important factor is the feed nutrition (Khalili Tilami and Sampels, 2017). At present, there have been many studies shown that supplementation of attractants, such as betaine (Yeşilayer and Kaymak, 2020), squid hydrolysate and squid meal (Novriadi et al., 2017), red seaweed eucheuma denticulatum (*Eucheuma denticulatum*) (Ragaza et al., 2015), taurine (Nguyen et al., 2020) to a low-fishmeal diet had no significant effect on fish chemical composition. However, the contents of the crude ash in FM30, FM30 + L, FM30 + A and FM30 + B groups were lower than that in FM40 group to varying degrees, and the FM30 + B group was significantly lower than that in FM40 group. Similar results were also shown in the study of Nile tilapia (*Oreochromis niloticus*) (Ahmad and Abdel-Tawwab, 2011) and juvenile tinfoil barb (*Barbonymus schwanenfeldii, Bleeker 1853*) (Nafees et al., 2022). In addition, previous study have shown that the crude ash content of fish decreases with the prolongation of starvation

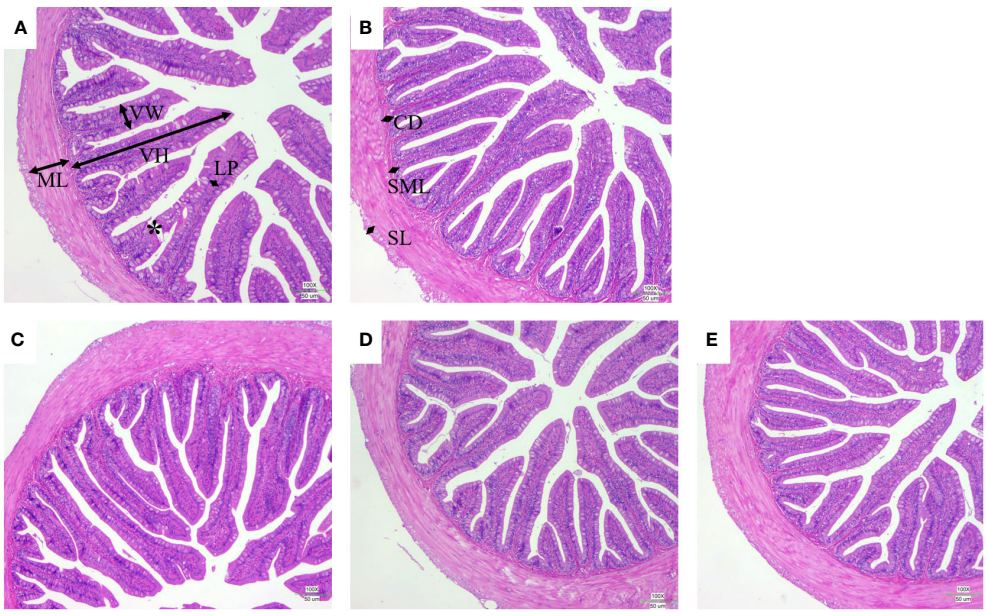

FIGURE 4
Effects of limonene, allicin and betaine on intestinal morphology of M. salmoides for 9 weeks. CD, crypt depth; LP, lamina propria; ML, muscular layer; SL, serous layer; SML, submucous layer; VH, villus height; VW, villus width. Scale bars = 50 μm. **(A)** FM30; **(B)** FM40; **(C)** FM30 + L; **(D)** FM30 + A; **(E)** FM30 + B.

TABLE 7 Histomorphometry of the intestine of *M. salmoides* fed different experimental diets.

Items	FM30	FM40	FM30 + L	FM30 + A	FM30 + B
VH (μm)	38.21 ± 3.01^{ab}	34.76 ± 2.88^{a}	43.09 ± 3.53^{ab}	38.17 ± 2.95^{ab}	44.64 ± 2.91^{b}
VW (μm)	9.15 ± 0.85	9.44 ± 1.03	12.43 ± 1.85	11.21 ± 1.24	12.59 ± 1.10
ML (μm)	14.70 ± 1.81	16.53 ± 3.10	18.26 ± 2.50	20.62 ± 1.64	20.09 ± 0.94
LP (μm)	2.99 ± 0.10^{a}	3.19 ± 0.19^{ab}	3.61 ± 0.26^{abc}	4.15 ± 0.47^{bc}	4.41 ± 0.40^{c}
CD (μm)	2.09 ± 0.24^{a}	3.43 ± 0.42^{b}	3.74 ± 0.44^{b}	3.85 ± 0.42^{b}	3.97 ± 0.58^{b}
SML (μm)	2.10 ± 0.18^{a}	2.66 ± 0.20^{ab}	3.21 ± 0.15^{b}	2.94 ± 0.24^{b}	2.61 ± 0.16^{ab}
SL (μm)	2.24 ± 0.23^{a}	2.43 ± 0.33^{a}	3.94 ± 0.47^{b}	3.16 ± 0.20^{ab}	2.86 ± 0.31^{a}

Values marked with different letters are significantly different ($P < 0.05$) between treatments.

time, (Abdel-Tawwab et al., 2006), thus, we speculate that largemouth bass fed a low fish meal diet were starved more quickly and fasted for longer, resulting in differences in the crude ash content of the whole fish. Thus, it has been perceived that growth performance, whole-body and muscle chemical composition as well as health parameters were not negatively affected by limonene, allicin, and betaine, even almost similar to FM40 group.

The SOD and CAT are typical antioxidant enzymes found in fish serum or liver that can prevent organisms from being harmed by reactive oxygen species (ROS), which can cause a variety of disorders by attacking macromolecules (Balaban et al., 2005). GSH is the most prominent non-enzymatic antioxidant in fish, as well as a free radical scavenger and detoxifier (Chen et al., 2015). MDA, a byproduct of lipid peroxidation that can interact with the free amino groups in protein causing cell damage (Xiao et al., 2022), and the contents of MDA in the fish liver can reflect the severity of the free radical attack on the liver or body cells (Całyniuk et al., 2016). Previous studies have demonstrated that limonene, allicin and betaine could improve the antioxidant capacity of fish (Abdel-Tawwab et al., 2021; Dong et al., 2021; Hamed et al., 2021; Ajiboye et al., 2016; Lopes et al., 2019; Lopes et al., 2020; Mohseni et al., 2021). In this study, the GSH and MDA contents variations in the liver as well as the MDA contents in the serum demonstrated the antioxidant effect of the aforesaid three attractants. On the contrary, the CAT activity in the liver and the GSH content as well as the SOD activity in the serum decreased in different group. This was because limonene, allicin and betaine could significantly reduce the oxidative stress damage, resulting in low concentration of catalytic substrates-free radicals, and SOD being unable to perform disproportionation reaction, whereas the CAT activity dropped as SOD activity declined. Furthermore, there were no significant differences between SOD in the liver and CAT in the serum, which might be because various antioxidant enzymes compete to respond with different degrees of antioxidative stress (Wang et al., 2019). Thus, it has been demonstrated that limonene, allicin, and betaine in a low fish meal diet may considerably minimize the degree of oxidative damage to body cells and our results are in line with the previous studies conducted on fruit fly (*Drosophila melanogaster*) (Nagpal and Abraham, 2017), Nile tilapia (*Oreochromis niloticus*) (Hamed et al., 2021), male rats (*Rattus norvegicus*) (Li et al., 2021b), broilers (Chen et al., 2021) and rats (Shan et al., 2021). These results indicated that limonene, allicin, and betaine might enhance *M. salmoides*' antioxidant capability by enhancing antioxidant enzymes and decreasing MDA levels.

Meat quality is an important feature for producers and consumers. In addition to sensory attributes (color, juiciness, and flavor) (Oliveira et al., 2017), the meat quality is reflected in its physicochemical parameters, such as WHC, pH, and nutrient composition (Maltin et al., 2007). The pH is one of the most important factors affecting many meat quality attributes, such as meat color, tenderness, the WHC and other characteristics of muscle (Cao et al., 2012). The fish meat tenderness decreased as the pH decline. Furthermore, fish color is one of the major criteria for determining freshness, with a significant influence on customer purchasing decisions (Truong et al., 2014). In this study, the pH of supplementation groups was all higher than that of the FM30 group, indicating that limonene, allicin and betaine could effectively maintain the relatively high pH in a short time, and improved the tenderness. Moreover, the a^* and b^* of fish fed the FM40 diet were significantly higher than that of fish fed the FM30 diet, whereas the a^* and b^* did not differ between the FM40 group and the supplementation groups, indicating that the low fish meal diet could affect the body color. However, limonene, allicin and betaine supplementation in low fish meal diet could restore a^* and b^* indices to the level of the normal fish meal diet, which were consistent with the earlier studies conducted on pigs (Lan et al., 2017) and chicks (Attia et al., 2009). Thus, we speculated that these are because of the antioxidant and antibacterial activity of the limonene and allicin (Bacanli et al., 2015; Costa et al., 2019; Dwivedi et al., 2019; Li D. et al., 2021). Limonene and allicin reduced the oxidation, degeneration and acidification rate of muscle, while increasing the pH. Betaine might improve the muscle pH by altering the anaerobic glycolysis and antioxidant capacity of muscle (Chen et al., 2020).

Water-holding capacity (WHC) is of great significance to the physical form, flavor and color of muscle, which can be

evaluated by thawing, refrigeration, centrifugal, cooking, drop and pressure loss. Our results showed that the refrigeration loss of fish fed FM30 + A and FM30 + B diets were significantly lower than that of fish fed FM30 and FM40 diets, and the cooking loss of fish fed FM30 + L diet was lower than that of fish fed FM30 diet, indicating that limonene, allicin and betaine could improve the flesh WHC. Earlier it has been illustrated that muscle WHC was positively correlated with the MDA content (Datta et al., 2015). Furthermore, studies have also been documented that the WHC of muscle is closely related to pH and decline in pH decreases results in lower electrostatic strength of muscle protein, which dipping the interaction between charges and the gap between myoprotein fiber and actin fiber. Water permeates from myofibrils to sarcoplasma and further into the extracellular space of muscle, resulting in increased water loss and lower WHC (Huff-Lonergan and Lonergan, 2005). In the study, the MDA content in the liver or serum in the supplementation groups were significantly lower than that of the FM30 group, and the pH was higher, indicating that limonene, allicin and betaine might improve the WHC by reducing the oxidative damage and increasing pH. Furthermore, the WHC had the highest cooking loss due to denaturation of muscle protein causing myofibril contraction, exposing more hydrophobic groups, and increasing water fluidity and eventually the water loss (Wang K. et al., 2020).

Intestinal health and integrity are directly connected to the precise fish physiological processes since it is a vital organ for nutrition absorption and utilization (Wang J. et al., 2020). In fish, intestinal villi are an important site for the secretion of digestive enzymes and nutrient absorption, therefore, the villus with regular shape and complete structure are the basic conditions to ensure fish intestinal health (Torrecillas et al., 2019; Yuan et al., 2019; Li W. et al., 2021). Crypt depth influence the process of migration, development and differentiation of tiny intestinal cells, hence influencing the process of digestion and absorption. Besides, the force of intestinal peristalsis generated from the contraction of smooth muscle, as the thickness of muscular layer increases, more will be the intestinal peristalsis which could improve the digestibility and absorption of the intestine. Previous studies have reported that allicin can improve digestion (Yan and Kim, 2013), intestinal microbiota and increase the beneficial microbiota of animals (Zhang et al., 2020; Guillamon et al., 2021). Furthermore, studies have also shown that betaine could improve intestinal barrier function (Shakeri et al., 2019) (Alhotan et al., 2021). In this study, compared with the FM30 group, lamina propria, muscular layer, serous layer, submucous layer, villus height, villus width and crypt depth of the supplementation groups were significantly higher, indicating that limonene, allicin and betaine could promote the development of intestinal villi and improve the structure of *M. salmoides* digestive tract by increasing the villi height, villi width, crypt depth and muscle thickness. However, further research is needed on whether limonene, allicin, and betaine improve intestinal structure by altering the intestinal microbiota of *M. salmoides*.

Conclusion

In conclusion, the results showed that the optimum attractant concentration of limonene, allicin and betaine was 0.2%. Adding limonene, allicin and betaine at concentration of 0.2% to the low fish meal feed could improve growth performance (increased final body weight, weight gain rate, and specific growth rate) of *M. salmoides* but only in 4 weeks. In addition, in 9th week, supplementation of limonene, allicin and betaine with concentration of 0.2% in low fishmeal feed improved antioxidant capacity in liver and serum (reduced the MDA contents.), meat quality (increased pH, a^*, and b^* values and decreased refrigeration loss, cooking loss values in the muscle) and intestinal morphology (increased villus height, lamina propria, crypt depth, submucous layer, and serous layer) of *M. salmoides*. Therefore, limonene, allicin and betaine may be recommended as promising attractants in the compound feed of *M. salmoides* and can alleviate the current shortage of fishmeal to a certain degree.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

All the experimental procedures including the animal experimentation were approved by the animal research committees of Foshan University Animal Ethics Committee (approval number: 2020056).

Author contributions

YuhuaY: conceptualization, data curation, writing - original draft. MC and XB: conducting a research and investigation process, specifically performing the experiments, or data/evidence collection. YingyY: conceptualization, supervision, methodology, writing - review & editing. WS: formulation or evolution of overarching research goals and aims. YL and YingY: writing - review & editing. HY: writing - review & editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Feeding juvenile largemouth bass (*Micropterus salmoides*) with carboxymethyl cellulose with different viscous: Impacts on nutrient digestibility, growth, and hepatic and gut morphology

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A 56-day trial investigated the impact of the dietary inclusion of cellulose with different viscosities on the growth, nutrient digestibility, serum biochemical indices, and the hepatic and gut morphology of largemouth bass juveniles. Four practical diets (42.50% protein and 13.70% lipid) were designed containing 8% microcrystalline cellulose (MC) and carboxymethyl cellulose (CMC) of 2,500, 5,000, and 6,500 mPa s dynamic viscosity [named MC, low-viscosity CMC (Lvs-CMC), medium-viscosity CMC (Mvs-CMC), and high-viscosity CMC (Hvs-CMC) groups, respectively]. Fish of a uniform size (6.0 g) were randomly assigned into 16 cages, with 40 fish per cage. The results showed that the protein and lipid deposition rates, specific growth rate, protein efficiency ratio, and the weight gain rate decreased significantly in the CMC groups compared to the MC group, whereas the feed intake and feed coefficient rate exhibited the opposite trend. Moreover, the intestinal Na^+/K^+-ATPase, alkaline phosphatase, and lipase activities significantly decreased in the Mvs-CMC and Hvs-CMC groups compared to the MC group, as well as the serum triglyceride, total cholesterol, and high-/low-density lipoprotein contents. The nutrient apparent digestibility significantly decreased in the CMC groups compared to the MC group. The viscerosomatic and intestinal length indices in the CMC groups and the villus height in the Hvs-CMC group were significantly lower than those in the MC group, whereas the number of gut goblet cells and muscular thickness in the Mvs-CMC and Hvs-CMC groups exhibited opposing results. The results also showed that dietary CMC damaged the hepatic and gut morphology and decreased the digestive enzyme activity, nutrient apparent digestibility, and

growth of largemouth bass. In summary, viscosity is the main anti-nutritional effect of dietary CMC and soluble non-starch polysaccharides.

KEYWORDS

carboxymethyl cellulose, viscous, growth performance, gut morphology, largemouth bass

Introduction

Non-starch polysaccharides (NSPs) mainly consist of hemicellulose, pectin, and cellulose, which compose the plant cell wall (Ma et al., 2017). Hence, plant feed ingredients usually contain high concentrations of NSPs (Choct, 2015; Cai et al., 2019). Recently, the increasing price of fishmeal has forced the addition of more plant-based feed ingredients in aquafeed to reduce production costs (Steinberg, 2022). In addition, some binders and fillers have also been used in feed formulations to improve the physical quality of the feed, such as wheat bran and rice bran. These strategies ultimately increased the contents of NSPs in the aquafeed (Deng et al., 2021). However, dietary NSPs cannot be directly digested by fish. They are trapped in the intestine, they inhibit nutrient digestion and absorption, and they reduce fish growth (Cai et al., 2019; Ren et al., 2020; Deng et al., 2021; Liu et al., 2022a; Liu et al., 2022b).

The physiological influences of dietary NSPs on aquatic animals have recently gained increasing attention. Commonly thought to be a class of anti-nutritional factors, these biomolecules have been shown to interfere with the absorption process, reduce the nutrient apparent digestibility, and induce metabolic disorders and metabolic organ damage in fish (Glencross et al., 2012; Gao et al., 2018; Cai et al., 2019; Deng et al., 2021). Based on the solubility of NSPs in natural buffers, they can be classified into insoluble and soluble types (INSP and SNSP, respectively), and differences in solubility lead to the varied viscosities of these two NSP types (Sinha et al., 2011). To date, many studies have found that dietary INSPs and SNSPs exhibit inconsistent physiological influence on aquatic animals, with dietary SNSPs typically exhibiting stronger anti-nutritional effects than dietary INSPs (Glencross et al., 2012; Deng et al., 2021; Jiang et al., 2022; Liu et al., 2022a). Recent studies have shown that the inclusion of 16.8% SNSPs extremely impaired gut health in rainbow trout (*Oncorhynchus mykiss*) compared to supplementation with 24.8% NSPs (Deng et al., 2021); moreover, supplementation with 30% SNSP (pectin) extremely decreased nutrient digestibility and induced intestine and liver impairments in yellow catfish (*Pelteobagrus fulvidraco*) compared to supplementation with 30% INSP (cellulose) (Cai et al., 2019). Thus, it can be speculated that the inconsistent physiological effects of dietary INSPs and SNSPs on fish may be associated with the differences in their physicochemical properties, including solubility and viscosity. However, there is limited information related to this issue in fish.

It is worth noting that dietary INSPs and SNSPs exert different effects on the physicochemical properties of the digesta. For example, dietary INSPs swelled with water have been shown to increase chyme volume, while dietary SNSPs tend to increase chyme viscosity (Sinha et al., 2011). The intestine is the main digestive organ for fish; therefore, dietary INSPs and SNSPs will inevitably affect the morphology and the development of the intestine. Although scholars have confirmed that dietary NSPs affect the intestinal development and morphology in fish (Leigh et al., 2018; Cai et al., 2019; Lin et al., 2020), the relationship between the viscosity of dietary NSPs and the digestive organ's morphology remains unclear.

Carnivorous fish have high dietary protein requirements, and fishmeal is usually added to their commercial feeds at more than 30% (Ma et al., 2020). For instance, the commercial feed of largemouth bass (*Micropterus salmoides*) contains 35%–50% fishmeal (Yang et al., 2022), while that of hybrid grouper (*Epinephelus fuscoguttatus*♀ × *E. lanceolatus*♂) is supplemented with 50% fishmeal, indicating that the commercial feed of carnivorous fish has broad potential for fishmeal substitution. Carnivorous fish are not equipped with the digestive physiology to cope with NSPs because their natural diet does not contain NSPs. Hence, dietary NSPs may have extreme impacts on carnivorous fish. However, there is limited knowledge on the physiological influences of dietary NSPs on carnivorous fish, and the correlation between the viscosity of dietary NSPs and their physiological effects is poorly understood. Therefore, it is necessary to investigate the correlation between the viscosity of dietary NSPs and their anti-nutritional effects in order to design feasible strategies for carnivorous fish to cope with the challenges of dietary NSPs. Toward this goal, the present trial investigated the influences of the physicochemical properties of dietary NSPs on the digestive enzyme activity, nutrient apparent digestibility, hepatic and gut morphology, and the growth of largemouth bass.

Materials and methods

Feed preparation

Four practical diets containing 8% microcrystalline cellulose (MC) and carboxymethyl cellulose (CMC) of 2,500, 5,000, and 6,500 mPa s [hereinafter MC, low-viscosity CMC (Lvs-CMC), medium-viscosity CMC (Mvs-CMC), and high-viscosity CMC (Hvs-CMC) groups, respectively] were designed (values in millipascal second denote the dynamic viscosity, which represents the internal friction force generated by the interaction of fluids between two 1-m^2 flat plates with a distance of 1 m when they move relative to each other at a speed of 1 m/s). The control group data have been published in a previous study (Liu et al., 2022b). All materials were first finely milled into powder, mixed thoroughly after being screened using a 0.30-mm diameter mesh, and then accurately weighed. Subsequently, the mixture was combined with the oil source following diet formulation (Table 1) and then 30% of pure water added to make a dough. Finally, using a double screw extruder (F-75; South China University of Technology, China), the dough was extruded into a moist feed (2.0 mm) and then stored at −20°C after air drying.

Fish and farming

The juvenile largemouth bass used in this trial were supplied by the Freshwater Aquaculture Base of Guangdong Ocean University. A total of 640 fish of similar size (6.00 ± 0.01 g) were randomly assigned to 16 net cages after being fasted for 24 h. The cages with dimensions of 1.2 m × 0.8 m × 1.0 m were set in a pool. For farmed water quality: temperature, average of 29.31°C; pH, average 7.02; ammonia nitrogen, <0.02 mg/L; nitrite, <0.05 mg/L; and dissolved oxygen, >6.00 mg/L. Fish were fed to satiation twice a day (0700 and 1700 hours), and fish mortality and feeding amount were accurately recorded during the feeding trial (56 days).

TABLE 1 Formulation and composition of the test diets.

Item	Group			
	MC	Lvs-CMC	Mvs-CMC	Hvs-CMC
Ingredients (%)				
Fish meal[a]	45.00	45.00	45.00	45.00
Corn gluten meal	10.00	10.00	10.00	10.00
Soy protein isolate	15.00	15.00	15.00	15.00
Fish oil	4.50	4.50	4.50	4.50
Soy oil	3.40	3.40	3.40	3.40
Soy lecithin	1.00	1.00	1.00	1.00
Starch	10.00	10.00	10.00	10.00
MC[b]	8.00	–	–	–
Lvs-CMC[b]	–	8.00	–	–
Mvs-CMC[b]	–	–	8.00	–
Hvs-CMC[b]	–	–	–	8.00
$Ca(H_2PO_4)_2$	1.00	1.00	1.00	1.00
NaCl	0.20	0.20	0.20	0.20
Choline chloride	0.30	0.30	0.30	0.30
Vitamin C	0.03	0.03	0.03	0.03
Vitamin and mineral premix[c]	1.50	1.50	1.50	1.50
Ethoxyquin	0.02	0.02	0.02	0.02
Yttrium(III) oxide	0.05	0.05	0.05	0.05
Proximate composition, dry matter (%)				
Crude protein	42.59	42.48	42.43	42.38
Crude lipid	13.75	13.81	13.70	13.72
Ash	9.70	9.66	9.73	9.70
Viscosity (mPa s)	5.14	182.15	320.48	440.65

MC, microcrystalline cellulose; Lvs-CMC, low-viscosity carboxymethyl cellulose; Mvs-CMC, medium-viscosity CMC; Hvs-CMC, high-viscosity CMC.
[a]Supplied by Zhanjiang Haibao Feed Co., Ltd. (Zhanjiang, China): fish meal, 65.81% crude protein and 7.69% crude lipid.
[b]Supplied by Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China).
[c]Supplied by Qingdao Master Biotech (Qingdao, China).

Digestibility test

The digestibility test was carried out in the feeding period using yttrium trioxide (Y_2O_3, 99.9% purity) as the indicator. Fecal collection was initiated 2 weeks after the fish had adapted to the diet. Feces at the bottom of the cages were collected daily using a 200-mesh brail net, with intact feces selected for subsequent analysis.

Sampling strategy

The fish were counted and weighed accurately after a 24-h fast after the fish had eaten their last meal, and then they were anesthetized using 100 mg/L of an MS-222 solution. From each cage, four fish were randomly chosen for the measurement of body length and weight, and then the fish were dissected on an ice plate. The visceral mass, gut, and liver were weighed accurately and the intestinal length measured. Another group of fish (n = 4 from each cage) was randomly selected for the collection of blood samples according to the method described by Liu et al. (2022b). The proximal and distal intestines of two fish from each cage were collected into separate Eppendorf (EP) tubes and stored at −80°C for subsequent analysis. Thereafter, another batch of fish (n = 3 from each cage) was randomly collected and stored at −20°C for whole-body chemical composition analysis.

Gut and hepatic morphological observation

One hindgut (1 cm) and liver sample per cage was collected into separate EP tubes and then fixed using 4% formaldehyde solution to prepare hematoxylin–eosin (HE) staining sections according to the method described by Liu et al. (2022b). HE-stained sections were observed using a Nikon Ni-U microscope imaging system (Nikon Ni-U, Tokyo, Japan) following the method described by Huang et al. (2022).

Furthermore, another hindgut tissue was collected per cage in the MC, Lvs-CMC, and Hvs-CMC groups and then fixed with 2.5% glutaraldehyde to prepare ultrathin sections according to the method described by Liu et al. (2022b). Finally, the ultrathin sections were examined using a transmission electron microscope (HT7600; Hitachi, Tokyo, Japan) according to the method of Huang et al. (2022).

Chemical analysis

Feces, whole-body, and the diet's approximate composition were measured using a laboratory method (AOAC, 2005), as follows: moisture, drying samples at 105°C until obtaining a constant weight; crude protein, using the Kjeldahl method; crude lipid, using the Soxhlet extraction method; and crude ash, burning the samples in a muffle furnace. Dietary viscosity was detected using a viscometer (LV-SSR type) with reference to the methods described in Liu et al. (2022b). The content of yttrium in the feed and fecal samples was measured using inductively coupled plasma mass spectrometry. Firstly, 100–200 mg sample was digested with a digestion solution (1 ml hydrogen peroxide and 6 ml nitric acid) in a microwave digestion apparatus (Multiwave PRO 41HVT56; Anton Paar, Graz, Austria). Thereafter, the digested solution of each sample was used to determine the yttrium content using mass spectrometry (7500cx; Agilent, Santa Clara, CA, USA).

Intestinal digestive enzyme activity analysis

Moist intestinal samples were first precisely weighed and then homogenized (IKA Works Asia, Bhd., Rawang, Malaysia) by adding 9x phosphate buffer (ice-cold, v/w) to obtain the supernatant for the analysis of enzyme activity. The activities of intestinal creatine kinase (CK), lipase, Na^+/K^+-ATPase, protease, alkaline phosphatase (AKP), and amylase and the concentration of protein were determined using commercial kits following the instructions of the manufacturer (ELISA; Shanghai Enzyme Link Biotechnology Co., Ltd., Shanghai, China).

Serum biochemical index analysis

The contents of serum low-/high-density lipoprotein cholesterol (LDL-C/HDL-C, respectively), malondialdehyde (MDA), blood urea nitrogen (BUN), triglyceride (TG), total amino acid (TAA), and total cholesterol (T-CHO) and the activities of serum superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and alanine and aspartate aminotransferase (ALT and AST, respectively) were examined using commercial kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Calculation and statistical analysis

The formulas used in the present study were as follows:

$$\text{Survival rate (SR, \%)} = 100 \times \left(\frac{\text{Final fish number}}{\text{Initial fish number}}\right)$$

$$\text{Weight gain rate (WGR, \%)} = \frac{100 \times (\text{Final body weight} - \text{Initial body weight})}{\text{Initial body weight}}$$

$$\text{Specific growth rate (SGR, \%/day)} = \frac{100 \times [\text{Ln(Final body weight)} - \text{Ln(Initial body weight)}]}{\text{Days}}$$

$$\text{Feed intake (FI, \% body}\frac{\text{weight}}{\text{day}}) = 100 \times \frac{2 \times \text{Feed consumption} \times \text{Days}}{(\text{Final body weight} + \text{Initial weight})}$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Feed intake}}{\text{Final body weight} - \text{Initial weight}}$$

$$\text{Protein efficiency ratio (PER)} = \frac{(\text{Final body weight} - \text{Initial body weight})}{\text{Protein intake}}$$

$$\text{Protein deposition rate (PDR, \%)} = 100 \times \frac{\text{Protein retention}}{\text{Protein intake}}$$

$$\text{Lipid deposition rate (LDR, \%)} = 100 \times \frac{\text{Lipid retention}}{\text{Lipid intake}}$$

$$\text{Condition factor (CF, g/cm}^3) = \frac{\text{Body weight}}{\text{Body length\^3}}$$

$$\text{Organ index (OI, \%)} = 100 \times \frac{\text{Organ weight}}{\text{Body weight}}$$

$$\text{Hepatosomatic index (HSI, \%)} = 100 \times \frac{\text{Liver weight}}{\text{Body weight}}$$

$$\text{Viscerosomatic index (VSI, \%)} = 100 \times \frac{\text{Intestinal weight}}{\text{Body weight}}$$

$$\text{Intestinal length index (ILI, \%)} = 100 \times \frac{\text{Intestinal length}}{\text{Body weight}}$$

$$\text{Apparent digestibility of dry matter (\%)} = 100 \times \left[1 - \frac{\text{Dietary Y content}}{\text{Fecal Y content}}\right]$$

$$\text{Apparent digestibility of dry nutrient (\%)} = 100 \times \left[1 - \left(\frac{\text{Dietary Y content}}{\text{Fecal Y content}}\right) \times \left(\frac{\text{Dietary Y content}}{\text{Fecal Y content}}\right)\right]$$

Experimental data were presented as the mean ± standard error of the mean (SEM). The percentage data were arcsine-transformed before analysis, and all data were subjected to one-way analysis of variance with SPSS software (version 22.0; Chicago, IL, USA). Tukey's multiple range test was performed when there was a significant difference between data ($p < 0.05$).

Results

Growth indices

The survival rate (SR) of largemouth bass was not significantly affected by the experimental diets ($p > 0.05$; Table 2). The protein efficiency ratio (PER), protein deposition rate (PDR), specific growth rate (SGR), and the weight gain rate (WGR) in the CMC groups were significantly lower than those in the MC group, whereas the feed intake (FI) and feed conversion ratio (FCR) in the CMC groups exhibited the opposite results ($p < 0.05$). Moreover, the lipid deposition rate (LDR) decreased significantly in the CMC groups compared to that in the MC group, and this parameter also decreased significantly with increasing CMC viscosity ($p < 0.05$).

Chemical composition and morphological parameters

The organ index (OI) and the whole-body crude protein and moisture contents were not significantly affected by the experimental diets ($p > 0.05$; Table 3). The condition factor (CF) in the Hvs-CMC group was significantly lower than that in the other groups. The hepatosomatic index (HSI) in the CMC groups was significantly lower than that in the MC group; moreover, this parameter significantly decreased with increased CMC viscosity ($p < 0.05$). The viscerosomatic index (VSI) and intestinal length index (ILI) in the CMC groups were significantly higher than those in the MC group, with the VSI showing an increasing trend with increased CMC viscosity ($p < 0.05$). Moreover, the whole-body crude lipid content decreased significantly in the CMC groups compared to that in the MC group, and this parameter decreased significantly in the Hvs-CMC group compared to the Lvs-CMC and Mvs-CMC groups ($p < 0.05$).

Dietary nutrient digestibility

Dietary crude lipid, crude protein, and the dry matter apparent digestibility coefficient in the CMC groups were significantly lower than those in the MC group ($p < 0.05$; Table 4). Additionally, the dietary dry matter apparent

TABLE 2 Effects of increasing dietary viscosity on the growth and feed utilization of juvenile largemouth bass.

Item	Group			
	MC	Lvs-CMC	Mvs-CMC	Hvs-CMC
Final body weight (g)	67.23 ± 1.26^{b}	60.87 ± 0.61^{a}	58.01 ± 0.94^{a}	57.79 ± 1.53^{a}
Survival rate (%)	98.75 ± 1.25	100.00 ± 0.00	98.75 ± 0.72	96.88 ± 1.88
Weight gain rate (%)	1,118.50 ± 20.53^{b}	1,012.28 ± 10.36^{a}	966.23 ± 16.00^{a}	962.55 ± 24.33^{a}
Specific growth rate (%/day)	4.31 ± 0.03^{b}	4.13 ± 0.02^{a}	4.05 ± 0.03^{a}	4.04 ± 0.04^{a}
Feed intake (% BW/day)	2.85 ± 0.05^{a}	3.11 ± 0.03^{b}	3.25 ± 0.05^{b}	3.26 ± 0.08^{b}
Feed coefficient rate	0.95 ± 0.02^{a}	1.06 ± 0.01^{b}	1.12 ± 0.02^{b}	1.13 ± 0.03^{b}
Protein efficiency ratio	2.46 ± 0.05^{b}	2.22 ± 0.03^{a}	2.11 ± 0.04^{a}	2.10 ± 0.06^{a}
Protein deposition rate (%)	38.57 ± 0.77^{b}	33.82 ± 0.40^{a}	33.11 ± 0.61^{a}	33.22 ± 0.96^{a}
Lipid deposition rate (%)	67.91 ± 1.29^{c}	52.40 ± 0.59^{b}	49.69 ± 0.88^{a}	41.60 ± 1.98^{a}

Values shown are the mean ± SEM (n = 4). Different superscript letters in the same row indicate significant difference between data ($p < 0.05$).
MC, microcrystalline cellulose; Lvs-CMC, low-viscosity carboxymethyl cellulose; Mvs-CMC, medium-viscosity CMC; Hvs-CMC, high-viscosity CMC; BW, body weight.

TABLE 3 Effects of increasing dietary viscosity on the morphological parameters and body composition of juvenile largemouth bass.

Item	Group			
	MC	Lvs-CMC	Mvs-CMC	Hvs-CMC
Morphological parameters				
Condition factor (g/cm^3)	2.20 ± 0.04^{b}	2.17 ± 0.04^{b}	2.13 ± 0.10^{b}	2.09 ± 0.03^{a}
Organ index (%)	8.04 ± 0.13	8.32 ± 0.16	8.15 ± 0.16	7.97 ± 0.18
Hepasomatic index (%)	1.86 ± 0.06^{d}	1.39 ± 0.05^{c}	1.05 ± 0.05^{b}	0.87 ± 0.04^{a}
Viserosomatic index (%)	0.69 ± 0.02^{a}	1.04 ± 0.03^{b}	1.16 ± 0.03^{c}	1.24 ± 0.02^{d}
Intestinal length index (%)	0.86 ± 0.01^{a}	0.94 ± 0.01^{b}	0.96 ± 0.02^{b}	0.95 ± 0.01^{b}
Body composition (%)				
Moisture	72.01 ± 1.06	72.59 ± 0.92	72.70 ± 1.12	73.73 ± 1.27
Crude protein	15.66 ± 0.20	15.29 ± 0.18	15.73 ± 0.17	15.79 ± 0.04
Crude lipid	8.53 ± 0.09^{c}	7.39 ± 0.08^{b}	7.33 ± 0.20^{b}	6.68 ± 0.03^{a}
Ash	4.06 ± 0.20	3.97 ± 0.12	4.10 ± 0.15	4.10 ± 0.12

Values shown are the mean ± SEM (n = 4). Different superscript letters in the same row indicate significant difference between data ($p < 0.05$).
MC, microcrystalline cellulose; Lvs-CMC, low-viscosity carboxymethyl cellulose; Mvs-CMC, medium-viscosity CMC; Hvs-CMC, high-viscosity CMC.

digestibility in the Lvs-CMC group was significantly higher than that in the Hvs-CMC group ($p < 0.05$).

Digestive and absorption enzyme activity

The activities of intestinal amylase and CK were not significantly affected by the experimental diets ($p > 0.05$; Table 5). The activities of intestinal AKP and lipase in the CMC groups were significantly lower than those in the MC group ($p < 0.05$). Additionally, the activities of intestinal Na^+/K^+-ATPase and protease in the Mvs-CMC and Hvs-CMC groups were significantly lower than those in the Lvs-CMC group ($p < 0.05$).

Serum biochemical indices

The concentrations of TAA and MDA and the activities of POD, SOD, and CAT in the serum were not significantly affected by the experimental diets ($p > 0.05$; Table 6). The concentrations of serum TG, HDL-C, LDL-C, and T-CHO in the CMC groups were significantly lower than those in the MC group ($p < 0.05$). Moreover, the serum HDL-C concentration in the Mvs-CMC and Hvs-CMC groups was significantly lower than that in the Lvs-CMC group ($p < 0.05$). Conversely, the activities of serum ALT and AST in the CMC groups were significantly higher than those in the MC group; the serum ALT activity increased significantly with increasing CMC viscosity ($p < 0.05$). The serum BUN content in the Hvs-CMC group was significantly higher than that in the other groups ($p < 0.05$).

TABLE 4 Effects of increasing dietary viscosity on the dietary apparent digestibility of juvenile largemouth bass.

Item	Group			
	MC	Lvs-CMC	Mvs-CMC	Hvs-CMC
Dry matter (%)	85.52 ± 0.32^{c}	82.64 ± 0.26^{b}	80.24 ± 0.14^{a}	80.56 ± 0.41^{a}
Crude protein (%)	91.32 ± 0.36^{b}	86.75 ± 1.12^{a}	86.32 ± 0.30^{a}	85.91 ± 0.42^{a}
Crude lipid (%)	90.88 ± 0.24^{b}	80.25 ± 0.17^{a}	80.39 ± 0.56^{a}	80.01 ± 0.60^{a}

Values shown are the mean ± SEM (n = 4). Different superscript letters in the same row indicate significant difference between data ($p < 0.05$).
MC, microcrystalline cellulose; Lvs-CMC, low-viscosity carboxymethyl cellulose; Mvs-CMC, medium-viscosity CMC; Hvs-CMC, high-viscosity CMC.

TABLE 5 Effects of increasing dietary viscosity on the intestinal digestive and absorptive enzyme activities of juvenile largemouth bass.

Item	Group			
	MC	Lvs-CMC	Mvs-CMC	Hvs-CMC
Proximal intestine				
Protease (U/g protein)	4.55 ± 0.14^{b}	4.29 ± 0.23^{b}	3.60 ± 0.23^{a}	3.51 ± 0.09^{a}
Lipase (U/g protein)	0.85 ± 0.04^{b}	0.68 ± 0.02^{a}	0.71 ± 0.03^{a}	0.69 ± 0.02^{a}
Amylase (U/g protein)	0.33 ± 0.04	0.40 ± 0.03	0.29 ± 0.05	0.26 ± 0.04
Distal intestine				
Creatine kinase (U/mg protein)	0.16 ± 0.02	0.12 ± 0.03	0.13 ± 0.03	0.16 ± 0.02
Na^{+}/K^{+}-ATPase (U/mg protein)	24.37 ± 1.44^{b}	23.28 ± 0.83^{b}	19.59 ± 0.54^{a}	18.55 ± 0.44^{a}
Alkaline phosphatase (U/g protein)	145.63 ± 5.69^{b}	124.82 ± 5.53^{a}	126.67 ± 4.74^{a}	125.08 ± 2.40^{a}

Values shown are the mean ± SEM (n = 4). Different superscript letters in the same row indicate significant difference between data ($p < 0.05$).
MC, microcrystalline cellulose; Lvs-CMC, low-viscosity carboxymethyl cellulose; Mvs-CMC, medium-viscosity CMC; Hvs-CMC, high-viscosity CMC.

Hindgut and liver morphology observation

Morphological observations of the gut and liver are presented in Figures 1–3. The measurement strategy is also indicated in the figures. The gut crypt depth and villus width were not significantly affected by the experimental diets ($p > 0.05$; Table 7). The gut microvillus height in the CMC groups was significantly lower than that in the MC group, and this parameter decreased significantly with increasing CMC viscosity ($p < 0.05$). Moreover, the gut villus height in the Hvs-CMC group was significantly lower than that in the other groups ($p < 0.05$). The gut muscular thickness and goblet cell number in the Mvs-CMC and Hvs-CMC groups were significantly lower than those in the MC and Lvs-CMC groups ($p < 0.05$).

Discussion

An increasing amount of reports confirmed that the physiological impacts of dietary NSPs on aquatic animals are associated with the type of dietary NSPs (either insoluble or soluble) (Sinha et al., 2011; Ren et al., 2020; Deng et al., 2021; Jiang et al., 2022). Several studies have shown that the anti-nutritional impacts of dietary NSPs are mainly caused by the SNSP component (Cai et al., 2019; Ren et al., 2020; Deng et al., 2021; Liu et al., 2022b). However, there is limited information on the correlation between the physicochemical characteristics of NSPs and their anti-nutritional effects. Our data demonstrated that dietary CMC exerts a greater anti-nutritional influence compared to dietary MC, suggesting that solubility and viscosity are the major anti-nutritional features of dietary NSPs. Similarly, dietary SNSPs negatively affected the growth of yellow catfish and rainbow trout compared to dietary INSPs (Cai et al., 2019; Deng et al., 2021), and dietary supplementation exceeding SNSP (guar gum) negatively affected the growth performance of mullet (*Mugil liza*) and striped catfish (*Pangasianodon hypophthalmus*) (Ramos et al., 2015; Tran-Tu et al., 2018).

Dietary SNSPs increase the viscosity of the digesta and slow down the passage of gastrointestinal emptying (Tran-Tu et al., 2019), which may, in turn, reduce the intake of fish feed. Additionally, dietary SNSPs can induce the production of glucagon-like peptides and peptide YY through bacterial fermentation, thereby enhancing satiety in fish (Lattimer and Haub, 2010). Therefore, the increase of dietary SNSP levels is usually accompanied by a decrease in the FI of fish (Sinha et al., 2011). In previous studies, an increase in dietary viscosity has been shown to decrease the FI of *M. liza* (Ramos et al., 2015), but increased the FI of rainbow trout (Deng et al., 2021). In this study, dietary CMC supplementation significantly increased the FI of

TABLE 6 Effects of increasing dietary viscosity on the serum biochemical indices of juvenile largemouth bass.

Item	Group			
	MC	Lvs-CMC	Mvs-CMC	Hvs-CMC
HDL-C (mmol/L)	5.11 ± 0.63^{c}	4.17 ± 0.13^{b}	3.00 ± 0.33^{a}	3.01 ± 0.12^{a}
LDL-C (mmol/L)	3.53 ± 0.22^{b}	2.29 ± 0.10^{a}	2.20 ± 0.13^{a}	2.41 ± 0.07^{a}
T-CHO (mmol/L)	10.75 ± 0.60^{b}	6.56 ± 0.38^{a}	6.20 ± 0.26^{a}	5.75 ± 0.25^{a}
TG (mmol/L)	10.17 ± 0.75^{b}	7.25 ± 0.20^{a}	7.74 ± 0.38^{a}	7.45 ± 0.35^{a}
TAA (mmol/L)	0.31 ± 0.03	0.31 ± 0.01	0.31 ± 0.01	0.31 ± 0.02
BUN (mmol/L)	2.05 ± 0.24^{a}	2.08 ± 0.04^{a}	2.34 ± 0.30^{ab}	2.80 ± 0.14^{b}
ALT (U/L)	3.84 ± 0.15^{a}	4.32 ± 0.42^{a}	6.12 ± 0.32^{b}	5.87 ± 0.37^{b}
AST (U/L)	15.75 ± 0.40^{a}	18.03 ± 1.63^{ab}	20.45 ± 1.33^{b}	19.89 ± 0.94^{b}
SOD (U/ml)	217.72 ± 10.52	208.04 ± 8.27	213.66 ± 6.73	209.64 ± 8.14
MDA (nmol/ml)	19.23 ± 1.20	18.92 ± 1.56	19.82 ± 1.80	18.02 ± 1.80
CAT (U/ml)	6.23 ± 0.26	6.20 ± 0.39	6.27 ± 0.11	6.67 ± 0.39
POD (U/ml)	1.31 ± 0.04	1.39 ± 0.05	1.26 ± 0.02	1.38 ± 0.09

Values shown are the means ± SEM (n = 4). Different superscript letters in the same row indicate significant difference between data ($p < 0.05$).
MC, microcrystalline cellulose; Lvs-CMC, low-viscosity carboxymethyl cellulose; Mvs-CMC, medium-viscosity CMC; Hvs-CMC, high-viscosity CMC; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; T-CHO, total cholesterol; TG, triglyceride; TAA, total amino acid; BUN, blood urea nitrogen; ALT, alanine aminotransferase; AST, aspartate aminotransferase; SOD, superoxide dismutase; MDA, malondialdehyde; CAT, catalase; POD, peroxidase.

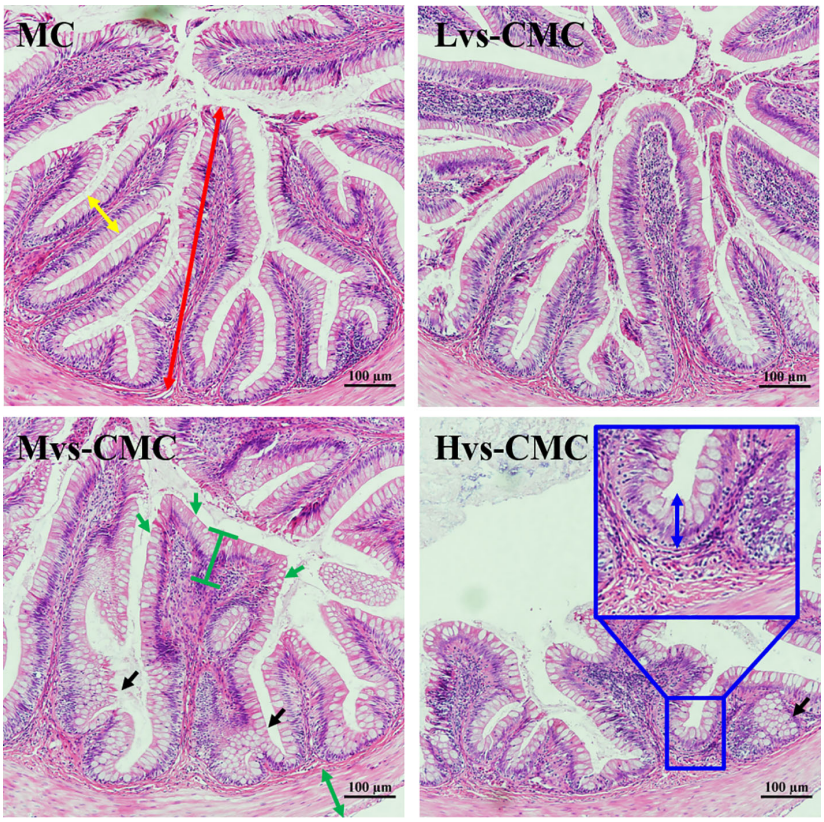

FIGURE 1
Hindgut hematoxylin–eosin (HE) staining of largemouth bass fed with the test diets (magnification, ×200). *Yellow double-sided arrow*, villus width; *black arrow*, crypt cell proliferation; *red double-sided arrow*, villus height; *green double-sided arrow*, muscular thickness; *green arrow*, goblet cell; *blue double-sided arrow*, crypt depth.

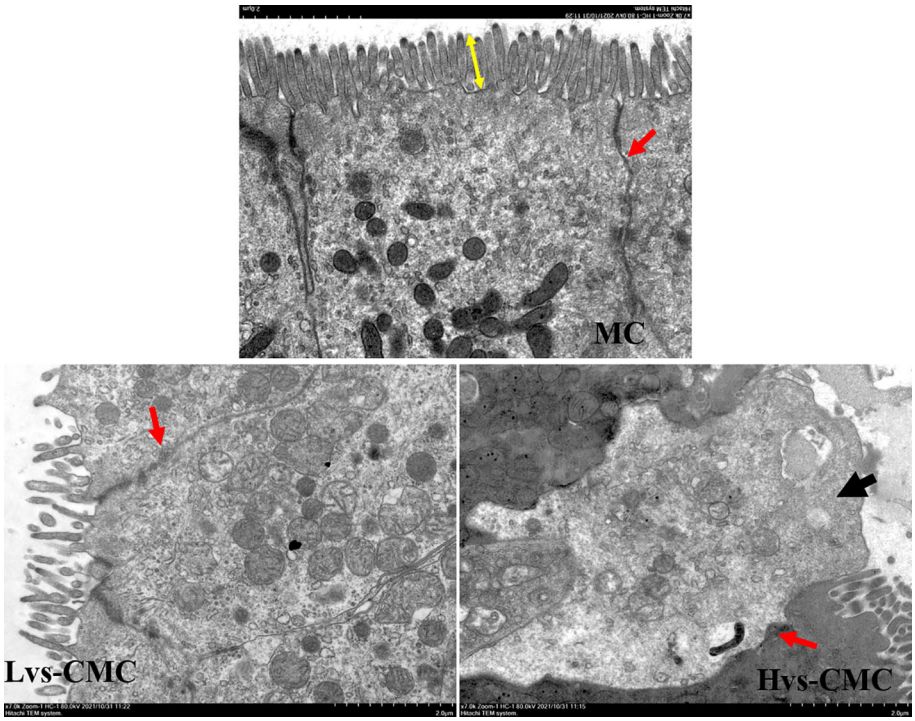

FIGURE 2
Hindgut transmission electron microscopy observation of juvenile largemouth bass fed with the test diets (magnification, ×7,000). *Black arrow*, epithelial cell death; *red arrow*, epithelial cell space; *yellow double-sided arrow*, microvillus height.

largemouth bass. The differences in these results suggest that the effect of dietary viscosity on the feeding rate of fish may be related to fish species.

Intestinal digestive enzymes play a crucial role in the absorption process of feed nutrients in fish, and their activity determines the nutrient absorption efficiency and growth rate of fish (Willora et al., 2022). On the other hand, digestive enzyme activity is inevitably influenced by the quantities and characteristics of feed ingredients (Zhang et al., 2021). Our data showed that dietary CMC extremely reduced the activities of the intestinal digestive enzymes compared to dietary MC, suggesting that soluble SNSPs are detrimental to dietary nutrient uptake. Moreover, the activities of intestinal protease and Na^+/K^+-ATPase exhibited a decreasing trend with increasing CMC viscosity, indicating that high-viscosity diets are more detrimental to nutrient digestion and absorption. A previous study indicated that dietary SNSPs bind to the enzymes in the gut, decrease the intestinal enzyme activities (Sinha et al., 2011), and may form some sticky granules that adhere to the intestinal villus, thereby interfering with the digestion and absorption processes (Nie et al., 2007). This evidence suggests that CMC diets may reduce the digestive enzyme activity through adhesion. Furthermore, AKP is also considered to be an important immune enzyme in fish, and a decrease in its activity represents a decreased immune status in fish (Yin et al., 2018; Yu et al., 2021). Combined with the poor gut morphology (epithelial cell death and increased cell intervals) (Figure 2) observed in the CMC groups, our results suggest that high dietary viscosity disrupts gut health.

Dietary NSPs have a large number of carboxyl and hydroxyl units that can interact with mineral elements (Ma et al., 2017), thereby accelerating the efflux of mineral components and reducing their absorption efficiency, especially for Na and K (Leenhouwers et al., 2006; Kraugerud et al., 2007; Leenhouwers et al., 2007). It is worth noting that the activity of Na^+/K^+-ATPase is affected by osmotic pressure (He et al., 2021) and is closely associated with the concentration of substrate ion (Gal-Garber et al., 2003). This evidence possibly explains the dramatic decrease in intestinal Na^+/K^+-ATPase activity in this study since a high-viscosity diet accelerates the excretion of Na, K, and other minerals.

Dietary proteins and lipids need to be broken down by protease and lipase before they can be absorbed and utilized by fish. Therefore, it can be hypothesized that the reduced apparent protein and lipid digestibility in the CMC groups is closely associated with the decreased activities of protease and lipase. In addition, endogenous nitrogen loss may also contribute to the decrease in apparent protein digestibility (Rgensen et al., 2003).

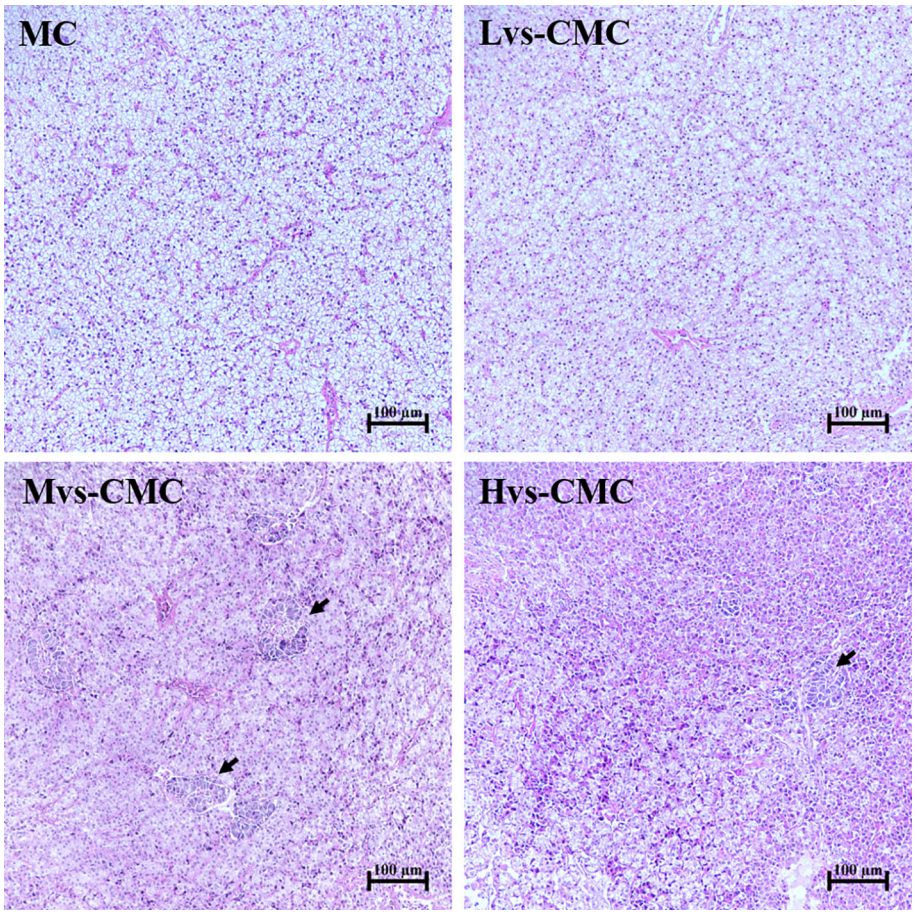

FIGURE 3
Hepatic hematoxylin–eosin (HE) staining of largemouth bass fed with the test diets (magnification, ×200). *Black arrow*, fibrosis of liver cells.

Furthermore, the poor apparent lipid digestibility ultimately reduced the serum TG concentration and whole-body crude protein content in the CMC groups. Similarly, increasing dietary viscosity significantly decreased the dietary dry matter and crude protein digestibility in catfish (*Clarias gariepinus*) and striped catfish (Leenhouwers et al., 2006; Tran-Tu et al., 2018; Tran-Tu et al., 2019).

ALT and AST are amino acid metabolizing enzymes that are mainly located in hepatocytes and enter the blood when liver damage occurs (Chaklader et al., 2021). Hence, the activities of

TABLE 7 Effects of increasing dietary viscosity on the hindgut morphology of juvenile largemouth bass.

Item	Group			
	MC	Lvs-CMC	Mvs-CMC	Hvs-CMC
Villus height (μm)	518.35 ± 24.40^{b}	549.44 ± 18.33^{b}	526.37 ± 12.25^{b}	457.92 ± 10.31^{a}
Villus width (μm)	103.45 ± 13.28	107.71 ± 8.84	107.71 ± 8.84	104.86 ± 9.49
Crypt depth (μm)	25.59 ± 3.14	24.18 ± 2.48	23.77 ± 2.28	24.89 ± 2.01
Muscular thickness (μm)	110.06 ± 6.48^{a}	102.48 ± 7.64^{a}	130.50 ± 5.57^{b}	131.80 ± 6.83^{b}
Goblet cell relative number (per 100 μm)	17.00 ± 0.50^{a}	14.00 ± 1.84^{a}	25.20 ± 3.07^{b}	24.83 ± 3.19^{b}
Microvillus height (μm)	1.31 ± 0.03^{c}	1.02 ± 0.04^{b}	–	0.82 ± 0.06^{a}

Values shown are the mean ± SEM (n = 4). Different superscript letters in the same row indicate significant difference between data ($p < 0.05$).
MC, microcrystalline cellulose; Lvs-CMC, low-viscosity carboxymethyl cellulose; Mvs-CMC, medium-viscosity CMC; Hvs-CMC, high-viscosity CMC.

serum ALT and AST can reveal the hepatic function status (Hanim et al., 2015). In this study, dietary CMC increased the activities of serum ALT and AST, with both ALT and AST activities in the Hvs-CMC group being significantly lower than those in the MC group; in contrast, a worse hepatic morphology was observed in the CMC groups (Figure 3). Our results suggest that dietary CMC disrupts hepatic health, with a highly viscous CMC exhibiting a stronger destructive impact than the low-viscosity CMC. Similarly, dietary SNSPs lead to hepatic damage in yellow catfish (Cai et al., 2019).

Fish gut morphology is inevitably affected by dietary components; hence, gut morphology is a widely used measure to evaluate the potential physiological impacts of dietary components on fish (Hartviksen et al., 2014; Huang et al., 2022). Furthermore, gut morphology is closely associated with its physiological functions (e.g., digestion and absorption) (Fang et al., 2019). For example, variations in the height of the intestinal villus and the number of folds and goblet cells may affect intestinal digestion and absorption (Sang and Fotedar, 2010). Generally, factors that can increase the digestive area promote intestinal digestion and absorption function. In this study, fish fed with Hvs-CMC diets had the shortest intestinal villus height, suggesting that a high-viscosity diet is unfavorable for gut digestive function. Muscular thickness can efficiently reveal the intestinal peristaltic capacity since it is closely related to intestinal motility (Huang et al., 2022). As aforementioned, dietary SNSPs increased the digesta viscosity and prolonged the digesta transit time in the intestine (Sinha et al., 2011). Therefore, it can be hypothesized that the increase in muscular thickness was intended to enhance intestinal motility, as an adaptive change to highly viscous diets. The mucin secreted by goblet cells is a crucial part of the intestinal mucosal immune barrier, which participates in maintaining the intestinal health of fish (Zheng et al., 2015; Martín et al., 2019; Tan and Sun, 2020). Thus, an increase in the number of goblet cells is beneficial for promoting intestinal health. Moreover, Sinha et al. (2011) suggested that increasing the digesta viscosity decreased intestinal oxygen tension, thereby promoting the proliferation of anaerobic microbiota. Moreover, anaerobic microbiota is generally detrimental to host health and even induces infections by producing toxic metabolites such as endotoxins, histamine, and trimethylamine N-oxide (Santos et al., 2014; Subramaniam and Fletcher, 2018; Cobo, 2021). This evidence suggests that the increased number of intestinal goblet cells in largemouth bass may be a response to the adverse effects of the high-viscosity diet, thereby maintaining intestinal health. Overall, combined with the decrease in digestive enzyme activity, feed utilization, and growth, as well as the unfavorable dietary nutrient digestibility and worse intestinal morphology aforementioned, our results demonstrated that the anti-nutritional effect of dietary SNSPs is mainly associated with their viscosity.

Conclusion

In conclusion, dietary CMC increases the dietary viscosity, decreases the digestive enzyme activities, and disrupts the intestinal morphology, thereby inhibiting dietary nutrient digestibility and reducing the growth of largemouth bass juveniles. Moreover, our data showed that solubility and viscosity are the dominant anti-nutritional features of NSPs and that the anti-nutritional effect of dietary SNSPs comes mainly from their viscosity.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Animal Research and Ethics Committee of Guangdong Ocean University.

Author contributions

YL: Conceptualization, formal analysis, data curation, and writing—original draft, review, and editing. JF, HZ, YZ, and HH: Methodology, project administration, and data curation. YC: Conceptualization, formal analysis, and data curation. WZ: Project administration and supervision. JD: Investigation, methodology, and resources. BT: Investigation, methodology, and resources. All authors contributed to the article and approved the submitted version.

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Conflict of interest

This study received funding from the Foundation of Tongwei Co., Ltd. The funder was not involved in the study design, sample collection and analysis, data analysis, paper writing, and paper publication decision.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Mulberry leaf extract improves non-specific immunity and antioxidant capacity of largemouth bass (*Micropterus salmoides*) fed a high-starch diet

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A 70-day experiment was conducted to investigate the effects of mulberry leaf extract (MLE) on growth, proximate compositions, antioxidant and plasma biochemical parameters, and indices of non-specific immunity of largemouth bass (*Micropterus salmoides*) fed a high-starch diet. Two hundred eighty largemouth bass (initial body weight, 68.00 ± 0.19 g) were randomly fed seven diets: basal-starch diet (BSD; 8.88% starch), high-starch diet (HSD; 11.42% starch), and HSD diets supplemented with 0.05%, 0.10%, 0.20%, 0.50%, and 1.00% MLE (MLE1, MLE2, MLE3, MLE4, and MLE5, respectively). The results indicated that HSD and MLE did not significantly affect the growth performance of largemouth bass during the experimental period compared with that of the BSD, but the supplementation with more than 0.20% (MLE3, MLE4, and MLE5) MLE significantly decreased the hepatosomatic index (HSI) values, and 1.00% (MLE5) MLE significantly decreased the viscerosomatic index (VSI) values. The whole-body moisture of fish fed an HSD decreased significantly, while the whole-body lipid content increased significantly in the HSD group compared with the BSD group. Compared with HSD, MLE supplementation significantly decreased the moisture and lipid contents in the whole body. Supplementation with more than 0.20% MLE (MLE3, MLE4, and MLE5) significantly decreased the moisture content in the muscle. Supplementation with 1.00% MLE significantly decreased the content of hepatic and muscle glycogen. The malondialdehyde (MDA) content of the HSD group was significantly increased compared to that of the BSD group, whereas more than 0.10% (MLE2, MLE3, MLE4, and MLE5) MLE significantly decreased the MDA content. Additionally, the total antioxidant capacity (T-AOC), catalase (CAT), and glutathione peroxidase (GSH-Px) activities of MLE5 were significantly higher than those of the HSD group. The complement-3 (C3) content and globulin (GLB) in the plasma of the HSD group were significantly lower than those of the BSD group. Plasma C3 levels in the MLE3, MLE4, and

MLE5 groups were significantly higher than those in the HSD group. In addition, glucose (GLU) levels in the MLE3, MLE4, and MLE5 groups were significantly lower than those in the HSD group. Supplementation with 0.50% (MLE4) MLE significantly increased the lysozyme (LYZ) content and decreased the activities of alanine transaminase (ALT) and aspartate transaminase (AST). Supplementation with 1.00% MLE significantly increased complement-4 (C4) and GLB contents and alkaline phosphatase (ALP) activity. Overall, these findings suggest that MLE could improve antioxidant capacity, immune function, and glycolipid metabolism, thereby alleviating the negative effects of a high-starch diet in *M. salmoides*.

KEYWORDS

mulberry leaf extract, growth performance, antioxidant, nonspecific immune indices, *Micropterus salmoides*

Introduction

Dietary starch is the cheapest and major source of energy in aquafeed, and it improves the efficiency of dietary protein and lipid utilization (Enes et al., 2009; Cui et al., 2010). Dietary starch is beneficial for the physical quality of feed (Sørensen et al., 2010). However, the application of starch is limited to most aquatic species, particularly carnivorous fishes (Taj et al., 2020). The largemouth bass (*Micropterus salmoides*) is a typical carnivorous fish and a major aquaculture freshwater species in China. The annual production of *M. salmoides* was estimated to have reached 619,000 tons in 2020. Numerous studies have evaluated the *M. salmoides* intake of dietary starch that exceeds 10%, which significantly impairs the antioxidant capacity and reduces the non-specific immunity, thus resulting in poor fish health and lower growth performance (Zhou et al., 2014; Ma et al., 2019; Li S et al., 2020). Long-term intake of a high-starch diet (HSD) can lead to disorders in glucolipid metabolism, non-specific immunity, and antioxidant capacity, which in turn compromises growth (Lee, 2002; Li et al., 2012). The addition of some functional additives to aquafeeds is an effective approach to prevent the detrimental effects of an HSD in fish. For example, berberine supplementation in an HSD can reduce hepatic lipid accumulation in black sea bream and improve liver health (Wang et al., 2020). Nicotinamide benefits the glucose and lipid metabolism of blunt snout bream (*Megalobrama amblycephala*) fed a high-carbohydrate diet (Shi et al., 2020). Bile acids supplementation can significantly improve growth performance and enhance liver function and immunity in *M. salmoides* fed a high-starch diet (Guo et al., 2020).

Mulberry leaves have been used in Chinese medicine for liver improvement as well as antihyperlipidemic and antihyperglycemic effects (El-Beshbishy et al., 2006; Kimura et al., 2007). Some studies have shown that mulberry contains abundant alkaloids, flavonoids, polysaccharides, phenols, and other active substances (Wang et al., 2010; Ou-yang et al., 2013; Gryn Rynko et al., 2016) such as 1-deoxynojirimycin, which is an alkaloid that can suppress the postprandial increases in plasma glucose (Wang et al., 2018) and reduce the α-glucosidase activity in humans (Kimura, 2011). Bioactive substances in mulberries have been shown to modulate glucose metabolism by correcting hyperglycemia, improving antioxidant status, and increasing insulin secretion in rats (Jeszka Skowron et al., 2014). To date, only a few studies on mulberry leaf extract (MLE) in aquaculture have been reported. Dietary MLE can improve the growth performance, feed utilization, digestive capacity, and hepatic antioxidant status of the Chinese giant salamander (Li Z et al., 2020). Mulberry leaf extract can also alleviate *Aeromonas hydrophila* infection of African catfish (*Clarias gariepinus*) (Sheikhlar et al., 2014; Sheikhlar et al., 2017). However, MLE supplementation has not been reported for *M. salmoides*. Thus, the aim of this study was to determine the effects of MLE supplementation in a high-starch diet on growth performance, antioxidant capacity, and immune parameters in *M. salmoides*.

Materials and methods

Preparation of mulberry leaf extract

First, the mulberry leaves were crushed, ground, and sieved through a 50-μm mesh. Then, mulberry leaf powder was placed in a 70% ethanol solution (v/v) on an ultrasonic frequency table at 100 kHz for 20 min at room temperature (ultrasound-assisted extraction). After the ultrasonic extraction, filtration was performed using a Boucher

funnel. The extracted liquid was freeze-dried to obtain the MLE that was used for diet preparation.

Experimental diets and experimental procedure

Mulberry leaf extract was supplemented to formulate seven experimental diets, including a control diet (BSD; 8.88% starch), HSD (11.42% starch), and HSD diets supplemented with 0.05%, 0.10%, 0.20%, 0.50%, and 1.00% MLE (MLE1, MLE2, MLE3, MLE4, and MLE5, respectively) (Table 1). All ingredients were ground and sieved through a 60-μm mesh before final mixing using a commercial food mixer and then mixed with oils. Then, 20% water was added to the mixture. The mixture was then pelleted (without injected steam) using a pellet machine (Valva-60; Guangzhou Weilawei Machinery Co., Ltd., Guangzhou, China), and the pellets were dried in a ventilated oven at 85°C for 30 min. After drying, diets were stored in sealed plastic bags at −20°C until use.

One thousand *M. salmoides* specimens were purchased from a commercial fish hatchery in Guangzhou, Guangdong Province, China. After acclimatization with commercial feed (Guangdong Junyou Feed Co., Ltd., Guangdong, China) for 14 days, the specimens were fasted for 24 h, then a total of 280 healthy *M. salmoides* (68.00 ± 0.19 g) were randomly distributed to 28 round plastic drums (237 L, 10 fish per drum) connected to a recirculating aquaculture system. Fish in each tank were randomly assigned to one of the seven experimental diets. Each diet was tested in four tanks. All fish were fed a certain proportion of their respective fish weights twice daily at 08:00 and 16:00, and feed consumption was recorded daily. The proportion of feeding was adjusted according to feeding conditions. During the 10-week feeding trial, the water temperature was kept at 24°C–28°C, dissolved oxygen was >6.8 mg/L, ammonia-nitrogen was<0.45 mg/L, and pH was between 7.5 and 8.0. The photoperiod was maintained at 12 h:12 h (light:dark).

TABLE 1 Formulation and chemical composition of experimental diets (g/kg, dry matter).

Ingredients (g/kg)	BSD	HSD	MLE1	MLE2	MLE3	MLE4	MLE5
Fish meal	440	440	440	440	440	440	440
Chicken meal	150	150	150	150	150	150	150
Blood meal	50	50	50	50	50	50	50
Soy protein concentrate	44	44	44	44	44	44	44
Cottonseed protein	40	40	40	40	40	40	40
Cassava starch	40	40	40	40	40	40	40
Flour	80	120	120	120	120	120	120
Fish oil	13	13	13	13	13	13	13
Soybean oil	12	12	12	12	12	12	12
Phosphatide oil	20	20	20	20	20	20	20
$Ca(H_2PO_4)_2$	22	22	22	22	22	22	22
Salt	2	2	2	2	2	2	2
Degelatinized bone dust	50.3	10.3	9.8	9.3	8.3	5.3	0.3
Mold inhibitor	1	1	1	1	1	1	1
Mulberry leaf extract[a]	0	0	0.5	1	2	5	10
Vitamin premix[b]	10	10	10	10	10	10	10
Mineral premix[c]	10	10	10	10	10	10	10
L-Lysine hydrochloride	4.4	4.4	4.4	4.4	4.4	4.4	4.4
DL-Met	2.7	2.7	2.7	2.7	2.7	2.7	2.7
L-Threonine	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Tau	1.6	1.6	1.6	1.6	1.6	1.6	1.6
Choline chloride	5.5	5.5	5.5	5.5	5.5	5.5	5.5
Proximate composition (g/kg)							
Protein	499.91	504.91	504.85	504.80	504.70	504.38	503.85
Lipid	108.19	107.71	107.68	107.66	107.60	107.44	107.17
Starch	88.82	114.22	114.22	114.21	114.21	114.20	114.19

a Mulberry leaf extract, obtained from Geneham Pharmaceutical Co., Ltd (Hunan, China). Actual mulberry leaf extract including 1.00% DNJ, 45.59% phenols, 1.80% flavonoids, and 5.7% polysaccharides.
b Vitamin premix (IU or mg/kg of diet): VA, 220,000 IU; VD_3, 75,000 IU; VB_1, 600; VB_6, 1,000; VB_{12}, 0.8; riboflavin, 420; inositol, 4,000; niacinamide, 2,300; folic acid, 75; biotin, 2; DL-α-tocopherol acetate, 4,800; menadione nicotinamide bisulfite, 180; l-ascorbic acid-2-phosphate, 8,500; calcium d-pantothenate, 850.
c Mineral premix (mg/kg of diet): Mg, 2,800; Cu, 350; Fe, 3,000; Zn, 8,500; Mn, 800; I, 250; Se, 15; Co, 60.

Sample collection

At the end of the feeding trial, all fish were fasted for approximately 24 h and then anesthetized with MS-222 (50 mg/L water), counted, and weighed. One fish per drum was randomly sampled and stored at −20°C for the body proximate analysis. Five fish per drum were used for the collection of blood samples using heparinized syringes after measuring the body weight and length. The fish were then dissected to obtain the viscera, liver, intestinal fat, and dorsal muscles. The viscera, liver, and intestinal fat weights of the five fish were measured to calculate the viscerosomatic (VSI), hepatosomatic (HSI), and intestinal fat (IFI) index values, respectively. In addition, the apparent condition of the liver was recorded and classified as normal or abnormal. The blood samples were centrifuged at 3,000 × *g* for 10 min (4°C) to obtain the plasma samples and stored at −80°C until used. Liver and dorsal muscles were immediately frozen in liquid nitrogen and stored at −80°C until analysis.

Chemical analysis

The proximate composition of feed ingredients, whole fish, and muscle was analyzed using the standard methods reported by the Association of Official Analytical Chemists (AOAC) (AOAC, 1995). Moisture was evaluated by oven drying at 105°C to a constant weight. Crude protein (N × 6.25) was determined according to the Kjeldahl method using the Kjeltec system (Kjeltec 8400, FOSS, Denmark). Crude lipid was quantified *via* ether extraction using a Soxhlet apparatus. Ash was detected using a muffle furnace at 550°C for 12 h. Glycogen in the liver and muscle samples was determined using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's protocol.

Activity quantification of antioxidant enzymes

Liver catalase (CAT), malondialdehyde (MDA), total antioxidant capacity (T-AOC), and glutathione peroxidase (GSH-Px) levels were measured using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's protocol.

Non-specific immune indices and plasma biochemical parameters

Plasma glucose (GLU), globulin (GLB), total triglyceride (TG), total cholesterol (TC), alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) levels were determined using an automatic biochemical analyzer (DT480, Dotopmed, Beijing, China). Plasma complement-3 (C3) and complement-4 (C4) levels were measured using commercially customized ELISA kits for fish according to the manufacturer's protocols (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China). Lysozyme (LYZ) activity was measured using a commercial kit (Shanghai Enzyme-linked Biotechnology Co., Ltd.).

Calculations and statistical analysis

$$\text{Survival rate (SR, \%)} = 100 \times \frac{N_t}{N_0}$$

$$\text{Weight gain (WG, \%)} = 100 \times \frac{W_t - W_0}{W_0}$$

$$\text{Specific growth rate (SGR, \%)} = (\text{Ln } W_t - \text{Ln } W_0)100/\text{days}$$

$$\text{Feed intake (FI, g } 100\text{g}^{-1}\text{BW day}^{-1}) = \text{Df} \times 100/((W_t - W_0)/2 \times t)$$

$$\text{Protein efficiency ratio (PER)} = \frac{W_t - W_0}{protein\ \ intake}$$

$$\text{Hepatosomatic index (HSI, \%)} = \frac{W_L}{W_t} \times 100;$$

$$\text{Viscerosomatic index (VSI, \%)} = \frac{W_V}{W_t} \times 100;$$

$$\text{Intestinal fat index (IFI, \%)} = \frac{W_{IF}}{W_t} \times 100;$$

where N_0 is the mean of the initial number of fish in each drum, and N_t is the mean final number of fish in each drum; W_t and W_0 represent the final and initial body weights (g), respectively; t is the experimental duration in days; D_f is the dry diet intake of each drum, W_L is the liver weight of the fish, W_v is the viscerosomatic weight of the fish, and W_{IF} is the mean intestinal fat content.

All data are presented as the mean ± standard error and were statistically analyzed using SPSS (version 26.0) after Tukey's test. All data were subjected to a one-way ANOVA. The level of significance was set at $p < 0.05$.

Results

Feed utilization and fish growth

The feed utilization and growth rate of the fish fed various diets supplemented with MLE are summarized in Table 2. No significant differences in WG, SGR, FI, or IFI were observed

among any of the experimental treatments ($p > 0.05$). However, weight gain was lower (7.12%) in the HSD group compared to the BSD group. For HSD, the MLE1, MLE2, and MLE5 groups showed an improved weight gain of 6.67%, 4.00%, and 7.59%, respectively. The MLE2–MLE5 groups showed significantly lower HSI values than HSD ($p< 0.05$), but no significant differences were observed between the BSD and HSD ($p > 0.05$). The HSI values of the MLE3, MLE4, and MLE5 groups were significantly lower than those of the BSD group ($p< 0.05$). The VSI values of the HSD group showed no significant differences compared to those of the BSD group ($p > 0.05$). The VSI value of the MLE5 group was significantly lower than that of the BSD group ($p< 0.05$). The percentage of the normal liver in HSD is the lowest (34.38%). The percentage of the normal liver in BSD is only 53.13%. In addition, the percentage of normal lives in MLE2, MLE3, MLE4, and MLE5 is higher than in BSD.

Proximate compositions

The proximate compositions of the fish fed various diets supplemented with MLE are summarized in Table 3. In this study, no significant differences were found in the body protein and ash among the dietary treatments ($p > 0.05$). However, the whole-body moisture of the HSD group was significantly lower than that of the BSD group ($p< 0.05$). Body moisture decreased significantly as the MLE levels increased ($p< 0.05$), and the body moisture content of the MLE5 group was significantly lower than that of the BSD group ($p< 0.05$). Body lipid levels were significantly higher in the HSD group than that in other groups ($p< 0.05$). The protein, lipid, and ash contents in the muscle did not show any statistical differences among dietary treatments ($p > 0.05$). The muscle moisture of the BSD and HSD groups was significantly higher than that of the MLE3, MLE4, and MLE5 groups ($p< 0.05$). There were no significant differences in hepatic and muscle glycogen contents between the HSD and BSD groups ($p > 0.05$). However, MLE decreased the content of hepatic and muscle glycogen, and a significant decrease was observed in the MLE5 group ($p< 0.05$).

Liver antioxidant indices

The activity quantification of antioxidant enzymes in the fish fed various diets supplemented with MLE is summarized in Figure 1. The hepatic MDA content in *M. salmoides* in the HSD group was significantly higher than that in the BSD group ($p< 0.05$), and the hepatic MDA content in *M. salmoides* in the MLE2, MLE3, MLE4, and MLE5 groups was significantly lower than that in the HSD group ($p< 0.05$); however, no significant difference was found in the MDA content among the MLE2, MLE3, MLE4, MLE5, and BSD groups ($p > 0.05$). The T-AOC, CAT, and GSH-PX of the HSD group showed no significant differences compared to those of the BSD group ($p > 0.05$). MLE improved the activities of T-AOC, CAT, and GSH-PX. In addition, fish from the MLE5 group had significantly higher T-AOC, CAT, and GSH-PX activities than those in the HSD group ($p< 0.05$).

Non-specific immune indices and plasma biochemical parameters

The non-specific immune indices and plasma biochemical parameters of the fish fed various diets supplemented with MLE are summarized in Figure 2. The lowest plasma LYZ, C3, C4, and GLB contents and the highest plasma ALT, AST, GLU, TG, and TC contents were observed in the HSD group, and C3 and GLB were significantly different from those of the BSD group ($p< 0.05$). Compared with the HSD group, adding 0.10%–1.0% (MLE2, MLE3, MLE4, and MLE5) MLE significantly decreased

TABLE 2 Effects of mulberry leaf extract on growth performance and feed utilization of *Micropterus salmoides* fed high-starch diet diets.

Items	BSD	HSD	MLE1	MLE2	MLE3	MLE4	MLE5
IBW (g)	68.02 ± 0.12	67.82 ± 0.06	68.02 ± 0.08	68.05 ± 0.06	68.05 ± 0.13	67.85 ± 0.08	67.92 ± 0.20
FBW (g)	200.21 ± 2.70	190.91 ± 8.04	199.7 ± 3.48	196.5 ± 6.73	189.5 ± 7.27	189.29 ± 5.36	200.58 ± 3.04
SR (%)	100	100	100	100	100	100	100
WG (%)	194.35 ± 3.92	181.44 ± 11.75	193.55 ± 4.76	188.71 ± 9.72	178.4 ± 10.42	178.92 ± 7.70	195.22 ± 4.74
SGR (%/day)	0.67 ± 0.00	0.64 ± 0.03	0.68 ± 0.10	0.66 ± 0.22	0.63 ± 0.24	0.64 ± 0.17	0.67 ± 0.10
FI (g 100 g^{-1} BW day^{-1})	0.76 ± 0.01	0.79 ± 0.03	0.75 ± 0.02	0.78 ± 0.03	0.81 ± 0.03	0.82 ± 0.03	0.77 ± 0.02
IFI (%)	1.38 ± 0.06	1.31 ± 0.08	1.52 ± 0.07	1.53 ± 0.08	1.36 ± 0.07	1.60 ± 0.10	1.30 ± 0.09
HSI (%)	1.90 ± 0.10^{ab}	2.12 ± 0.21^{a}	1.95 ± 0.16^{ab}	1.73 ± 0.07^{bc}	1.52 ± 0.08^{cd}	1.55 ± 0.08^{cd}	1.26 ± 0.10^{d}
VSI (%)	5.60 ± 0.11^{a}	5.65 ± 0.27^{a}	5.70 ± 0.21^{a}	5.57 ± 0.15^{ab}	5.20 ± 0.13^{ab}	5.59 ± 0.16^{a}	4.97 ± 0.15^{b}
The percentage of normal liver (%)	53.13%	34.38%	50.00%	68.75%	73.33%	78.13%	96.88%

Values (means ± SEM, n = 4) within a row with different letters are significantly different from the other dietary groups ($p< 0.05$).
IBW, initial body weight; FBW, final body weight; SR, survival rate; WG, weight gain; SGR, specific growth rate; FI, feed intake; IFI, intestinal fat index; HSI, hepatosomatic index; VSI, viscerosomatic index.

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TABLE 3 Effects of mulberry leaf extract on experimental diets and proximate compositions of *Micropterus salmoides* fed high-starch diet diets.

Items	BSD	HSD	MLE1	MLE2	MLE3	MLE4	MLE5
Feed (g/100 g)							
Moisture	14.18	15.22	14.17	14.51	15.82	14.38	14.62
Protein	47.67	46.88	47.65	47.78	47.42	47.99	47.91
Lipid	10.32	10.15	10.27	10.23	10.06	10.22	10.17
Starch	8.47	10.89	11.01	10.95	11.05	11.15	10.98
Whole body (g/100 g)							
Moisture	74.3 ± 0.28^{a}	73.51 ± 0.45^{bc}	74.50 ± 0.19^{a}	74.26 ± 0.13^{ab}	73.72 ± 0.18^{abc}	73.70 ± 0.44^{abc}	73.13 ± 0.27^{c}
Protein	16.19 ± 0.14	16.36 ± 0.32	16.55 ± 0.16	16.32 ± 0.17	16.55 ± 0.18	16.23 ± 0.45	16.57 ± 0.25
Lipid	4.77 ± 0.24^{b}	6.94 ± 0.74^{a}	5.03 ± 0.15^{b}	5.46 ± 0.32^{b}	5.83 ± 0.32^{b}	5.08 ± 0.27^{b}	4.93 ± 0.22^{b}
Ash	4.19 ± 0.07	4.35 ± 0.07	4.23 ± 0.08	4.27 ± 0.07	4.14 ± 0.21	4.16 ± 0.08	4.44 ± 0.08
Muscle (g/100 g)							
Moisture	77.55 ± 0.20^{a}	77.49 ± 0.20^{a}	77.15 ± 0.21^{abd}	77.20 ± 0.17^{ac}	76.66 ± 0.17^{bd}	76.48 ± 0.11^{e}	76.60 ± 0.25^{d}
Protein	20.50 ± 0.32	20.34 ± 0.24	20.52 ± 0.29	20.51 ± 0.27	20.75 ± 0.17	21.23 ± 0.20	21.17 ± 0.13
Lipid	1.07 ± 0.17	1.21 ± 0.17	1.17 ± 0.07	1.08 ± 0.12	1.43 ± 0.15	1.37 ± 0.16	1.34 ± 0.14
Ash	1.31 ± 0.02	1.30 ± 0.01	1.35 ± 0.03	1.35 ± 0.02	1.29 ± 0.01	1.34 ± 0.03	1.30 ± 0.02
Glycogen content (mg/g)							
Hepatic glycogen	131.2 ± 8.96^{a}	130.90 ± 6.96^{a}	123.01 ± 10.48^{ab}	117.93 ± 7.19^{ab}	112.17 ± 6.77^{ab}	129.82 ± 5.41^{a}	104.8 ± 7.85^{b}
Muscle glycogen	0.99 ± 0.14^{ab}	1.15 ± 0.11^{a}	0.99 ± 0.18^{ab}	0.92 ± 0.33^{ab}	0.77 ± 0.08^{ab}	0.87 ± 0.19^{ab}	0.67 ± 0.15^{b}

Values (means ± SEM, n = 4) within a row with different letters are significantly different from the other dietary groups ($p < 0.05$).

the activity of ALT ($p < 0.05$). Adding more than 0.20% (MLE3, MLE4, and MLE5) MLE significantly decreased the GLU content and increased the C3 content ($p < 0.05$). Adding more than 0.50% (MLE4 and MLE5) MLE significantly decreased AST activity and TC content and significantly increased LYZ content ($p < 0.05$). The addition of 1.00% (MLE5) MLE significantly increased the C4 and GLB contents and ALP activity ($p < 0.05$).

Discussion

Carbohydrates are an important energy source for vertebrates, and starch is one of the most common carbohydrate sources in fish feed. Scientific evidence has recently indicated that the source and level of starch play a decisive role in fish growth (Xia et al., 2018; Li et al., 2019; Zhao et al., 2020). *M. salmoides* is a carnivorous fish, and the level of starch in its feed requires serious consideration. Recent studies have shown that the growth performance of *M. salmoides* is impaired when its dietary starch content is >10% (Lin et al., 2018; Ma et al., 2019; Zhang et al., 2020a; b). This study showed that *M. salmoides* fed with 8.88% and 11.42% dietary starch had no significant influence, indicating that this range of starch (8.88%–11.42%) content can be tolerated by *M. salmoides* in the short-term without a visible reduction in production performance.

The viscera are crucial for fish metabolism. The VSI and HIS values of the HSD group showed no significant differences compared with those of the BSD group after the 70-day feeding trial, which was similar to a previous report (Zhang et al., 2020b). Zhao et al. (2020) demonstrated that VSI values are not significantly affected by feeding juvenile golden pompano (*Trachinotus ovatus*) different levels of corn starch, and HSI values were significantly higher when the corn starch level was >20%. Zhou et al. (2015) also reported that the HSI values of fish fed a 22.4% carbohydrate diet were significantly higher than those of fish fed a 0%–11.2% carbohydrate diet. Modern pharmacology shows that MLE contains polysaccharides, flavonoids, alkaloids, and other active ingredients (Sánchez-Salcedo et al., 2015; Yuan et al., 2015), and previous studies have shown that MLE protects the liver by regulating glucose and lipid metabolism (Chang et al., 2013; Ou et al., 2013; Sheikhlar et al., 2017). This study showed that *M. salmoides* fed a high-starch diet supplemented with > 0.1% MLE had lower HSI values than fish fed a high-starch diet alone, which indicates that MLE may be conducive to liver health. Therefore, MLE could repair and improve liver function in fish fed a high-starch diet.

Excessive dietary starch levels can lead to excessive glycogen and lipid deposition (Lin et al., 2018; Ma et al., 2019; Zhang et al., 2020b). After the 70-day feeding trial, whole-body lipid levels were significantly higher in the HSD group than in the BSD group. This is in agreement with previous studies on the golden pompano (Zhou et al., 2015), blunt snout bream (Xia et al., 2018), and grass carp (*Ctenopharyngodon idella*) (Tian et al., 2011). However, hepatic and muscle glycogen contents in this study were not correlated with dietary starch content. It has been reported that hepatic and muscle glycogen contents were significantly affected when dietary starch content was >15% in

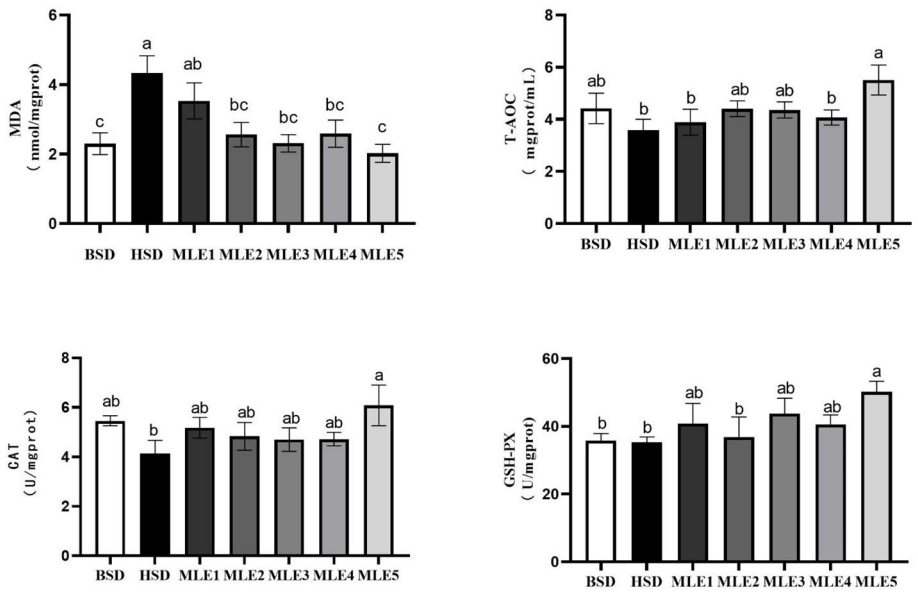

FIGURE 1
Effects of mulberry leaf extract on antioxidant enzymes of *Micropterus salmoides* fed high-starch diet diets. Values in each column with different superscripts have significant differences ($p < 0.05$).

M. salmoides (Ma et al., 2019). The possible reason may relate to the starch levels in the diet. The results showed that *M. salmoides* in the MLE5 group had significant decreases in hepatic and muscle glycogen contents. Dietary MLE significantly decreased the whole-body lipid content. Jeszka Skowron et al. (2014) found that bioactive substances in mulberry leaves regulate glucose metabolism by correcting hyperglycemia and increasing insulin secretion in the streptozotocin-induced non-obese diabetic rat model. Dietary MLE has also been reported to inhibit lipid accumulation by reducing lipogenesis and promoting hepatic lipid clearance (Chang et al., 2013). Moreover, Hou et al. (2019) found that mulberry leaf meal reduced liver lipid content by suppressing the isolation and proliferation of adipocytes. In this experiment, dietary MLE lowered the contents of GLU, TG, and TC of *M. salmoides* fed a high-starch diet. Therefore, high starch diet supplemented with MLE decreased lipid and glycogen deposition by enhancing glucose and lipid metabolism. In addition, the whole-body moisture and muscle were significantly decreased in the MLE groups, which was consistent with results previously obtained in the Chinese giant salamander (*Andrias davidianus*) (Li Z et al., 2020).

Antioxidant enzyme systems are an important defense mechanism of organisms, which can reduce peroxide in the body into less harmful substances (Bogdan et al., 2000). A previous study indicated that hyperglycemia after consuming a high-starch diet is associated with oxidative stress (Rains and Jain, 2011). MDA is the final product of lipid peroxidation and reflects the degree of lipid peroxidation (Koruk et al., 2004). SOD, GSH-Px, and CAT are three important members of the antioxidant system that work against the formation of reactive oxygen species (ROS), protect cell membranes and intracellular nucleic acids, and reflect the growth and development of the organism, changes in the metabolic state *in vivo*, and environmental stress (Zimmermann et al., 1973; Holmblad and Söderhäll, 1999). Additionally, T-AOC is the main index used to determine the total antioxidant level of an organism, which reflects a compensatory mechanism of the organism under the stimulation of oxidative stress (Decker et al., 2000). This study suggests that significantly higher MDA levels might be induced in *M. salmoides* fed a high-starch diet but with no significant effect on the activities of SOD, GSH-Px, CAT, and T-AOC. Some recent studies have indicated that oxidative stress is not directly related to dietary starch (Wang et al., 2014) and that appropriate starch levels can improve antioxidant capacity (Wu et al., 2015). However, excess starch can induce strong oxidative stress in fish (Zhou et al., 2013; Zhao et al., 2020). A previous study indicated that 5% and 10% starch diets had no negative effects on *M. salmoides* (Lin et al., 2018). Zhang et al., (2020a) also found that suitable dietary starch levels (0–100 g/kg) had no negative effect on antioxidant capacity. In this study, the higher MDA content in the HSD group may indicate fatty liver injury. Mulberry leaf extract contains enriched polyphenolics, flavone, and 1-deoxynojirimycin, which has been shown to scavenge free radicals (Radojković et al., 2012) and enhance antioxidant enzyme activities in mammals (Bae et al., 2013; Lee et al., 2016). In addition, the antioxidative effects of MLE have been

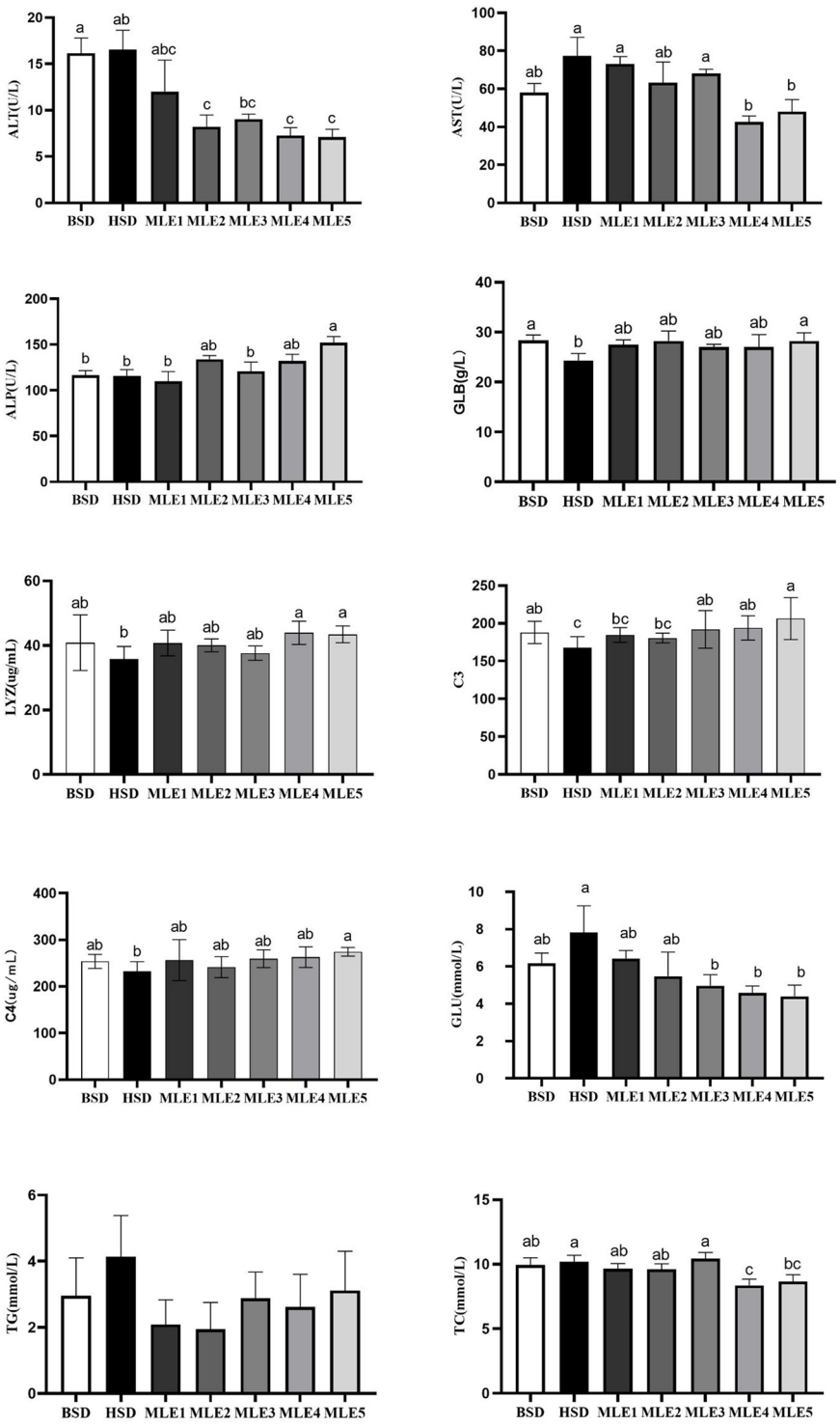

FIGURE 2
Effects of mulberry leaf extract on non-specific immune indices and plasma biochemical parameters of *Micropterus salmoides* fed high-starch diet diets. Values in each column with different superscripts have significant differences ($p < 0.05$).

demonstrated in African catfishes (Sheikhlar et al., 2017). In this study, significantly higher antioxidant capacities of *M. salmoides* were observed after 1.0% MLE was added to the high-starch diet. Thus, MLE improved liver function by suppressing oxidative stress.

Hematological parameters are vital physiological indicators that reflect the metabolic and physiological states of the body; therefore, they are beneficial for disease diagnosis (Ahmdifar et al., 2011). Lysozyme activity is an important immune parameter that protects against microorganisms (Jiang et al., 2009). Complement is also an important component of non-specific immunity, which mediates inflammatory and immune responses (Han and Ulevitch, 2005; Boshra et al., 2006). The inhibition of lysozyme and complement activity has been confirmed in some fish fed a high-starch diet (Wu et al., 2015; Xia et al., 2018; Li S et al., 2020). In this study, the lysozyme and C3 content in the HSD group were lower than those in the BSD group, and C4 was significantly lower. These results suggested that starch levels could impact the immune system. Several studies have suggested that MLE has anti-bacterial and anti-inflammatory effects (Wang et al., 2009; Forato Anhê et al., 2014). In addition, MLE can improve hepatic injury and inflammation induced by a high-sugar and high-fat diet through various pathways (Ou et al., 2013; Park et al., 2013). In the experiment, MLE supplementation in a high-starch diet enhanced the non-specific immunity of *M. salmoides*. AST and ALT are two crucial aminotransferases that mainly exist in cells and are rarely present in the plasma. The activities of AST and ALT were higher in cardiomyocytes and hepatocytes than those in other organs. Therefore, the activities of AST and ALT in plasma can reflect the health status of the liver and heart (Cho et al., 1994; Liu et al., 2010). ALP is a hallmark enzyme of lysosomal integrity, is involved in the transfer and metabolism of phosphoric groups in organisms, and plays a crucial role in immunity and growth (Oner et al., 2008; Yan et al., 2014). In this study, no differences were observed in plasma levels of ALT, AST, and ALP between the HSD and BSD groups. However, there was approximately 46.87% observable liver damage in the BSD group during sample collection. Thus, *M. salmoides* could not adapt to an 8.88% starch-formulated diet. Alternatively, formulated diets of *M. salmoides* must be optimized (Huang et al., 2017; Ma et al., 2019; Ma et al., 2020). In this study, the plasma ALT and AST activities in the MLE5 group were significantly lower than those in the HSD group, indicating that 1.0% MLE can alleviate liver damage of *M. salmoides* fed a high-starch diet. GLB is an important part of non-specific immunity. The plasma GLB level in the MLE5 group was significantly higher than that in the HSD group. This result indicated that mulberry leaf extract improved the non-specific immunity of *M. salmoides* fed a high-starch diet. The dietary starch level markedly affects the metabolism of carbohydrates and lipids. In this experiment, increased levels of GLU, TG, and TC were observed in the HSD group compared to the BSD group. Similar results have been observed in the blunt snout bream, golden pompano, and *M. salmoides* (Xia et al., 2018; Zhang et al., 2020a; Zhao et al., 2020). Mulberry leaf extract contains DNJ as a competitive inhibitor, which improves control and decreases plasma glucose content in mammals (Shang et al., 2012; Wang et al., 2018). In addition, mulberry leaf extract inhibits lipid accumulation by reducing lipogenesis and promoting hepatic lipid clearance (Chang et al., 2013). In this experiment, dietary MLE lowered the contents of GLU, TG, and TC in this study. These results suggest that MLE can improve the transport of glucose and lipids in the liver of *M. salmoides* fed a high-starch diet.

Conclusion

In conclusion, no significant difference in growth in the 8.88% starch level was observed, whereas it significantly affected proximate compositions, liver antioxidant activity, and non-specific immunity in *M. salmoides*. Dietary supplementation with 1.0% MLE and 11.42% starch decreased the moisture, lipids, and glycogen content in the body. Moreover, MLE improved immune and liver function. This study shows that MLE has positive effects on the health of *M. salmoides* when combined with an 11.42% starch diet. Mulberry leaf extract may also play a protective role by regulating glycolipid metabolism. The underlying mechanisms of MLE on glycolipid metabolism in *M. salmoides* warrant further exploration.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by The Animal Ethics Committee of Southwest University.

Author contributions

JT: methodology and design. LH: methodology and investigation. HJ: design. LZ, LY, JH, ZX and KZ: carried out the chemical analysis. SW and HM: carried out the data analysis and statistical analysis. YH and YHJ: supervision and project administration. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author HJ is employed by Guangzhou A Share Aquatic Science and Technology Co. Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effect of dietary cholesterol on ovarian development of Chinese mitten crabs (*Eriocheir sinensis*)

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This study was conducted to evaluate the effect of cholesterol (CHO) on ovarian development of *Eriocheir sinensis* through a feeding trial. Crabs (average weight 43.35 ± 0.05 g) were randomly fed with one of three diets: 0% CHO, 0.4% CHO and 1.6% CHO for 16 weeks. Results indicated that the hepatosomatic index (HSI) and gonad index (GSI) of crabs fed with 0.4% CHO diet were significantly higher than those in the control group. ($P < 0.05$). Meanwhile, 0.4% CHO can significantly increase the gene expression of *vtg* and *vgr* in ovary ($P < 0.05$), thus promoting the accumulation of Vtg. The protein expression of MEK1/2, ERK, p-ERK1/2, Cyclin B and CDC2 were significantly increased in the 0.4% CHO group, therefore dietary CHO might promote oocyte maturation by activating MAPK signal pathway and cooperating with maturation promoting factor (MPF). Further exploration results showed that 0.4% CHO was able to significantly up-regulate the protein expression of STAR and SR-B1 to promote the transport of CHO to ovary, thereby providing sufficient substrates for estradiol (E_2) synthesis. In addition, the results *in vivo* and *in vitro* shown that CHO could regulate the activities of enzymes such as CYP11A1 and CYP19A1 through the cAMP-PKA-CREB/SF-1 signal pathway and then affects the level of E_2 in the organism. In summary, supplementing the appropriate amount of CHO in the diet can improve the E_2 level of the organism, thus increasing the accumulation of nutrients in the ovary, promoting the completion of meiosis, and finally achieving the purpose of promoting ovarian maturation.

KEYWORDS

Eriocheir sinensis, cholesterol, ovarian development, estradiol synthesis, vitellogenesis

Introduction

As one of the most important economic crustaceans, the Chinese mitten crab (*Eriocheir sinensis*) has a vast consumer market in China, and its output and economic benefits have been increasing continuously in the past few years (Wang et al., 2016; Chinese Fishery Statistical Yearbook, 2021). As the most popular edible part, the maturing status of ovary directly affects the economic value of *Eriocheir sinensis* (Long et al., 2020). In China, the most popular time for *Eriocheir sinensis* consumption is mainly around the Mid-Autumn Festival. But, during this period, compared with crabs fed with formula feed, the crabs fed with trash fish have higher gonad maturity, which significantly affected farmers' income and thus hindered the promotion of formula feed (Djunaidah et al., 2003; Zheng et al., 2020; Fang et al., 2021). However, feeding trash fish for a long time will lead to environmental pollution and ecological damage (Wu et al., 1994). Therefore, looking for a nutrient that can promote the ovarian development, and then preparing a more comprehensive formula feed is of great significance to the sustainable development of the crab industry.

In fact, the ovarian maturation of *Eriocheir sinensis* is actually a process of nutrient accumulation, of which the most important substance is yolk protein (Wu et al., 2007). Therefore, the accumulation of yolk protein directly affects the maturity and economic value of *Eriocheir sinensis* (Wouters et al., 2001). The main component of yolk protein is vitellin (Vn), which can be processed by vitellogenin (Vtg), lipids and proteins (Okuno et al., 2002; Wu et al., 2017). Presently, there is still some controversy about the sites of Vtg synthesis in crustaceans, but for most shrimp and crabs, hepatopancreas and ovary are still the main sites of Vtg synthesis (Subramoniam, 2011). According to the different sites of Vtg synthesis, vitellogenesis can be divided into two stages: endogenous vitellogenesis and exogenous vitellogenesis (Wu et al., 2017). In the endogenous vitellogenesis stage, Vtg is mainly synthesized in the ovary, and in the exogenous vitellogenesis stage, the hepatopancreas is the main site of Vtg synthesis (Rani and Subramoniam, 1997; Lu et al., 2018). The Vtg synthesized in the hepatopancreas will be transported to the ovary through hemolymph after combining with steroid hormones, vitamins and lipids, and binds to vitellogenin receptor (VgR) on the surface of oocytes, then stored in oocytes through endocytosis (Soroka et al., 2000).

Besides the accumulation of nutrients, meiosis is also a critical event in the process of ovarian maturation (Song et al., 2014). However, during gametogenesis, immature oocytes will be blocked in the germinal vesicle (GV) stage, and the recovery of meiosis is a process of cell cycle transition induced by complex signal pathways (Cau et al., 1988; Lanot and Cledon, 1989). As the main promoter of cell cycle transformation, maturation promoting factor (MPF) is a heterodimeric protein kinase composed of the regulatory subunit Cyclin B and the catalytic subunit CDC2, which can catalyze the phosphorylation of various proteins, and then promote the transformation of oocytes from G2 phase to M phase (Wang et al., 2013; Song et al., 2014). Mitogen-activated protein kinase (MAPK) signaling pathway is a highly conservative signal transduction pathway, which widely exists in all kinds of eukaryotic cells. Many studies have shown that MAPK cascade signaling pathway through synergy with MPF and activate each other by positive feedback mechanism to jointly regulate meiotic cell cycle transition (Bodart et al., 2002; Ohashi et al., 2003).

The ovarian development of *Eriocheir sinensis* is regulated by many factors, among which hormone regulation plays a vital role (Malati et al., 2013). Estradiol (E_2), as one of the most active reproductive hormones, has been identified in many decapod crustaceans, and the promoting effect on the ovarian maturation of crustaceans has also been reported (Martins et al., 2007; Huang et al., 2009). Previously, there was some controversy about whether crustaceans have the ability to synthesize E_2. However, with the discovery of related enzymes in the process of E_2 synthesis in crustaceans such as *Macrobrachium rosenbergii*, *Procambarus clarkii* and *Eriocheir sinensis*, it is widely believed that crustaceans can synthesize E_2 with CHO as raw material under the action of a series of enzymes. (Teshima and Kanazawa, 1971; Warrier et al., 2001; Summavielle et al., 2003). Therefore, it may be an excellent method to solve the problem of delayed ovarian maturation of *Eriocheir sinensis* by supplementing exogenous nutrients to regulate the secretion of endogenous hormones.

CHO, as a necessary nutrient for aquatic animals, and the effects of growth performance has been widely studied (Suprayudi et al., 2012; Tian et al., 2020; Guo et al., 2022; Su et al., 2022). Meanwhile, as a substrate for the synthesis of E_2, CHO could be of great significance for ovarian development (Teshima et al., 1983; Kumar et al., 2018). However, there are few reports about the effect of CHO on the ovarian development of *Eriocheir sinensis* yet. The synthesis of E_2 occurs in the mitochondrial intima of the ovary (Jefcoate et al., 1992). In crustaceans, high-density lipoprotein (HDL) and scavenger receptor class B type 1 (SR-B1) are responsible for the transport of CHO into oocytes, and CHO in oocytes needs to be further transported into the inner mitochondrial membrane under the action of steroidogenic acute regulatory protein (STAR). (Becker et al., 1993; Miller, 2007). Subsequently, CHO produces pregnenolone under the action of CHO side-chain cleavage enzyme (CYP11A), which goes through a series of reactions to testosterone, and then to E_2 under the further action of aromatase (CYP19A1). Therefore, STAR, CYP11A1 and CYP19A1 are three key substances catalyzed E_2 synthesis (Miller, 2007; Zheng et al., 2020). cAMP/PKA is an important signal pathway for regulating E_2 synthesis in the organism, SF-1 and CREB are the response factors of the cAMP/PKA signal pathway, which is responsible for regulating the activity of various enzymes in the process of E_2 synthesis (Carlone and Richards, 1997; Stocco, 2001; Qu et al., 2008). In some aquatic

animals, it has been reported that by controlling the substrate to regulate secretion of estrogen to influence the ovarian development, but this has been not reported in crustaceans (Jefcoate et al., 1992). Therefore, this experiment aims to evaluate whether the exogenous CHO can regulate the secretion of E_2 through the cAMP-PKA-CREB/SF-1 signal pathway and then affect the ovarian maturation. Thereby solving the problems of delayed ovarian maturation of *Eriocheir sinensis*.

Materials and methods

The experimental design and all experimental operations of this study was approved by the Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, China) (permit number: SYXK (Su) 2011–0036).

Experimental diets

Our previous study results showed that the CHO deposition in the hepatopancreas of *Eriocheir sinensis* was about 0.4%, so we designed a group with 0.4% CHO (Guo et al., 2022). In addition, in order to explore whether excessive CHO will affect ovarian development, a 1.6% CHO supplement group was set up. Therefore, three isonitrogenous and isolipid diets were formulated to contain CHO 0%, 0.4% and 1.6% (actually 0.06%, 0.44%, 1.64%) at the cost of soybean oil. The preparation method of experimental diets according to the previous study of our laboratory (Guo et al., 2022). Firstly, all raw materials were smashed and passed through a 60-mesh percolator, then mixed step by step. Put the weighed CHO and oil of each group into a beaker and mix them thoroughly with a glass rod. Subsequently, about 30% deionized water was added to the mixture to make a dough. At last, the feed pellets (2.5-mm diameter) were squeezed out through a single-screw meat grinder extruder. After being air-dried (27°C) for 24 hours, four diets were packed in vacuum plastic bags and stored at -20°C. The ingredient formulation and proximate composition of the diet was shown in Table 1.

Experimental crabs and feed trial

The experimental crabs were obtained from a local farm in Pukou, Nanjing, China. This experiment was implemented in the Aquatic Teaching Base of Nanjing Agricultural University (Nanjing, Jiangsu, China). A total of 160 healthy female crabs (average weight, 43.35 ± 0.05 g) were randomly assigned to 16 cement pools (10 crabs in each cement pool, each treatment contains four cement pools). 12 pipes (20 cm long and 10 cm diameter) were put into each cement pool as shelters for crabs to hide. Prior to the 16-week breeding experiment, the crabs were fed in the cement pool (1.0×1.0×0.8m, L:W:H) for a week to adapt to the new environment. After a week of acclimation, crabs of each treatment were fed with their respective diets once daily (18:00) with 3%-5% of total body weight till obvious satiation. During the experiment period, the residual feed of the previous day was removed with a dirt absorber, and 1/3 volume of water should be changed in cement pool. The water quality was detected daily and maintain the water temperature at 24-28°C, dissolved oxygen at 5.0-7.0 mg/L, pH at 7.3-8.4, and ammonia nitrogen under 0.05 mg/L.

Sample collection

At the end of 16-week feeding trial, all crabs were starved for 24 hours. Subsequently, four individuals were randomly selected from each cement pool to collect hemolymph with a 1mL syringe according to the method in our laboratory (Cheng et al., 2020). Thereafter, the crabs were anesthetized on ice, then the ovaries and hepatopancreas were quickly collected and weighed to calculate hepatosomatic index (HSI) and gonad index (GSI). After weighing, part of the ovaries was fixed with 4% paraformaldehyde solution for histological analysis. The remaining samples were quickly frozen with liquid nitrogen and stored at -80°C for determining biochemical indicators and enzymes activities and protein expression. The calculation formulas of related parameters were as follows:

$$\text{HSI}, \% = \frac{\text{hepatopancreas weight (g)}}{\text{final body weight (g)}} \times 100$$

$$\text{GSI}, \% = \frac{\text{gonad weight (g)}}{\text{final body weight (g)}} \times 100$$

Proximate composition analysis

The content of crude protein, ash and moisture in the diet were determined according to the standard method (AOAC, 1995). The details detection of crude protein, ash and moisture were the same as Guo et al. (2022). The content of CHO in the feed was detected through the high-performance liquid chromatography (HPLC) method (Agilent ZORBAX Eclipse Plus, column C18 5 μm 4:6 × 150 mm). 100% methanol was used as a mobile phase pumped with a flow rate at 1 mL/min. The specific measurement method refers to Guo et al. (2022).

TABLE 1 Ingredient formulation and proximate composition (% dry matter) of the diet in this feeding trial.

	Cholesterol supplementation (%)		
	0	**0.4**	**1.6**
Ingredients (%)			
defatted fish meal [a]	18.50	18.50	18.50
Soybean meal	15.00	15.00	15.00
Rapeseed meal	2.50	2.50	2.50
Cottonseed meal	3.00	3.00	3.00
Peanut meal	28.50	28.50	28.50
α-Starch	19.00	19.00	19.00
EPA oil: DHA oil (1:1) [b]	1.20	1.20	1.20
Soybean oil	5.20	4.80	3.60
Carboxymethyl cellulose	1.00	1.00	1.00
$Ca(H_2PO_4)_2{\cdot}H_2O$	2.20	2.20	2.20
Cholesterol (purity 99%) [c]	0.00	0.40	1.60
Lecithin	0.20	0.20	0.20
Zeolite	0.40	0.40	0.40
Premix [d]	1.00	1.00	1.00
Mixture [e]	2.30	2.30	2.30
Total	100.00	100.00	100.00
Proximate composition (%)			
Crude protein	36.21	36.31	36.24
Crude lipid	7.95	7.98	7.96
Crude ash	7.15	7.07	7.16

[a]Fishmeal had been skimmed from 0.38% to 0.11%. cholesterol.
[b]DHA oil and EPA oil (DHA content, 70% of oil; EPA content, 70% of oil) was purchased from Shanxi Pioneer Biotech Co., Ltd., Xian, Shanxi, China.
[c]Cholesterol (purity 99%) was purchased from Shanxi Pioneer Biotech Co., Ltd., Xian, Shanxi, China.
[d]P Premix supplied the following minerals (g/kg) and vitamins (IU or mg/kg): $CuSO_4{\cdot}5H_2O$, 2 g; $FeSO_4{\cdot}7H_2O$, 25 g; $ZnSO_4{\cdot}7H_2O$, 22 g; $MnSO_4{\cdot}4H_2O$, 7 g; Na_2SeO_3, 0.04 g; KI, 0.026 g; $CoCl_2{\cdot}6H_2O$, 0.1 g; Vitamin A, 900,000 IU; Vitamin D, 200,000 IU; Vitamin E, 4500 mg; Vitamin K3, 220 mg; Vitamin B1, 320 mg; Vitamin B2, 1090 mg; Vitamin B5, 2000 mg; Vitamin B6, 500 mg; Vitamin B12, 1.6 mg; Vitamin C, 10,000 mg; Pantothenate, 1000 mg; Folic acid, 165 mg; Choline, 60,000 mg; Biotin, 100 mg; Myoinositol 15,000 mg.
[e]Mixture includes the following ingredients (%): choline chloride 4.75%; antioxidants 1.72%; mildew-proof agent 2.35%; salt 22.06%; Lvkangyuan 59.30% and biostimep 9.51%.

Gene expression analysis

The total RNA of the ovary and hepatopancreas were extracted using RNAiso Plus (TakaRa, Dalian, China), and treated with gDNA wiper Mix and HisScript III Qrt SuperMix (Cat. No. R323–01, Vazyme Biotech Co, China) to eliminate genomic DNA. Then, the purity of total RNA was estimated by spectrophotometry on the basis of OD 260/280 using a Nano Drop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Subsequently, 1 μg RNA was reverse transcribed into first-standard cDNA immediately using HisScript III Qrt SuperMix (Vazyme Biotech Co, China) according to Wang et al. (2020). After reverse transcription, the target cDNA was amplified by the ChamQ Universal SYBR qPCR Master Mix Kit (Vazyme Biotech Co, China). The specific sequences of primers are given in Table 2. The reaction and protocol were set according to Guo et al. (2022). *β*-actin was the reference to do relative quantification of the target gene

TABLE 2 Nucleotide sequences of the primers for real-time quantitative PCR.

Gene	Position	Primer sequence (5′-3′)	Length	Product size (bp)	Reference
vtg	Forward	AAGGTCCGCAGCAAGCAGAT	20	181	Lin et al., 2020
	Reverse	GGCGAGGCACGAGGTAGAAT	20		
vgr	Forward	GCAACGCCTTCCTTCTGGTA	20	193	Lin et al., 2020
	Reverse	GGCACGGTGTTCGCTATCAT	20		
β-actin	Forward	TCGTGCGAGACATCAAGGAAA	21	178	KM244725.1
	Reverse	AGGAAGGAAGGCTGGAAGAGTG	22		

vtg, vitellogenin; *vgr*, vitellogenin receptor.

transcripts and the target gene expression of crabs in the group with 0% CHO as the correct factor. The transcript levels of target genes were analyzed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Ovarian histology analysis

The ovaries were fixed in 4% paraformaldehyde for 24 hours, then placed in 70%, 75%, 80%, 90%, 95% and absolute ethanol for gradient dehydration. After that, transparently treated with xylene and embedded in paraffin. The embedded ovarian tissue was sliced with a microtome (LeicaRM2016, Berlin, Germany) according to the thickness of 6 μm. Then the slices were routinely dewaxed and rehydrated in absolute ethanol, 95% ethanol and 80% ethanol. After rehydration, soak in ultrapure water for 5 minutes and then hematoxylin-eosin (H&E) staining was performed according to Zheng et al. (2020). Finally, the film was sealed with neutral gum, and four individuals were observed under the optical microscope (Nikon Eclipse 80i, Tokyo, Japan), captured by a digital camera (Nikon DS-U2, Tokyo, Japan). Only oocytes with obvious nuclei were measured by Image-Pro Plus 6.0 (media cybernetics, USA). The parameters include the short diameter of an oocyte (SO), the long diameter of an oocyte (LO), the short diameter of nuclei (SN), the long diameter of nuclei (LN). The volume of an oocyte (VO), the volume of a nuclei (VN), and the nucleo-cytoplasmic ratio (NCR) were calculated according to the formula as follows (Wu et al., 2017; Zheng et al., 2020):

$$VO = 0.523 \times W_o^{\ 2} \times L_o$$

$$VN = 0.523 \times W_n^{\ 2} \times L_n$$

$$NCR = VN/VO$$

Where W_o is the maximum width of oocyte, L_o is the maximum length of oocyte, W_n is the maximum width of nuclei, L_n is the maximum length of nuclei.

Fluorescence assay of cholesterol

The ovarian was embedded in the optimal cutting temperature compound (OCT, Bioss, C2076), and then fixed on the sample holder. After the temperature of the microtome stabilized at -20°C, the tissue was cut into 6 μm thin slices, dipped in the slices with glass slides, and then washed away the excess OCT with PBS. The FILIPIN dye was dropped on the slices and incubated at room temperature for 30 minutes. In the end, washing away the excess staining solution with PBS, the slides were sealed and observed under confocal laser scanning microscope (ZEISSLMS900, Berlin, Germany).

Biochemical analysis of ovary

The ovarian samples were made into homogenate, centrifuged at 4°C and 6000 rpm for 5 minutes, and the supernatant was taken to detect the following indexes: total CHO (TC) (TC, Kit, NO. ml094937), estradiol (E_2) (mouse E_2 ELISA Kit, NO. ml001962), vitellogenin (Vtg) (crab Vtg ELISA Kit, NO. ml003467), Cytochrome P450 Family 11 Subfamily A Member 1 (CYP11A1) (insect CYP11A1 ELISA Kit, NO.ml122176), Cytochrome P450 Family 19 Subfamily A Member 1 (CYP19A1) (insect CYP19A1 ELISA Kit, NO. ml036941). The commercial kits (Shanghai Enzyme Bioengineering Co., Shanghai, China) were used to detect the above indicators, specific operation steps are strictly implemented in accordance with the instructions.

Western blot analysis

The process of western blot refers to Cao et al. (2019). Total protein of ovary was extract with a glass tissue grinder (Kimble Chase) on ice and lysed with RIPA lysis buffer (#ab156034, Abcam, United Kingdom). The protein concentration was detected by BCA protein assay kit (Beyotime Biotechnology, China). Heat denatured protein lysate (2 μg/μL) of 10 μL was loaded into each well, separated on 4-20% gradient SDS-PAGE electrophoresis gels for 30-35 minutes at 150 V using a Mini-Protean System (Bio-Rad, United States). The protein was transferred to PVDF membrane by electrophoresis at 105 V for 65 minutes. Each PVDF was blocked with 5% BSA in TBST (0.1% Tween 20, 500 mM NaCl, 20 mM pH=7.4 Tris-HCl) at room temperature for 2 hours, then incubated overnight at 4°C, with primary antibodies against STAR (#AC026, ABclonal), SR-B1 (#DF6479, Affinity), cAMP (#ab76238, Abcam), PKA (#ab75991, Abcam), SF-1 (#AF7895, Affinity), p-CREB (#AF3189, Affinity), CYP19A1 (#AF5229, Affinity), CYP11A1 (#DF4697, Affinity), MEK1/2 (#AF6385, Affinity), ERK1/2 (#AF0155 Affinity), p-ERK1/2 (#AF1015, Affinity), Cyclin B (#DF6786, Affinity), CDC2 (#DF6024, Affinity), VTG (#abs119855, Absin), α-Tubulin (#AF7010, Affinity). The next day, PVDF membranes were incubated with secondary antibodies (Goat Anti-Mouse IgG H&L: #ab6728, Abcam; Goat Anti-Rabbit IgG H&L: #ab6721, Abcam) for 2 hours at room temperature. The bands of the protein were detected by a ECL reagent (Beyotime Biotechnology, China), and captured with a luminescent image analyzer (Fujifilm LAS-3000, Japan). The intensity of target bands was analyzed by image J software (U.S. National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence

Refer to the above steps of making paraffin sections, and treat the ovaries to the step of dewaxing and rehydrating. Then the slices were immersed in 0.01 M citrate solution and heated at 95°C for 5 minutes for antigen repair. After cooling to room temperature, rinse with PBS three times for 5 minutes each time. Then 5% goat serum was dripped and sealed at room temperature for 2 hours, and the excess sealing solution was removed. The first antibody was dripped and incubated in a wet box at 4°C for 12 hours. After returning to room temperature, rinsed with PBS three times for 5 minutes each time. Drop the fluorescent secondary antibody to cover the sample completely, incubate at 37°C for 1 h. Subsequently, DAPI was added and dye for 10 minutes at room temperature in the dark, then wash the excess dye off with PBS, seal the film and observed under confocal laser scanning microscope (ZEISSLMS900, Berlin, Germany).

In vitro tissue culture

The crabs were obtained from the Aquaculture Base of Nanjing Agriculture University. Before dissection, the female crabs were anesthetized on ice and disinfected with 75% ethanol. Ovaries were quickly taken out in a sterile environment and rinsed 8 times with PBS solution containing penicillin (100 IU/mL) and streptomycin (100 ug/mL), meanwhile, hemolymph and connective tissue were removed. Cut the ovaries into small pieces of ~1 mm^3 and place into a 24-well cell culture plate with 0.5 mL L-15 medium. After 30 minutes of tissue attachment, the previous culture medium was sucked off, and 400 μL of L-15 medium containing double antibody was added again. The ovarian tissue was cultured in a constant temperature incubator at 26°C. The CHO was resuspended in ethanol absolute (EA) and mixed thoroughly with L-15 medium. Based on the pre-experiment, the final concentrations of CHO were set to 0 mg/L, 50 mg/L and 200 mg/L, the media and the EA were control group. Each treatment contains four replicates, after culturing at 26°C for 24 hours, removing the culture medium, then rinse the ovaries with PBS for three times, and quickly extract the total protein for western blot analysis.

Statistical analysis

The statistical analysis of the data in this study were analyzed using SPSS 23.0 (Chicago, IL USA). All date were presented as mean ± S.E.M. (standard error of the mean). Before analysis, the normality and homogeneity of data were evaluated by Kolmogorov-Smirnov test and Levene's tests. Subsequently, a one-way analysis of variance was performed, and then, Duncan's multiple comparison test to estimate the significant differences ($P < 0.05$).

Results

Growth performance

The parameters of growth performance were shown in Figure 1. Compared to the control group, crabs fed the diet with 0.4% CHO resulted in a significantly higher HSI and GSI, while no significant difference was observed in 1.6% CHO treatment group ($P < 0.05$).

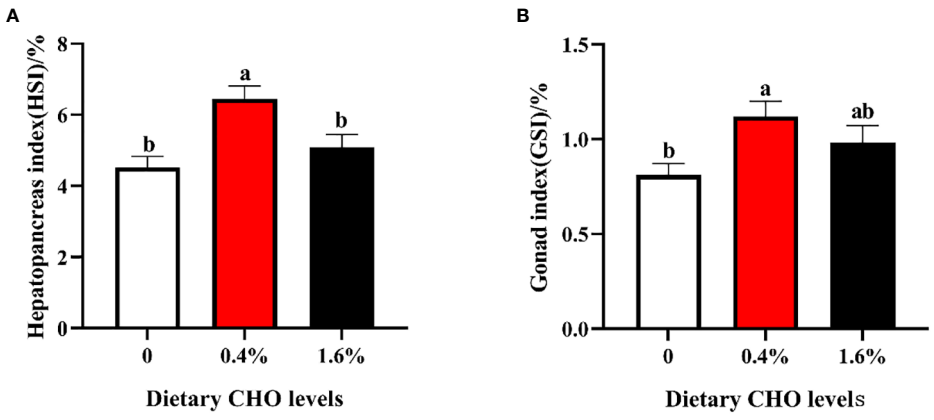

FIGURE 1
Effects of dietary CHO on **(A)** hepatopancreas index (HSI); **(B)** gonad index (GSI). The values are the Means ± SEM (n = 4). Different letters indicate significant differences ($P < 0.05$).

Histological analysis

The effect of dietary CHO on the ovarian histology of crabs was shown in Figure 2. Compared with the group without CHO, the volume of oocytes in the group supplemented with CHO was larger, and the oocytes were filled with yolk granules. Further observation showed that when the dietary CHO was 0.4%, the oocytes were squeezed and deformed, and the volume of oocytes and yolk granules was obviously larger.

The related parameters of oocytes were shown in Table 3. Compared with the control group, the LO, SO and VO in CHO treatment group were significantly increased ($P < 0.05$), and the maximum value appeared in the group with 0.4% CHO. For the nucleus, adding CHO has no effect on LN, but when the amount of CHO is 0.4%, SN and VN are significantly higher than the control group ($P < 0.05$). In contrast, after adding CHO, NCR was significantly lower than that of the control group ($P < 0.05$).

The expression and transport of VTG in the hepatopancreas and ovary

Dietary CHO had no significant effect on the content of VTG in the hepatopancreas (Figure 3B). While for the ovary, when the amount of CHO is 0.4%, the content of VTG was significantly increased ($P < 0.05$), and when crabs were fed the diet with 1.6% CHO, the content of VTG was significantly lower than the control group ($P < 0.05$) (Figure 3A). Compared with the control group, 0.4% CHO could significantly increase the mRNA expression level of *vtg* in ovary, but had no significant effect on the expression of *vtg* in hepatopancreas ($P < 0.05$) (Figures 3C, D). Furthermore, in 0.4% CHO group, the gene expression of *vgr* significantly increased in the ovary. In comparison, the expression of *vgr* shows a downward trend when the content of CHO reached 1.6% ($P < 0.05$) (Figure 3E). The results of protein expression showed that the protein expression level of VTG was significantly increased by adding CHO, and the protein

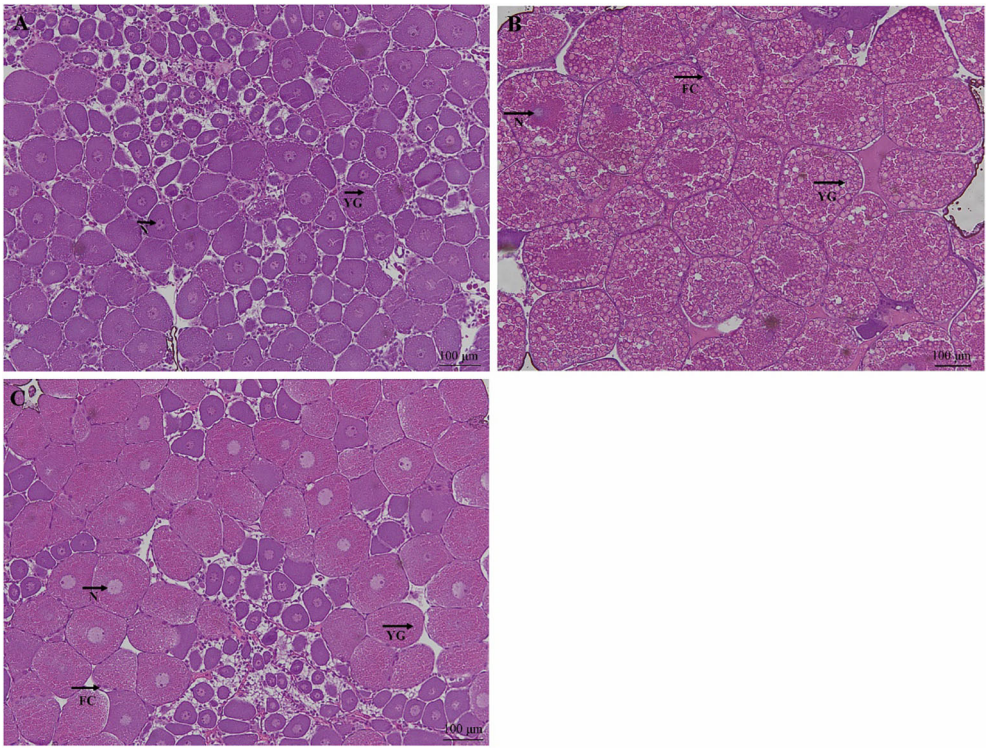

FIGURE 2
H&E staining of ovarian tissue from *E. sinensis* subjected to different levels of dietary CHO (means ± SEM, n = 4). Photomicrographs (10×) and scale bar (100 μm). **(A)**: crabs fed with 0% CHO diets; **(B)**: crabs fed with 0.4% CHO diets; **(C)** crabs fed with 1.6% CHO diets. N: nucleus; YG: yolk granule; FC: follicle cell.

TABLE 3 Sizes of oocytes and nuclei in *Eriocheir sinensis* subjected to different levels of dietary CHO.

Parameters	0%	0.4%	1.6%
LO (μm)	123.94 ± 4.68^a	190.34 ± 4.92^b	183.42 ± 5.41^b
SO (μm)	94.81 ± 3.98^a	156.90 ± 3.32^b	155.36 ± 4.39^b
VO (10^5 μm^3)	5.89 ± 0.61^a	24.65 ± 1.66^b	23.37 ± 1.96^b
LN (μm)	30.22 ± 1.05^a	31.04 ± 0.62^a	29.12 ± 0.48^a
SN (μm)	24.41 ± 1.10^a	28.01 ± 0.59^b	25.43 ± 0.51^a
VN (10^5 μm^3)	0.094 ± 0.010^a	0.128 ± 0.007^b	0.099 ± 0.005^a
NCR	0.016 ± 0.002^a	0.005 ± 0.001^b	0.004 ± 0.001^b

LO means long diameter of an oocyte; SO means short diameter of an oocyte; VO means volume of an oocyte; LN means long diameter of a nucleu; SN means short diameter of a nucleu; VN means volume of a nucleu; NCR means nucleus-cytoplasmic ratio.
Values (Mean ± S.E.M. of 20 replications) in the same column with different superscripts are significantly different at $P < 0.05$.

expression level of VTG was the highest in the group with 0.4% CHO (Figures 3F, G).

The expression of oocyte division-related proteins

As shown in Figure 4, with the increase of dietary CHO level, the expression levels of MEK1/2, ERK1/2, p-ERK1/2, Cyclin B and CDC2 showed a tendency to increase first and then decrease. And when crabs were fed the diet with 0.4% CHO, the expression of the above proteins in the ovary was significantly higher than that in the control group ($P < 0.05$).

The analysis of cholesterol transport

The results of western blot and immune fluorescence showed that CHO can significantly affect the protein expression of SR-B1 and STAR ($P < 0.05$) (Figures 5A–D). Compared with the control group, 0.4% CHO can significantly promote the protein expression of SR-B1 and STAR ($P < 0.05$), while 1.6% CHO will significantly suppress the expression of SR-B1 ($P < 0.05$). Through the staining and detection of CHO in the ovaries, it was found that after feeding a diet with 0.4% CHO, the amount of CHO deposition in the ovaries was significantly higher than that of other groups ($P < 0.05$).However, when the diet containing 1.6% CHO was fed to crabs, the content of CHO in the ovaries was significantly lower than that in the control group ($P < 0.05$) (Figures 5E, F).

The analysis of estradiol synthesis

The detection of E_2 synthesis-related proteins found that with the increase of CHO content from 0% to 1.6% in the diet, the expression levels of cAMP, PKA, p-CREB, SF-1, CYP11A1 and CYP19A1 showed a tendency to increase first and then decrease ($P < 0.05$) (Figures 6A–F). In addition, 0.4% CHO can significantly increase the activity of enzymes CYP11A1 and CYP19A1 in the ovary compared to the control group ($P < 0.05$) (Figures 6G, H). The detection of E_2 in the ovary showed that the content of E_2 in CHO treated groups was significantly higher than that in the control group ($P < 0.05$), and the maximum value appeared in the group with 0.4% CHO (Figure 6I).

Protein expression *in vitro*

The proteins expression of cAMP, PKA, p-CREB, SF-1, CYP11A1 and CYP19A1 in the ovary exposed to 0, 50 and 200 mg/L CHO were shown in Figure 7. Compared with the control group, the expression levels of cAMP and PKA in the CHO treated groups were significantly increased (Figures 7A, B) ($P < 0.05$). Moreover, 50 mg/L CHO could significantly up-regulate the expression levels of p-CREB, SF-1, CYP11A1 and CYP19A1 ($P < 0.05$), while when the concentration of CHO reached 200 mg/L, the expression of p-CREB, SF-1, CYP11A1 and CYP19A1 decreased significantly ($P < 0.05$) (Figures 7C–F).

Discussion

CHO, as an essential nutrient for aquatic animals, widely exists in various animal tissues. Especially for crustaceans, which have limited ability to synthesize CHO on their own and can only obtain it from food to meet their own needs (Kumar et al., 2018). Therefore, most previous studies on CHO in crustaceans have focused on survival, growth, and lipid metabolism (Sheen et al., 1994; Teshima et al., 1997; Niu et al., 2012; Tian et al., 2020). Our previous study showed that 0.27% CHO could significantly improve the weight gain and survival ratio of *Eriocheir sinensis*. However, 0.27% CHO may be able to meet the growth requirement of *Eriocheir sinensis*, but may be insufficient for the demand of ovarian development (Tao et al., 2014; Guo et al., 2022). For *Eriocheir sinensis*, the HSI increases gradually before the gonad enters the rapid development stage. However, when the gonad enters the rapid development period,

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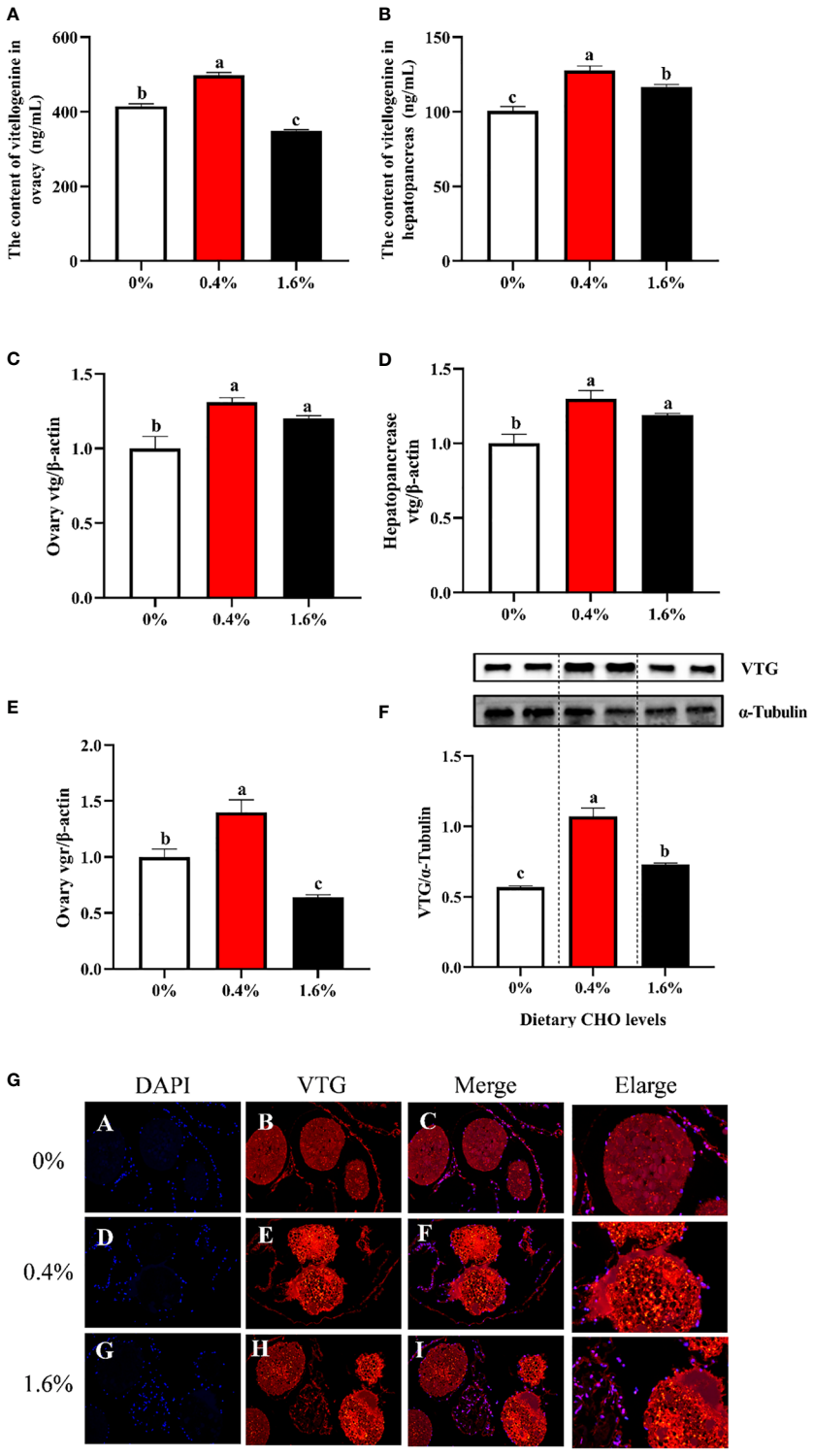

FIGURE 3
Effects of dietary CHO on **(A)** the content of Vtg in ovary; **(B)** the content of Vtg in hepatopancreas; **(C)** the mRNA expression of *vtg* in ovary; **(D)** the mRNA expression of *vtg* in hepatopancreas; **(E)** the mRNA expression of *vgr* in ovary; **(F)** the protein expression of VTG in ovary. **(G)** the immunofluorescence of VTG in ovarian tissue of *E.sinensis* after fed with diets supplemented different CHO levels (blue: nucleus; red: VTG). The values are the Means ± SEM (n = 4). Different letters indicate significant differences ($P < 0.05$).

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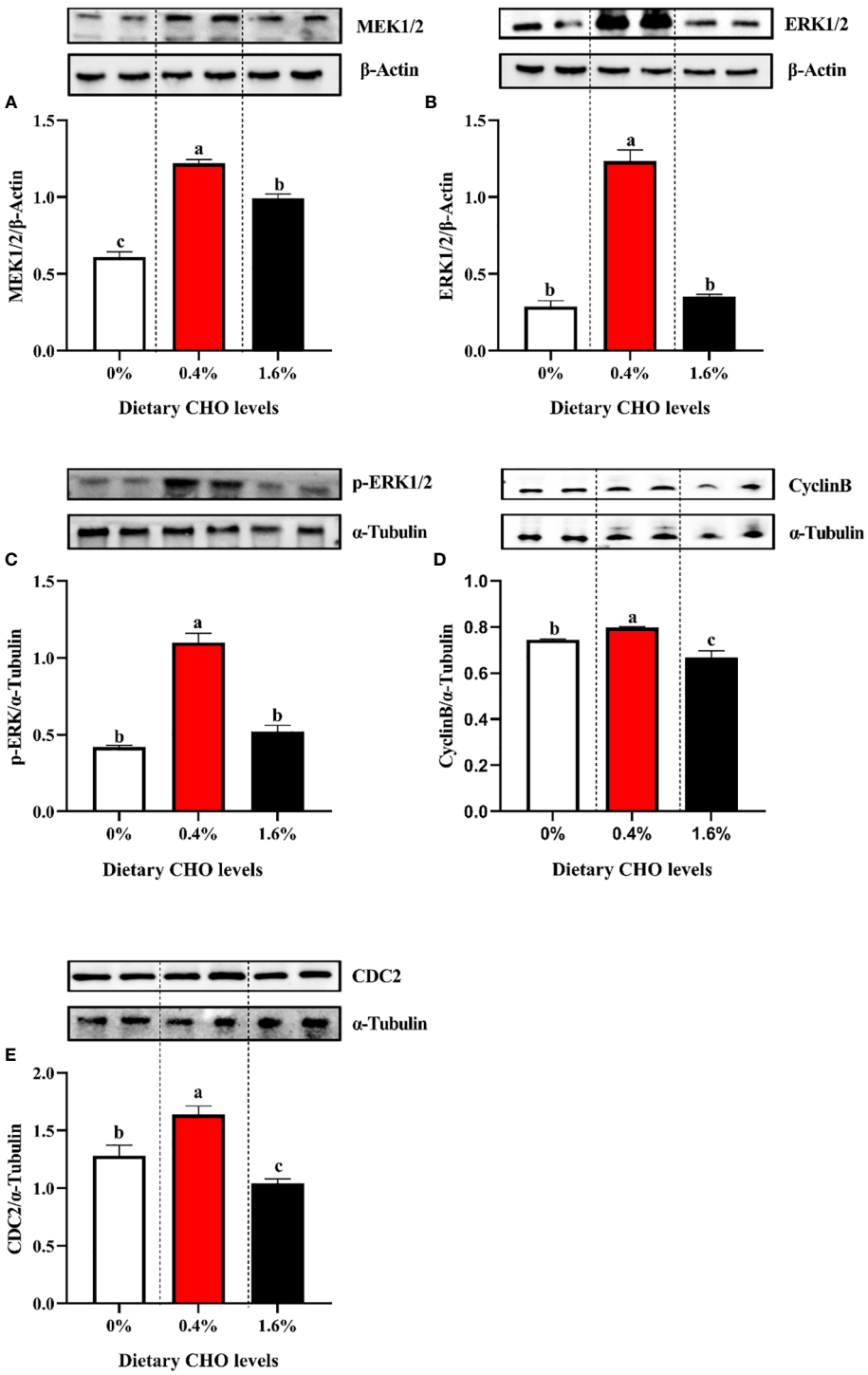

FIGURE 4
Western blot analysis of **(A)** the protein expression of MEK1/2 in ovary; **(B)** the protein expression of ERK1/2 in ovary; **(C)** the protein expression of p-ERK1/2 in ovary; **(D)** the protein expression of Cyclin B in ovary; **(E)** the protein expression of CDC2 in ovary. The values are the Means ± SEM (n = 4). Different letters indicate significant differences ($P < 0.05$).

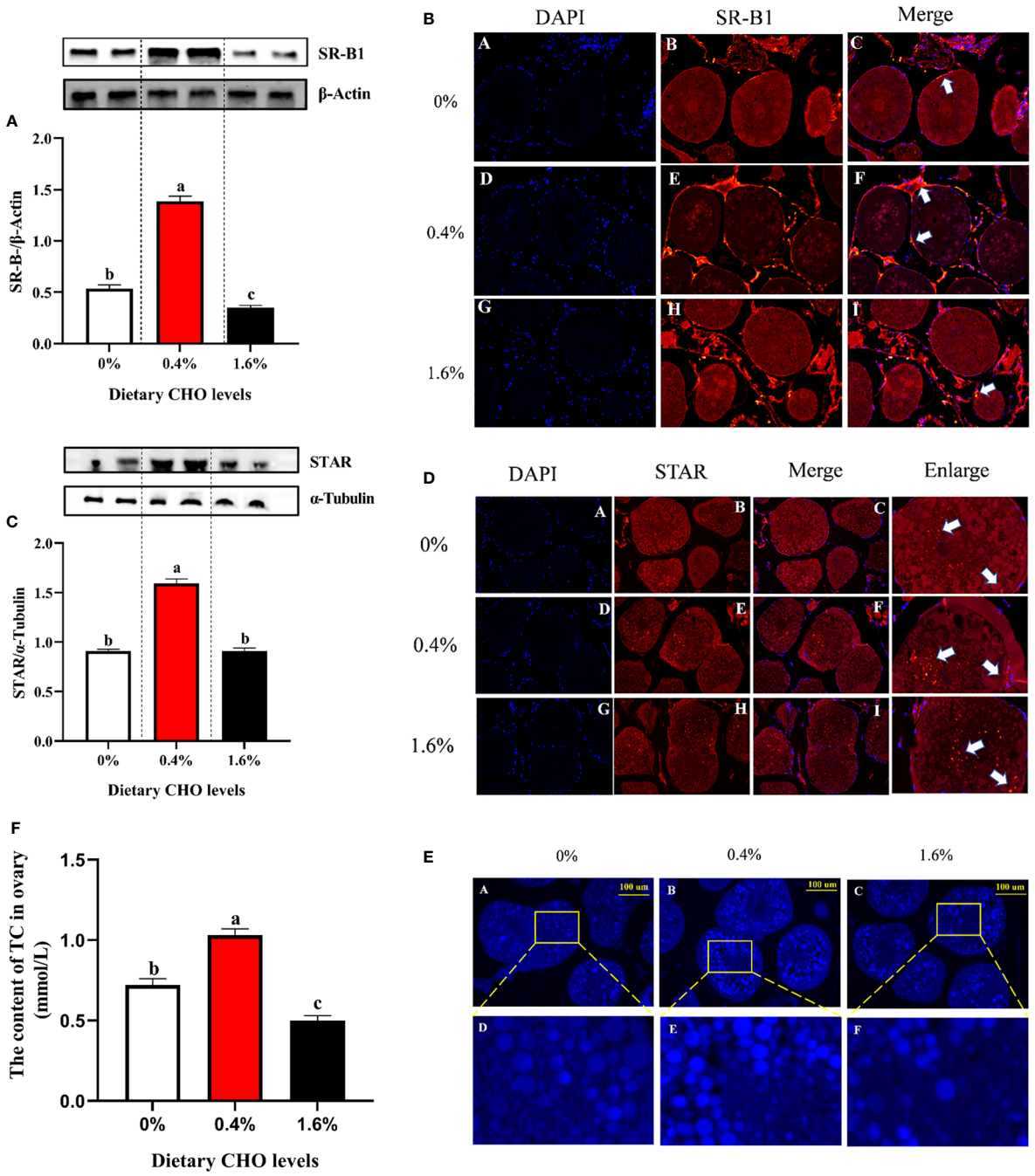

FIGURE 5
Effects of dietary CHO on **(A)** the protein expression of SR-B1 in ovary; **(B)** the immunofluorescence of SR-B1 in ovary (blue: nucleus; red: SR-B1); **(C)** the protein expression of STAR in ovary; **(D)** the immunofluorescence of STAR in ovary (blue: nucleus; red: STAR). The content of ovary CHO after crabs was fed diets with different CHO levels. **(E)** Representative confocal microscopic image of ovarian CHO stained by FILIPIN (blue), photomicrographs (200×) and scale bar (100 μm); **(F)** The content of TC in ovary. The values are the Means ± SEM (n = 4). Different letters indicate significant differences ($P < 0.05$).

the GSI will gradually increase, while the HSI will decrease accordingly. Therefore, it is generally believed that the nutrients in hepatopancreas are the energy pool to support the gonad development of crab (Wu et al., 2017; Jiang et al., 2022). In this study, the GSI and HSI in the group that fed diet containing 0.4% CHO were significantly higher than those in the control group, while this difference was no longer significantly at high doses of CHO. This result may be because dietary CHO affects the E_2

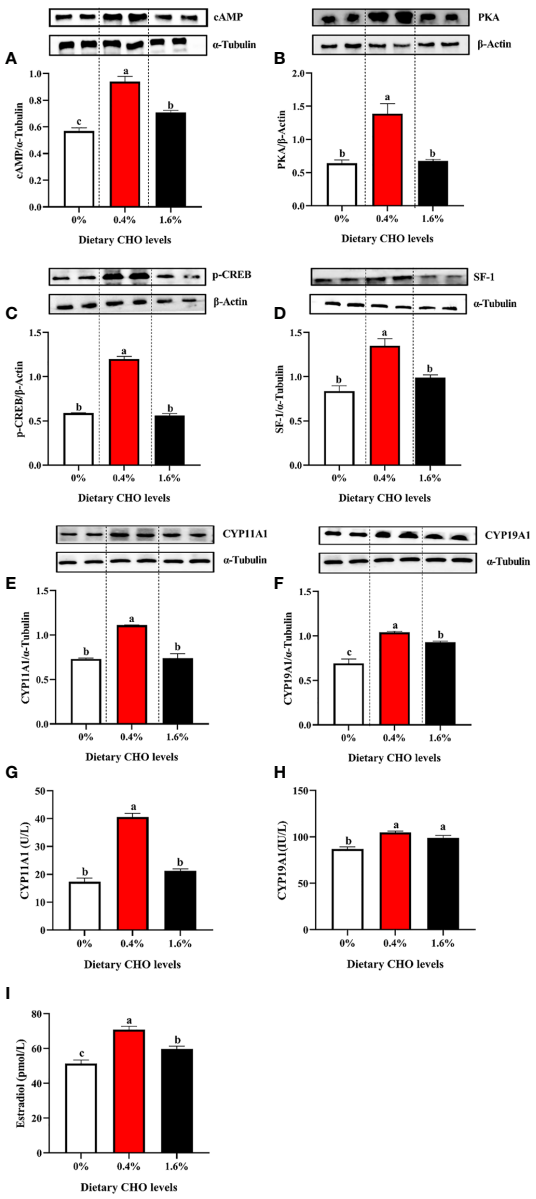

FIGURE 6
The protein expression of **(A)** cAMP; **(B)** PKA, **(C)** p-CREB, **(D)** SF-1, **(E)** CYP11A1 and **(F)** CYP19A1 in the ovary of *Eriocheir sinensis* fed with the experimental diets. **(G)** The activity of CYP11A1 in the ovary; **(H)** the activity of CYP19A1 in ovary; **(I)** the content of E_2 in ovary. The values are the Means ± SEM (n = 4). Different letters indicate significant differences ($P < 0.05$).

synthesis, leading to the difference in ovarian development (Fairs et al., 1990; Janer and Porte, 2007; Pan et al., 2018).

The ovarian maturation of *Eriocheir sinensis* is often accompanied by increased oocyte volume (Chen et al., 2004; Zheng et al., 2021). In this study, under the same magnification of light microscope, the number of oocytes observed in 0.4% CHO group was much less than that in the other two groups, and the yolk granules were fuller. Further measurement results also showed that the LO, SO and VO of oocytes in 0.4% CHO group were significantly higher than those in the other two groups. This further proves that an appropriate amount of CHO plays an important role in promoting ovarian maturation. In crustaceans, it has been reported that the increase of oocyte size is closely related to vitellogenesis (Wu et al., 2017). For example, substantial quantities of vitellin accumulates in the oocyte resulted in the active growth of oocytes in *Macrobrachium rosenbergii* (Du et al., 1995; Lee and Chang, 1997). In this experiment, 0.4% CHO significantly increased the content

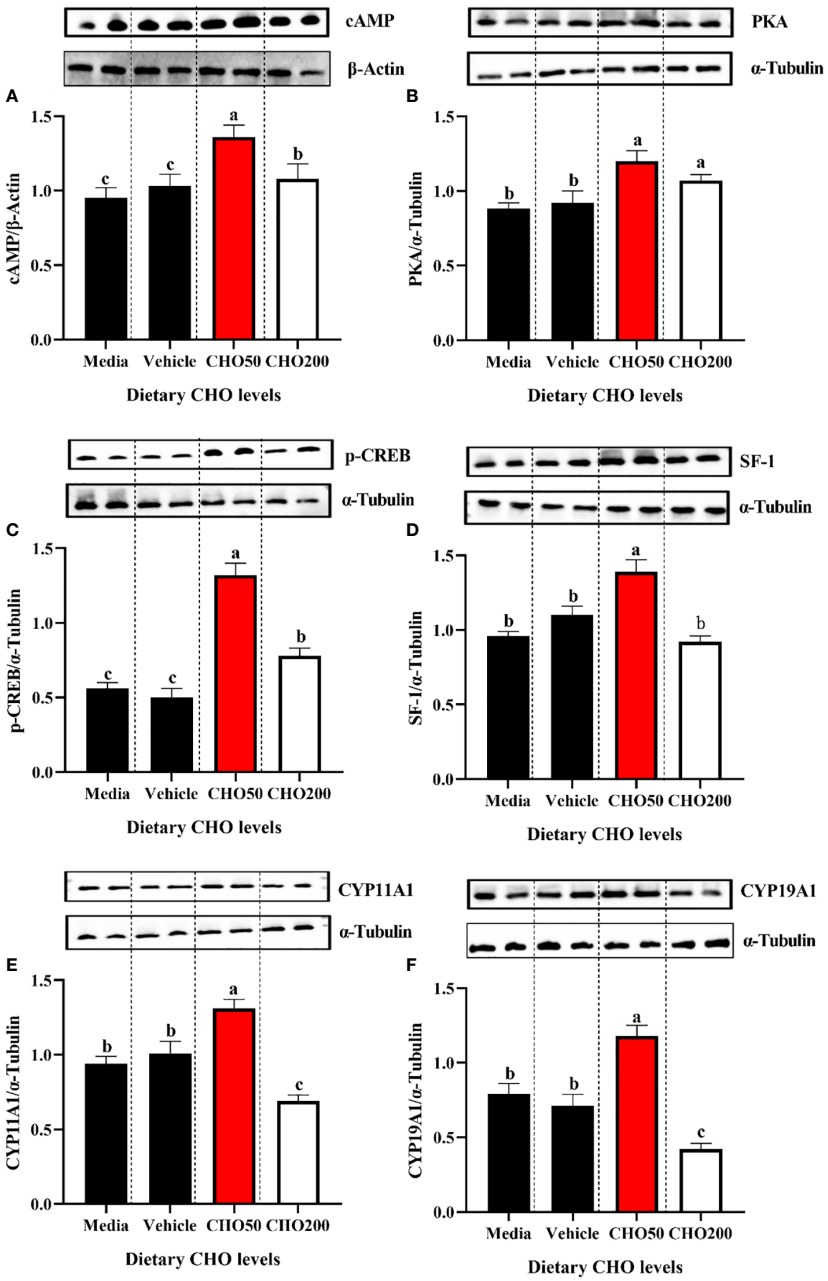

FIGURE 7
The protein expression of **(A)** cAMP; **(B)** PKA, **(C)** p-CREB, **(D)** SF-1, **(E)** CYP11A1 and **(F)** CYP19A1 in the ovary exposed to 0, 50 and 200 mg/L CHO. The values are the Means ± SEM (n = 4). Different letters indicate significant differences ($P < 0.05$).

VTG in the ovary. According to this, this study speculated that the reason why adding an appropriate amount of CHO makes oocytes grow actively by promoting the deposition of VTG in ovaries (Xue et al., 1987; Zheng et al., 2020).

In terms of nutrition, the development of ovary in crustaceans is actually a process of nutrient accumulation, of which the main nutrient is Vtg (Yano and Hoshino, 2006; Matozzo et al., 2008). In the process of vitellogenesis, Vtg can provide nutrients such as lipids, protein, vitamins and mineral salts for oocytes to meet the needs for ovarian development. Therefore, the content of Vtg is usually considered as an important indicator for judging the ovarian development of crustacean (Meusy, 1980; Subramoniam, 2011). The results of the present study showed that the addition of 0.4% CHO could significantly promote the transcription level of *vtg* and *vgr* and thus increase the content of Vtg in the ovary, while when the

amount of CHO reached 1.6%, it no longer had promoting effect, which was consistent with the results of protein expression, and further confirmed that the appropriate amount of CHO could promote the ovarian development of *Eriocheir sinensis* by promoting the accumulation of VTG. We speculated that there might be two reasons for this result. On the one hand, CHO, as an important component of Vtg, is crucial to the formation of VTG (Okuno et al., 2002). Previous studies have reported that CHO in hemolymph decreases rapidly during vitellogenesis in female ghost crab and *Litopenaeus Vannamei*, at the same time the content of CHO in the ovary increases significantly, which may be due to the organism transporting CHO to the ovaries to compensate for CHO depletion (Teshima et al., 1986; Vinagre et al., 2007). On the other hand, as a substrate for synthetic E_2, CHO may promote Vtg synthesis and transport by affecting the synthesis of endogenous hormones (Kumar et al., 2018). Although there is no report on CHO promoting ovarian development of *Eriocheir sinensis* at present, the promoting effect of estrogen on vitellogenesis of crustaceans has been demonstrated. Warrier et al. (2001) found a positive correlation between the level of E_2 in hemolymph of crustacean and the stage of vitellogenesis. Yano and Hoshino (2006) reported that exogenous E_2 could promote ovarian development in *Litopenaeus vannamei* and *Portunus trituberculatus* by *in vivo* injection. Therefore, we believe that the appropriate amount of CHO can promote the synthesis of VTG, then promote the transport of various nutrients to the ovary, and finally achieve the goal of promoting ovarian maturation.

The development and maturity of oocytes require the coordinated regulation of various signal pathways in the cells, in which the activation of MPF is the central link to initiate oocyte maturation (Gavet and Pines, 2010). As a key substance to promote oocyte maturation, MPF mainly exists in mitotic cells and is highly conservative among different species. The dephosphorylation of CDC2 and the recruitment of Cyclin B are two important factors to maintain the activity of MPF (Bodart et al., 2002). CDC2 contains three phosphorylation sites: Thr-14, Tyr-15 and Thr-161. The dephosphorylation of Thr-14 and Thr-15 is the prerequisite for activating MPF. In the cell cycle, CDC2 through phosphorylation and dephosphorylation, combined with Cyclin B to regulate the activity of MPF (Pirino et al., 2009; Feng et al., 2020). In this study, 0.4% CHO treatment group has the highest ovarian maturity,and the protein expression levels of Cyclin B and CDC2 are significantly higher than those in the control group. Previous study on *Penaeus monodon* has found that estrogen can increase the expression of Cyclin B, promote the dephosphorylation of Thr-14 and Thr-15, and then induce the combination of cyclin B and dephosphorylated CDC2 to transform oocytes from G2 phase to M phase (Loukaci et al., 2001; Pirino et al., 2009). In this experiment, after 0.4% CHO treatment, the level of E_2 in the organism was significantly increased. Therefore, we speculate that CHO may promote ovarian maturity by promoting E_2 synthesis. Besides MPF, MAPK also plays an irreplaceable role in the regulation of oocyte cycle. MAPK belongs to serine/threonine protein kinase family, ERK1/2 is one of the subtribes that is mainly responsible for the recovery of meiosis (Fan et al., 2009). Previous studies have pointed out that the maturation promoting effect of MAPK is to enhance the activity of MPF and then promote the germinal vesicle breakdown (GVBD), because the activation of MAPK is highly consistent with the synthesis of Cyclin B in time (Frank-Vaillant et al., 2001; Dupré et al., 2011). After MAPK is activated, the translation of Cyclin B increases immediately, MAPK can positively regulate the translation and synthesis of Cyclin B, and stabilize the activity of MPF by inhibiting the degradation of Cyclin B (Frank-Vaillant et al., 2001; Maller et al., 2001). The results of this study show that compared with the other two groups, 0.4% CHO can significantly increase the protein expression of MEK1/2. which is a specific protein kinase. MEK1/2 is a specific protein kinase, which uses MAPK as the only substrate and catalyzes the phosphorylation of threonine and tyrosine, thus ensuring the activation of MAPK. Activated MAPK can enter the nucleus and regulate the expression of cell cycle-related proteins such as Cyclin B and CDC2 (Gotoh and Nishida, 1995; Kishimoto, 2018). Zheng et al. (2020) reported that estrogen could promote the activation of MAPK signal pathway in the ovary of *Eriocheir sinensis*. Therefore, an appropriate amount of CHO may promote E_2 synthesis, then induce the activation of the MAPK signal pathway, and cooperate with MPF to start ovarian maturation (Ohashi et al., 2003; Nagahama and Yamashita, 2008).

As a substrate for the synthesis of E_2, CHO must be transported from the storage site to the ovary through hemolymph circulation in the organism (Rodenburg and Van der Horst, 2005). In crustaceans, lipid transport through the hemolymph is accomplished by HDL (Komatsu and Ando, 1998). HDL combines with CHO in peripheral tissues to form high-density lipoprotein CHO (HDL-C), which is transported to the ovary through hemolymph. Previous studies have reported that exogenous chemicals can reduce the content of HDL in hemolymph, then affect the content of CHO transported to the ovary, thus interfere with the synthesis of E_2 (Sharpe et al., 2007; Reading et al., 2014). SR-B1 is the first HDL receptor to be identified, and it is an important substance to maintain the homeostasis of CHO metabolism in the organism. When SR-B1 gene is knocked out in mouse, it will lead to the disorder of CHO metabolism, and the female will be infertile (Kolmakova et al., 2010). In this experiment, 0.4% CHO can significantly increase the content of total CHO in the ovary, and when the amount of CHO reached 1.6%, the content of CHO in the ovary shows a downward trend, which may be due to the inhibition of SR-B1 expression by high doses CHO, thereby reducing the endocytosis of CHO (Ji et al., 1997). Due to the synthesis of E_2 on the inner mitochondrial membrane, CHO in oocytes needs to be

transported to the inner mitochondrial membrane by STAR to start the E_2 synthesis pathway, this process which is a speed limit step for E_2 synthesis (Jefcoate et al., 1992). STAR is a transporter of CHO, which is mainly involved in the absorption and metabolism of CHO. The damage of STAR will lead to E_2 synthesis paths being blocked, thus the level of E_2 in the organism will drop sharply (Stocco, 2000). In addition, the expression level of STAR is easily affected, for example, exposing goldfish to 200 μg/g β-sitosterol will significantly reduce the transcription level of STAR, thus reducing the content of CHO in the mitochondrial inner membrane (Sharpe et al., 2007). The results of this experiment also found that an appropriate amount of CHO could promote the transcription of STAR, while high doses of CHO would block the transcription of STAR. In short, an appropriate amount of CHO can promote the expression of proteins such as SR-B1 and STAR to provide sufficient raw materials for the synthesis of E_2.

The conversion of CHO to E2 requires the catalysis of a series of enzymes, among which CYP11A1 and CYP19A1, two members of CYP450 family, are key enzymes (Ankley et al., 2012). CYP11A1 through cleaving the side chain of CHO, catalytic generated a precursor of steroid hormones. CYP19A1 is another rate-limiting enzyme in E_2 synthesis, which is responsible for the conversion of testosterone to E_2 (Hu et al., 2010). Ankley et al. (2012) found that the addition of non-specific inhibitors of CYP450 can significantly reduce the activity of different CYPs in the E_2 synthesis pathway, of which CYP11A1 and CYP19A1 are the primary targets. In this experiment, 0.4% CHO can significantly increase the activity of CYP11A1 and CYP19A1, thus increasing the level of estradiol in the organism. The activity regulation of CYP11A1 and CYP19A1 depends on cAMP/PKA signal transduction pathway. When the cAMP/PKA signal pathway is activated, it can regulate the expression of steroidogenic enzymes related to transcription factors such as SF-1 and CREB (Lapointe and Boerboom, 2011; Manna and Stocco, 2011). It has been confirmed that CREB can regulate the activity of CYP19A1 through phosphorylation and dephosphorylation of serine at position 133. SF-1 can activate the expression of all CYPs involved in steroid hormone production, and SF-1 is also involved in regulating CHO transport in steroid-producing cells. Therefore, SF-1 is the key regulatory factor of CYP11A1, CYP19A1, STAR and other substances (Huang et al., 2022). Zheng et al. (2020) reported that the addition of icariin can regulate the expression level of p-CREB and SF-1, thereby affecting estradiol synthesis in *Eriocheir sinensis*. In this study, the results of *in vivo* and *in vitro* experiments showed that after appropriate CHO treatment, the cAMP/PKA signaling pathway was activated, and the protein expression levels of p-CREB and SF-1 were significantly increased. Therefore, we speculate that appropriate CHO can regulate the activity of key enzymes in the process of steroid hormone production through cAMP-PKA-CREB/SF-1 signaling pathway, and then affect the secretion of E_2 in the organism.

Conclusion

In conclusion, the results of this study show that 0.4% CHO in the diet can promote the gene and protein expression of VTG in the ovary and hepatopancreas, thus promoting the accumulation of nutrients. On the other hand, an appropriate amount of CHO can induce the activation of the MAPK signal pathway, improve the activity of MPF, promote the completion of meiosis of oocytes, and then promote the ovarian maturation of *Eriocheir sinensis*. Further studies have found that an

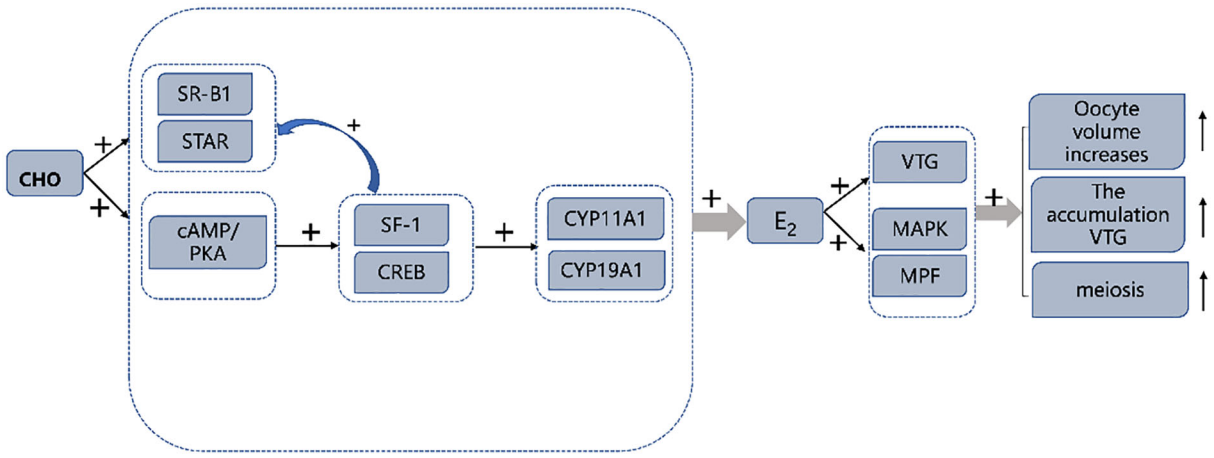

FIGURE 8
Graphical summary of CHO promotes ovarian maturation in *Eriocheir sinensis*. "+" symbol: expression was up-regulated by CHO.

appropriate amount of CHO can increase the activity of E_2 synthesis-related enzymes such as CYP11A1 and CYP19A1 *in vivo* and *in vitro* by increasing the expression of cAMP/PKA pathway related-proteins to increase the level of E_2 in the organism, which may be the main reason for the improvement of ovarian maturity of *Eriocheir sinensis* (Figure 8).

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, KM244725.1.

Ethics statement

The animal study was reviewed and approved by the Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, China) (permit number: SYXK (Su) 2011–0036).

Author contributions

HG, GJ and WL designed the experiment. HG and MW completed the breeding experiment. XW and KX assisted in data analysis. HG drafted the manuscript. KA, WX, HH and YH polished the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effect of *Schizochytrium limacinum* supplementation to a low fish-meal diet on growth performance, lipid metabolism, apoptosis, autophagy and intestinal histology of *Litopenaeus vannamei*

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In this experiment, we aimed to evaluate the relationship between the addition of *Schizochytrium limacinum* to low fish meal diets on growth performance, apoptosis, autophagy, lipid metabolism, and intestinal health of *Lipenaeus vanamei*. The diet containing 25% fish meal was used as a positive control (FM) and the other three diets contained 15% fish meal and were supplemented with 0, 0.3, and 0.6% *S. Limacinum* (LF, LFLD, LFHD). The shrimp (0.22 ± 0.00 g) were divided into four replicates of 40 shrimp per tank and fed four times daily to apparent satiation for 8 weeks. Results showed that the final weight (FBW) and weight gain rate (WGR) of shrimp fed FM and LFHD diets were significantly increased compared to those fed the LFLD diet ($P<0.05$), and there was no significant difference in survival rate (SR) and feed conversion rate (FCR) among the groups ($P>0.05$). Supplementation of *S. Limacinum* in low fish meal diets had no effects on shrimp body composition ($P<0.05$). There were significant differences ($P<0.05$) in low-density lipoprotein (LDL-C) glucose (GLU), triglycerides (TG), and total cholesterol (TC) in the hemolymph of shrimp fed the LF diet compared to those fed the LFLD and LFHD diets. HE staining and transmission electron microscopy (TEM) results showed that the microvilli height, mucosal folds height, mucosal folds width and muscle layer thickness in the intestine of shrimp fed the LF diet were significantly reduced compared to those fed the other three diets ($P<0.05$). Swelling of the endoplasmic reticulum and irregular mitochondria in the gut of shrimp fed the LF diet was also observed by TEM, and the endoplasmic reticulum and mitochondria of shrimp fed the LFHD diet returned to a healthy state. Hepatopancreas genes expression results were showed that the gene expression of 5′ -AMP-activated

protein kinase (*ampk*), stearoyl-CoA desaturase (*scd1*), acetyl-CoA carboxylase 1 (*acc1*), and malonyl-CoA decarboxylase (*mcd*) of shrimp fed the LF diet was significantly increased compared to those fed the FM diet ($P<0.05$). The gene expression of sterol regulatory element-binding protein (*srbep*) and carnitine palmitoyl transferase 1 (*cpt-1*) of shrimp fed the LFLD diet was significantly increased compared to those fed the LF diet ($P<0.05$). The gene expression of acc1, mcd and scd1 of shrimp fed the LFHD diet was significantly reduced compared to those fed the LF diet ($P<0.05$). Results of genes expression associated with apoptosis in the hepatopancreas showed that the gene expression of B lymphocytoma-2 (*bcl-2*), BCL2 associated X apoptosis regulator (*bax*) and cysteinyl aspartate specific proteinase 8 (*caspase 8*) of shrimp fed the LF diet was significantly reduced compared to those fed the FM diet ($P<0.05$). The gene expression of bcl-2 of shrimp fed the LFHD diet was significantly reduced compared to those fed the LF diet ($P<0.05$). Genes related to autophagy in the hepatopancreas showed that the expression of autophagy-related protein 12 (*atg 12*), autophagy-related protein 13 (*atg 13*) and beclin1 of shrimp fed LF the diet was significantly reduced compared to those fed the FM diet ($P<0.05$). The gene expression of *atg 12* and *atg 13* of shrimp fed the LFHD diet was significantly increased compared to those fed the LF diet ($P<0.05$). In summary, reducing fish meal is detrimental to the growth performance and intestinal health of shrimp, and 0.6% S. *Limacinum* supplementation can improve the growth performance, promotes hepatopancreas lipid metabolism, reduces apoptosis, promotes autophagy and improve intestinal health of *Litopenaeus vannamei*.

KEYWORDS

***Litopenaeus vannamei*, soy protein concentrate, *Schizochytrium limacinum*, lipid metabolism, apoptosis, autophagy, intestinal health**

Introduction

Litopenaeus vannamei has the characteristics of fast growth, strong disease resistance, and delicious taste, which has important economic value, and the annual production has up to 5.8 million tons in 2020 (FAO, 2022; Xu et al., 2022a). Fish meal is rich in amino acids, vitamins, and minerals which are necessary for the growth of fish, shrimp, and crab, and has a special flavor that makes it good palatability (An et al., 2018). Nonetheless, the high cost of fish meal has increased the cost of feed Previous studies found that fish meal in aquatic animal feed can be substituted by different protein sources, such as soy protein peptide (Lin et al., 2022), soy protein concentrate (Zhu et al., 2020), fermented soy pulp (Kari et al., 2022), hydrolyzed fish protein powder (Hlordzi et al., 2022), bacterial protein meal (Chen et al., 2021b), concentrated dephenolization cottonseed protein (Zhao et al., 2021), blood meal (Kirimi et al., 2016), black soldier fly (Huang et al., 2022), meat and bone meal (Ai et al., 2006), and hydrolyzed feather meal (Campos et al., 2017). Plant proteins are widely available and inexpensive, which were favored by researchers for partial replacement of fish meal in aquatic animal feed (Liao et al., 2022). However, plant protein generally has the disadvantages of amino acid imbalance and high content of anti-nutritional factors (Jannathulla et al., 2019), and adding excessive plant protein can also affect the growth performance, intestinal microecology, and nutrition metabolism of aquatic animals (Ray et al., 2020). In our previous study, we found that the essential nutrient balance in low fish meal diets improved growth properties and intestinal fitness of shrimp (Xie et al., 2016; Xie et al., 2020c).

Fish meal contains high levels of fish oil, which is rich in n-3 long-chain polyunsaturated fatty acids, especially for docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Cho and Kim, 2011). Consequently, the content of high-unsaturated fatty acids decreases accordingly in the low fish meal diets. Polyunsaturated fatty acids are essential fatty acids for fish and crustaceans, which cannot be synthesized *in vivo* and must be obtained from food (Dyall et al., 2022). DHA is a polyunsaturated fatty acid, which has various functional roles in regulating the physiological health of the body and has important regulatory influences on growth, inflammatory response, development, immune regulation, and antioxidation in aquatic animals (Ruyter et al., 2022). DHA is an essential fatty acid for many fish, shrimp, and shellfish larvae, previous study

proved that dietary supplementation of DHA in the low fish meal diet could improve the immune system response and intestinal fitness of *Penaeus monodon* (Xie et al., 2020b).

Schizochytrium Limacinum (*S. Limacinum*), a type of marine algae, is rich in the highly unsaturated fatty acid DHA (Li et al., 2018). In contrast to other algae, *S. Limacinum* is produced by employing heterotrophic propagation and was regarded as microalgae with the potential to produce DHA in an industrial chain (Sarker et al., 2016; Osmond et al., 2021). Numerous studies have been conducted to demonstrate the improvement of the growth performance of aquatic animals by feeding diets containing *S. Limacinum*, such as *Litopenaeus vannamei* (Wang et al., 2017), *Salmo Salar* (Sprague et al., 2015), *Ictalurus punctatus* (Li et al., 2009), *Danio rerio* (Byreddy et al., 2019), *Trachinotus Ovatus* (Xie et al., 2019), *Epinephelus Lanceolatus* (García-Ortega et al., 2016). In addition, there have been several studies showing that dietary supplementation of *S. Limacinum* be significantly enhanced the non-specific immunity of *Trachinotus Ovatus* (Xie et al., 2019) and improve the intestinal health of *Oreochromis niloticus* (Souza et al., 2020) and *Oncorhynchus mykiss* (Lyons et al., 2016). The results of another study suggest that *S. Limacinum* can be a promising low-level substitute for fish meals, which could improve the fillet texture of largemouth bass (Liao et al., 2022).

Few studies have been reported on the effect of *S. Limacinum* addition in the low fish meal diet of *Litopenaeus vannamei*. Thus, the intention of this experiment was to assess the effects of low fish meal diet supplementation with *S. Limacinum* on growth performance, hemolymph biochemistry, intestinal health, lipid metabolism, apoptosis, and autophagy of *Litopenaeus vannamei*.

Materials and methods

Diet preparation

Two diets with different levels of the fish meal were formulated as the positive control (FM) and negative control (LF), and two levels of *S. Limacinum* were added to the LF, which were labeled as LFLD and LFHD. The nutritional composition of the four diets was shown in Table 1. Ingredients were crushed and passed through an 60 mesh sieve, weighed accurately, and blended well (M-256, South China University of Technology, Guangzhou), next were stirred well with pre-weighed distilled water, fish oil, soybean oil, soy lecithin, and pre-configured coated crystal amino acids as described by Xie et al. (Xie et al., 2020c). The 1.0 mm and 1.5 mm feeds were extruded by a twin-screw extruder (F-26, South China University of Technology, Guangzhou), followed by being heated in an oven at 90°C for 60 min and air-drying at room temperature, the diets were storage at -20°C until use (Li W. et al., 2022).

Experimental shrimp and management conditions

Juvenile *Litopenaeus Vannamei* were purchased from Zhanjiang Yuehai Seed Co. The shrimp was transferred to a pre-disinfected specimen pond for one month and acclimatized for one week to the experimental conditions before the start of the experiment. Then 640 healthy shrimp with similar body sizes (0.22 ± 0.00g) were randomly assigned to 16 fiberglass tanks (500 L) with 40 shrimp per tank. The shrimp were periodically fed four times a day (7:00, 11:00, 16:00, 21:00) for 8 weeks. During the time of trial, water temperature and salinity were to be measured daily, with the water temperature at 28.0-30.0°C, salinity at 26-30‰, pH at 7.6-8.1, and ammonia nitrogen level below 0.05 mg/L (He et al., 2017). In addition, each tank was aerated to ensure enough oxygen, and the water was changed by approximately 60% at 2h after the first feeding every day.

Sample collection and analysis

Growth indexes

At the end of 8 weeks of feeding, shrimp were starved and treated for 24 h before being anesthetized using MS-222 (Zhou et al., 2019; Xu et al., 2021). Samples were then collected, and shrimp in each tank was counted and weighed to determine the initial weight (IBW), final weight (FBW), survival rate (SR), feed conversion rate (FCR), and weight gain rate (WGR). Immediately after weighing, 10 shrimp were randomly selected from each tank, and blood was taken using a 1 mL sterile syringe, and placed in a 1.5 mL sterile centrifuge tube. The hemolymph was gathered by centrifugation at 1500 x g for 10 min at 4°C through a benchtop high-speed frozen centrifuge (JIDI-20R, Guangzhou JIDI Instruments Ltd. formula). Six shrimps were randomly detected from each tank, which were stored at -20°C for the shrimp body composition analysis using standard methods (Feldsine et al., 2002). Moisture was determined in a constant-weight oven at 105°C. The content of crude protein and Crude lipid were determined using the Kjeldahl method (Kjeltec^{TM}8400, Sweden) and the Soxhlet extraction method (extractant petroleum ether), respectively, according to the description of (Zhang et al., 2018; Liu et al., 2021).

Hemolymph biochemical indexes and enzyme activity analysis

Hemolymph biochemical indicators were measured using kits developed by Nanjing Jiancheng Institute of Biology (China), the content of high-density lipoprotein (HDL-C),

TABLE 1 Formulation and proximate composition of experimental diets (% dry matter).

Ingredient	Treatments			
	FM	LF	LFLD	LFHD
Fish meal	25	15	15	15
Soybean meal	25	25	25	25
Peanut meal	12	12	12	12
Soy protein concentrate	0	10	10	10
Flour	20	20	20	20
Brewer's yeast	2	2	2	2
Shrimp shell powder	2	2	2	2
Chicken Powder	3	3	3	3
Fish Oil	1.5	2.3	2.3	2.3
Soybean Oil	2	1.9	1.9	1.9
Choline	0.2	0.25	0.25	0.25
Soy lecithin	1	1	1	1
Vitamin and mineral premixes [a]	1	1	1	1
Calcium dihydrogen phosphate	1	2	2	2
Vitamin C	0.1	0.1	0.1	0.1
Micro Nutrients Mix	0	0.05665	0.05665	0.05665
Microcrystalline cellulose [c]	3.2	0.65	0.35	0.05
L selenomethionine type II [b]	0	0.007	0.007	0.007
Sodium carboxymethyl cellulose	1	1	1	1
Docosahexaenoic acid	0	0	0.3	0.6
Proximate composition				
Dry matter				
Crude protein	40.02	40.57	40.84	40.62
Crude lipid	7.61	7.55	7.41	7.53

[a]Vitamin and Mineral Premix ((kg) 1 of diet): thiamine, 5 mg; riboflavin, 10 mg; vitamin A,5000 IU; vitamin E, 40 mg; vitamin D3, 1000 IU; menadione, 10 mg; pyridoxine, 10 mg; biotin, 0.1 mg; cyanocobalamin, 0.02 mg; calcium pantothenate, 20 mg; folic acid, 1 mg; niacin, 40 mg; vitamin C, 150 mg; iron, 100 mg; iodine, 0.8 mg; cupper, 3 mg; zinc, 50 mg; manganese, 12 mg; selenium, 0.3 mg; cobalt, 0.2 mg.
[b]L selenomethionine type II Sichuan New Yimei Biotechnology Co., Ltd, Selenium Power II (L-selenomethionine ≥ 0.5%, selenium ≥ 0.2%)
[c]Micro Nutrients Mix (kg^{-1} of wet weight diet): threonine, 0.5g; glycine, 1g; alanine, 1.5g; methionine, 1.5g; lysine, 2g; γ-aminobutyric acid, 0.2g; taurine, 0.6g; ornithine, 0.12g; phytase, 0.4g; vitamin B2, 1mg; vitamin B12, 10mg; niacin, 10mg; ferrous Glycinate, 60mg; zinc amino acid complexes, 30mg; purchased from Shanghai Aladdin Biochemical Technology Co. and Guangzhou Chengyi Aquaculture Co.

low-density lipoprotein (LDL-C), glucose (GLU), triglyceride (TG), total protein (TP), and total cholesterol (TC), and the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) were measured using a full-wavelength enzyme marker (Thermo, Multiskan GO 1510). The commercial kit IDs were as follows: A112-1-1, A113-1-1, A154-1-1, A110-1-1, A045-2, A111-1, C009-2-1, and C009-2-1, respectively. The methods of the kit were tested strictly according to the description of (Gui et al., 2019; Wu et al., 2021).

Quantitative real time PCR analysis

Total RNA was obtained from the hepatopancreas and intestine of *Litopenaeus vannamei* using the TransZol Up Plus RNA kit (Transgen, China), and the extracted RNA was assayed by NanoDrop2000 (Thermo USA), and the massification of RNA was determined by the A260/280 ratio (Zhu et al., 2021; Zhang et al., 2022a). Reverse transcription was performed using the PrimeScriptTM RT kit (Takara, Japan) according to the manufacturer's instructions. Oligo dt (18T) primer random 6 mers, PrimeScriptTM RT enzyme mixture I, 5× PrimeScriptTM buffer, and RNase-free water were used to reverse transcribe 1000 ng of RNA at 37°C for 15 min, followed by inactivation at 85°C for 5 s (Li Y. et al., 2022; Xu et al., 2022c).

The polymerase chain reaction was performed using SYBR® Green Premix Pro Taq HS qPCR Kit II (Accurate Biotechnology (Hunan) Co., Ltd.) and the system was quantified fluorescently on a LightCycler 480 (Roche Applied Science) according to the set procedure for 10 The fluorescence quantification of the samples was performed on a LightCycler 480 (Roche Applied Science) according to a set procedure. The 10 μl system consisted of 0.5 μM of forward and reverse specific primers, 5 μl of 2× SYBR® Green Pro Taq HS Premix II, 10 ng of cDNA

template and RNase-free water (Chi et al., 2017). The denaturation step lasted for the 30s at 95°C, followed by 40 amplification cycles, denaturation at 95°C for 5s, and annealing at 60°C for 30s before analysis of the solubility curves. The relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method using *ef-1α* as the internal reference gene (Chen et al., 2021a; Wang et al., 2022). (The information of the primers used in this study was shown in Table 2).

Intestinal tissue analysis

Intestine of four shrimp was randomly selected from each tank for hematoxylin-eosin (H&E) stain and transmission electron microscopy (TEM) analysis. The intestine was stored in Bouin's solution for 24 h and then dehydrated in 75% ethanol. Then the tissue was dehydrated and washed with xylene, The samples were cleaned in toluene followed by embedding in paraffin to make solid wax blocks. A rotary microtome was used to cut the solid wax blocks into transverse section blocks into 5 μm sections followed by mounting on the slide and staining with hematoxylin-eosin (H&E). The slides were observed under a microscope (Olympus, BX51, Tokyo, Japan) and the built-in software was used to measure the thickness of the muscle layer, the height of the mucosal fold, and the width of the mucosal fold. TEM was performed as the method described before (Xie et al., 2018): intestines were fixed with 2.5% glutaraldehyde for 2h, washed and fixed with osmium acid for 3h, dehydrated and embedded with resin, and ultrathin sections were made of resin blocks, followed by staining with saturated uranyl acetic acid solution for 30 min and distilled water washing followed by lead citrate for 30 min. Finally, the cytoarchitecture was observed by TEM (Hitachi HT7700 TEM, Japan) and the length of microvilli was measured with Image-Pro Plus 6.3 software (Media Cybernetics, Inc., Rockville, USA).

TABLE 2 Primers used for quantitative real-time PCR.

Gene name	Sequence of primer (5′ -3′)	sources
caspase3 F	ACATTTCTGGGCGGAACACC	AGL61582.1
caspase3 R	GTGACACCCGTGCTTGTACA	
caspase8 F	CACGGAAGCTCTCCCTACAG	(Yin et al., 2021)
caspase8 R	GAAGACCTTGGGTTTCCCCC	
bcl-2 F	CCTTGCTTGACACAGTCGGA	(Yin et al., 2021)
bcl-2 R	CAGACAAGGTCGTGAGGTGG	
bax F	GGTGGAATCACAAGAGAGCGA	(Yin et al., 2021)
bax R	TGTTCTCCACGGTGTCTCAC	
atg13 F	GAGACTTTTTACCGCTTCGC	XM_027375959.1
atg13 R	ATCCTGCTGGACCTCTATGG	
atg12 F	GAGAAGGAGAAAACTGCGAC	XM_0273638831
atg12 R	CTACCCAACCTACTGGCTTC	
beclin1 F	CTGTCTGAGGTGGAGGCTGA	MH797016.1
beclin1 R	ATGTGGAAGGTGGTGTTGAA	
ampk F	TCAGAGGAGGAGCAGGAAC	KP272117.1
ampk R	CCCGAGGTCTAATAGGCAC	
srbep F	ACTGAGCTCAACACCTTCCG	MG770374·1
srbep R	TGCTGGTGAAGAGCTGTCTG	
acc1 F	TGCATAGAAACGGCATTGCG	XM_027360190·1
acc1 R	TTTGACACCTGAGCCAGACC	
mcd F	AAGACCACAGGAAGGGACCA	XM_027376735·1
mcd R	GACACTTGAGATGCCACCCA	
fas F	CAGGTGGAGATGCTCCTCGTGTT	HM595630.1
fas R	GGTGACTAGCTCGGCTACATGGTT	
cpt-1 F	CAACTTCTACGGCACTGAT	XM_027361886.1
cpt-1 R	GTCGGTCCACCAATCTTC	
scd1 F	TGTCTTACACCTTATCAATGGC	XM_027374708·1
scd1 R	CGTTCGTATGTTCCTCTTCGTC	
ef-1α F	GTATTGGAACAGTGCCCGTG	JF288785.1
ef-1α R	ACCAGGGACAGCCTCAGTAAG	

caspase 3, cysteinyl aspartate specific proteinase 3; caspase 8, cysteinyl aspartate specific proteinase 3; bcl-2, B lymphocytoma-2; bax, BCL2 associated X apoptosis regulator; Atg13, autophagy-related protein 13; atg12, autophagy related protein 12; ampk, 5′ -AMP-activated protein kinase; srebp, sterol regulatory element-binding protein; acc1, acetyl-CoA carboxylase 1; mcd, malonyl-CoA decarboxylase; fas, fatty acid synthase; cpt-1, carnitine palmitoyl transferase 1; scd1, stearoyl-CoA desaturase; ef-1α, elongation factor 1α.

Calculations and statistical analysis

These statistics are obtained as follows:

$$\text{Survival rate (SR, \%)} = \frac{\text{final number of shrimp}}{\text{initial number of shrimp}} \times 100$$

$$\text{Weight gain rate (WGR, \%)} = \frac{\text{final body weight} - \text{initial body weight}}{\text{initial body weight}} \times 100$$

$$\text{Feed conversion rate (FCR)} = \frac{\text{feed consumed (g)}}{\text{final body weight} - \text{initial body weight}}$$

The results are expressed as mean ± SEM. There was a one-way ANOVA for all data and a Duncan's multiple tests was performed using SPSS 21.0 to identify significant between treatment differences. The probability value of $P<0.05$ is statistically significant and indicates a significant difference in the results.

Result

Growth performance

The results in Table 3 show that the FBW and WGR of shrimp fed the LFLD diet were significantly lower than those fed the FM diet ($P < 0.05$), dietary supplementation of 0.6% *S. Limacinum* significantly increased the growth of shrimp ($P<0.05$). The SR and FCR of shrimp were similar among the four groups.

Analysis of whole shrimp body composition

The results in Table 4 show the whole shrimp body composition.

Hemolymph biochemical indexes

As shown in Table 5, the HDL-C content of shrimp fed the LF diet was significantly increased compared to those fed the FM and LFHD diets ($P < 0.05$). The LDL-C content of shrimp fed the LF and FM diets were significantly increased compared to those fed the LFLD and LFHD diets ($P < 0.05$). The GLU content of shrimp fed the LFLD and LFHD diets were significantly lower than those fed the FM and LF diets ($P < 0.05$). The TG content in the hemolymph of shrimp fed the LF diet was significantly lower than those fed the LFDH diet ($P < 0.05$), and the TG content of shrimp fed the LFLD diet was significantly lower than those fed the other diets ($P < 0.05$). AST and ALT activity in the hemolymph of shrimp fed the LF diet were significantly increased compared to those fed the other diets ($P < 0.05$). TC levels in the hemolymph of shrimp fed the FM diet were significantly increased compared to those fed the other diets ($P < 0.05$).

The expression levels of the lipid metabolism, apoptosis, and autophagy-related genes

Lipid metabolism-related gene expression levels were shown in Figure 1. The gene expression of *ampk* of shrimp fed the LFLD

TABLE 3 Effect of low fish meal diet supplemented with *Schizochytrium limacinum* on the growth performance of *Litopenaeus vannamei*.

Index	FM	LF	LFLD	LFHD
IBW	0.22 ± 0.00	0.22 ± 0.00	0.22 ± 0.00	0.22 ± 0.00
FBW	4.67 ± 0.23^{b}	4.55 ± 0.04^{ab}	4.30 ± 0.21^{a}	4.59 ± 0.10^{b}
SR	79.38 ± 3.75	76.88 ± 5.15	83.13 ± 5.15	77.50 ± 6.77
WGR	2104.77 ± 9.71^{c}	1986.33 ± 4.74^{ab}	1930.01 ± 29.19^{a}	2014.02 ± 21.33^{b}
FCR	1.94 ± 0.06	1.96 ± 0.05	2.06 ± 0.01	2.03 ± 0.02

Values in the table are the mean of four replicates of treatment and are expressed as mean ± SEM (n=4). Values in the same row with different superscript letters are significantly different (P<0.05).
Where: FM, high fish meal diet; LF, low fishmeal diet; LFLD, LF supplemented with 0.3% S. Limacinum; LFHD, LF supplemented with 0.6% S. Limacinum; IBW, initial body weight (g); FBW, final body weight (g); SR, survival rate (%); WGR, weight gain rate (%); FCR, Feed conversion rate.

TABLE 4 Effect of low fish meal diet supplemented with *Schizochytrium limacinum* on the body composition of *Litopenaeus vannamei*.

Index	FM	LF	LFLD	LFHD
Moisture (%)	75.28 ± 0.36	74.90 ± 0.86	76.63 ± 2.11	76.27 ± 0.49
Crude lipid (%)	3.81 ± 1.39	3.74 ± 1.13	3.32 ± 1.66	3.48 ± 1.45
Crude protein (%)	17.66 ± 3.07	17.31 ± 1.45	16.51 ± 1.67	16.49 ± 0.90

Values in the table are the mean of four replicates of treatment and are expressed as mean ± SEM (n=4). Values in the same row with different superscript letters are significantly different (P<0.05).
Where: FM, high fish meal diet; LF, low fishmeal diet; LFLD, LF supplemented with 0.3% S. Limacinum; LFHD, LF supplemented with 0.6% S. Limacinum.

TABLE 5 Effect of low fish meal diet supplemented with *Schizochytrium limacinum* on hemolymph biochemical parameters of *Litopenaeus vannamei*.

Index	FM	LF	LFLD	LFHD
HDL-C (mmol L^{-1})	0.40 ± 0.01^{bc}	0.32 ± 0.01^{a}	0.36 ± 0.02^{ab}	0.45 ± 0.01^{c}
LDL-C (mmol L^{-1})	1.72 ± 0.24^{c}	0.95 ± 0.05^{b}	0.45 ± 0.03^{a}	0.67 ± 0.10^{a}
GLU (mmol L^{-1})	1.60 ± 0.07^{b}	1.53 ± 0.03^{b}	1.24 ± 0.09^{a}	1.35 ± 0.06^{a}
TG (mmol L^{-1})	2.00 ± 0.22^{bc}	1.73 ± 0.11^{b}	1.23 ± 0.12^{a}	2.29 ± 0.24^{c}
AST (U L^{-1})	33.23 ± 1.25^{c}	36.96 ± 0.37^{d}	29.65 ± 0.12^{b}	26.92 ± 0.72^{a}
ALT (U L^{-1})	36.11 ± 0.11^{a}	44.18 ± 0.55^{c}	39.52 ± 0.41^{b}	35.17 ± 0.55^{a}
TC (mmol L^{-1})	1.84 ± 0.06^{d}	1.39 ± 0.13^{c}	0.66 ± 0.05^{a}	0.86 ± 0.14^{b}

Values in the table are the mean of four replicates of treatment and are expressed as mean ± SEM (n=4). Values in the same row with different superscript letters are significantly different ($P<0.05$).
Where: FM, high fish meal diet; LF, low fishmeal diet; LFLD, LF supplemented with 0.3% S. Limacinum; LFHD, LF supplemented with 0.6% S. Limacinum.

diet was significantly increased compared to those fed the FM and LFHD diets ($P<0.05$). The gene expression of *srbep* was significantly higher in shrimp fed the LFLD and LFHD diets than those fed the FM and LF diets, which is decreased with the dietary *S. Limacinum* supplementation ($P<0.05$). The gene expression of *acc1* and *scd1* of shrimp fed the LFLD and LFHD diets were significantly increased compared to those fed the FM diet, which is decreased with the dietary *S. Limacinum* supplementation ($P<0.05$). The gene expression of *mcd* and *cpt-1* of shrimp fed the LFLD diet was significantly increased compared to those fed the FM and LFHD diets ($P<0.05$). the gene expression of *fas* of shrimp fed the LFLD and LFHD diets were significantly reduced compared to those fed the FM and LF diets ($P<0.05$).

Apoptosis and autophagy-related gene expression levels were shown in Figure 2. The gene expression of *bax* and *caspase 8* of shrimp fed the other three diets were significantly reduced compared to those fed the FM diet ($P<0.05$). The gene expression of *caspase 3* of shrimp fed the LF diet was significantly reduced compared to those fed the other three diets ($P<0.05$). The gene expression of *bcl-2* of shrimp fed the LF and LFHD diets were significantly reduced compared to those fed the FM diet ($P<0.05$). The gene expression of *beclin1* of shrimp fed the other three diets were significantly reduced compared to those fed the FM diet ($P<0.05$). The gene expression of *atg 12* of shrimp fed the LFLD and LFHD diets were significantly reduced compared to those fed the FM diet, which is decreased with the dietary *S. Limacinum* supplementation ($P<0.05$). The gene expression of *atg 13* of shrimp fed the LF and LFLD diets was significantly reduced compared to those fed the FM and LFHD diets ($P<0.05$).

Intestinal histology

The statistical results of the intestinal histology are shown in Table 6. The microvilli height, mucosal fold height, mucosal fold width, and muscle layer thickness of shrimp fed the LF diet were significantly reduced compared to those fed the other diets ($P<0.05$). The mucosal fold height, mucosal fold width, and muscle layer thickness of shrimp fed the LFLD diet were higher than those fed the other diets. As shown in Figure 3, TEM results showed that with the decrease in dietary fish meal levels, the endoplasmic reticulum and mitochondria of shrimp fed the LF diet were found to be swollen, in which the mitochondrial matrix became irregularly arranged and tended to dissolve. After the supplementation of 0.3% *S. Limacinum* in the low fish meal diet, the endoplasmic reticulum recovered to its original state, but the mitochondria were irregular and the mitochondrial matrix was marginalized. After supplementation with 0.6% *S. Limacinum*, the mitochondria recovered to the level of fish meal.

Discussion

SPC has been proven as a high-quality protein source to replace fish meal protein in the feed of a wide range of aquatic animal species (Paripatananont et al., 2001; Chen et al., 2019; Zhang et al., 2022b). Several researches have pointed out that satisfactory growth and feed utilization were obtained in juvenile cobia (El-Saidy and Gaber, 2003) and juvenile starry flounder (Li et al., 2015) when dietary SPC inclusion was below 60%, and even further increased SPC content in the diet can cause lower dietary efficiency and higher death rates in fish. However, Zhao et al. showed that the SR and SGR of Nile tilapia were not affected even if the fish meal was totally replaced with SPC (Salze et al., 2010; Zhao et al., 2010). Earlier studies reported that it was possible to reduce the dietary fish meal content from 20% to 5% with SPC without negatively affecting the growth of *Litopenaeus vannamei* (Ray et al., 2020). The results of Paripatananont et al. showed that 50% substitution of fish meal with SPC in the diet of *Penaeus monodon* could support the normal growth of shrimp (Paripatananont et al., 2001), which was similar to the results of the current research. Results of this trial showed that the FBW of *Litopenaeus vannamei* was not influenced when the dietary fish meal content was reduced from 25% to 15%, after

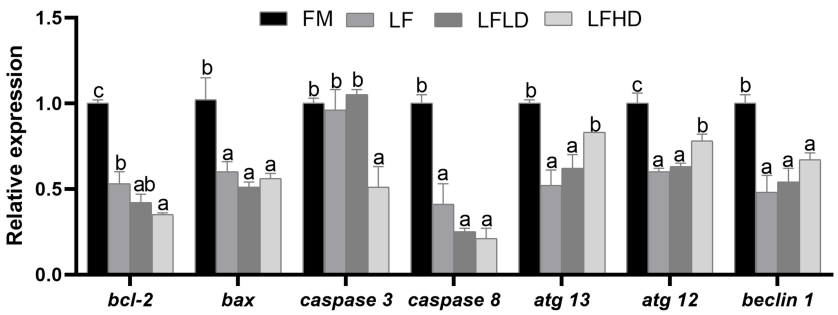

FIGURE 1
Effect of low fish meal diet supplemented with *Schizochytrium Limacinum* on the expression levels of hepatopancreas lipid metabolism-related genes in *Litopenaeus vannamei*. Vertical bars represent the mean ± SEM (n = 5). Data marked with letters differ significantly (P < 0.05) among groups. Where: FM, high fish meal diet; LF, low fishmeal diet; LFLD, LF supplemented with 0.3% *S. Limacinum*; LFHD, LF supplemented with 0.6% *S. Limacinum*.

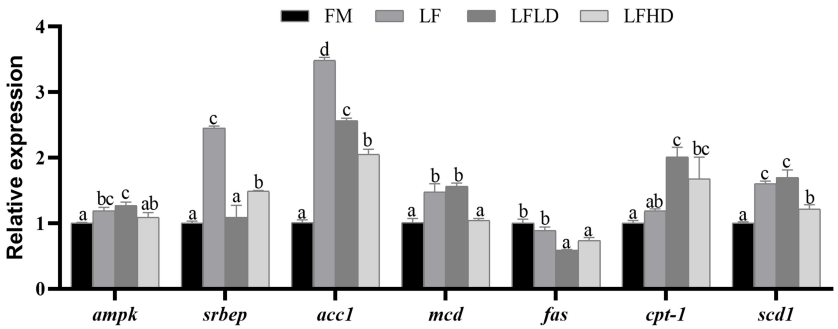

FIGURE 2
Effect of low fish meal diet supplemented with *Schizochytrium Limacinum* on the expression levels of hepatopancreas apoptosis and autophagy-related genes in *Litopenaeus vannamei*. Vertical bars represent the mean ± SEM (n = 5). Data marked with letters differ significantly (P < 0.05) among groups. Where: FM, high fish meal diet; LF, low fishmeal diet; LFLD, LF supplemented with 0.3% *S. Limacinum*; LFHD, LF supplemented with 0.6% *S. Limacinum*.

TABLE 6 Effect of low fish meal diet supplemented with *Schizochytrium limacinum* on the intestinal tissues of *Litopenaeus vannamei*.

Parameters	Diets			
	FM	LF	LFLD	LFHD
Microvilli height (μm)	2.54 ± 0.01^{d}	1.46 ± 0.01^{a}	1.67 ± 0.04^{b}	2.26 ± 0.08^{c}
Mucosal folds height (μm)	57.45 ± 3.68^{b}	41.60 ± 2.73^{a}	89.91 ± 4.35^{c}	76.01 ± 6.42^{c}
Mucosal folds width (μm)	49.88 ± 3.20^{bc}	36.18 ± 1.92^{a}	53.18 ± 1.06^{c}	45.76 ± 1.45^{b}
Muscle layer thickness (μm)	56.21 ± 3.21^{b}	36.80 ± 2.09^{a}	83.47 ± 2.86^{c}	78.71 ± 9.51^{c}

Values in the table are the mean of six replicates of treatment and are expressed as mean ± SEM (n=6). Values in the same row with different superscript letters are significantly different (P<0.05).
Where: FM, high fish meal diet; LF, low fishmeal diet; LFLD, LF supplemented with 0.3% S. Limacinum; LFHD, LF supplemented with 0.6% S. Limacinum.

amino acids, micronutrients, and fish oil was supplemented in the low fish meal diet to balance the nutritional profile. Several similar studies have also been done on *Epinephelus lanceolatus*, *Salmon*, and *Seriola rivoliana* (Ai et al., 2006; Perez-Velazquez et al., 2018; Katerina et al., 2020). Some studies reported that dietary supplementation with 4% *S. Limacinum* improved the growth performance of *Litopenaeus vannamei* (Wang et al., 2016), 3% *S. Limacinum* improved the growth performance of *Trachinotus ovatus* (Xie et al., 2019). Interestingly, (Xie et al., 2020b) found that 0.75% *S. Limacinum* supplementation in a low fish meal diet

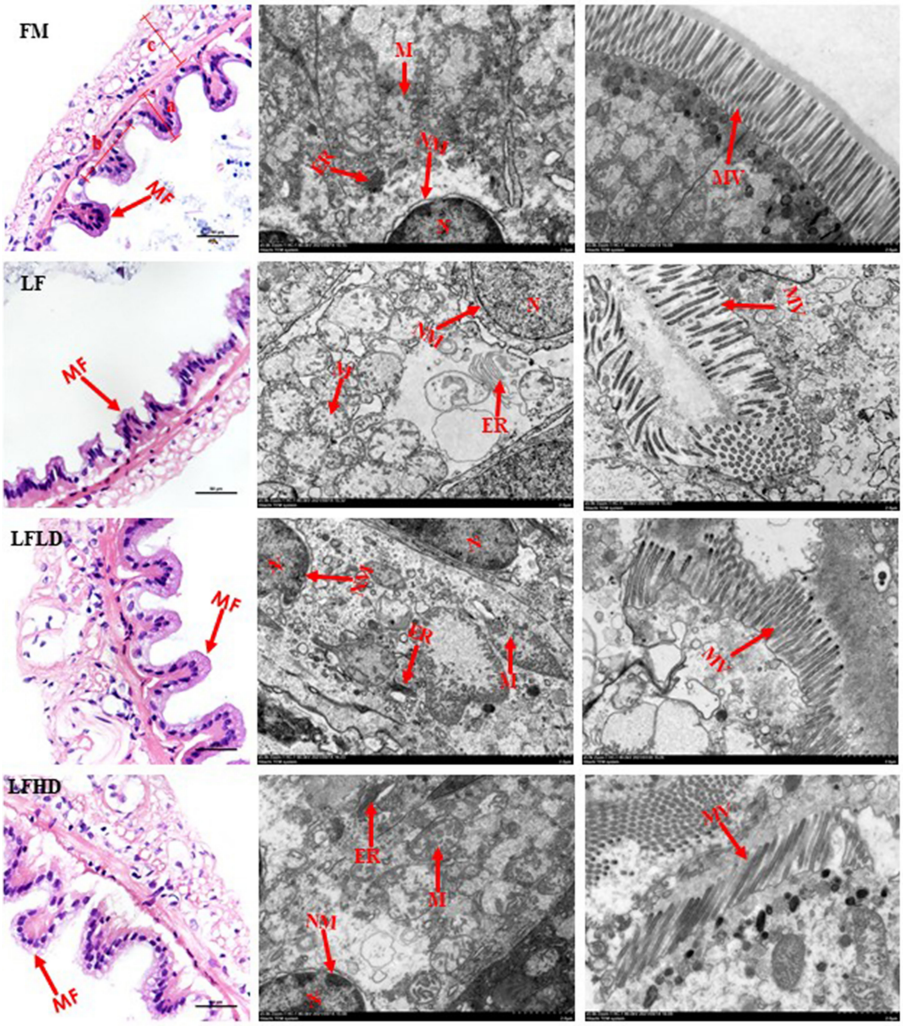

FIGURE 3
Effect of low fish meal diet supplemented with *Schizochytrium limacinum* on the histology of the midgut of *Litopenaeus vannamei*. Where: FM, high fish meal diet; LF, low fishmeal diet; LFLD, LF supplemented with 0.3% *S. Limacinum*; LFHD, LF supplemented with 0.6% *S. Limacinum*. MF, mucosal folds; a, width of mucosal folds; b, height of mucosal folds; c, thickness of the annular sarcolemma; MV, microvilli; ER, endoplasmic reticulum; N, nucleus; NM, nuclear membrane; M, mitochondria.

improved the growth performance of *Penaeus monodon*. High levels of nutrients in *S. Limacinum* such as DHA, docosapentaenoic acid (EPA), and carotenoids may contribute to the growth of animals (Xie et al., 2019). However, some studies have also found that supplementation with high levels of *S. Limacinum* is detrimental to the growth performance of *Litopenaeus vannamei* and blunt snout bream (Wang et al., 2016; Wang C. et al., 2020). Our results showed that the supplementation of 0.6% *S. Limacinum* had a positive effect on the FBW and WGR of shrimp.

Hemolymph is important for fat absorption and transport, and TG is transported as an energy substance between adipose tissue and the liver *via* hemolymph (Gyan et al., 2021). The fitness of aquatic animals which can be assessed using blood parameters (Lemaire et al., 1991). Several studies have shown that SPC substitution for fish meal affects blood indicators (Zhang et al., 2019; Wang J. et al., 2020; Zhang Q. et al., 2021). TG and TC are important indexes of lipid sedimentation in animals. LDL-C is responsible for transporting liver cholesterol to tissue cells throughout the body, and HDL-C is responsible for transporting excess cholesterol from blood or tissues to the liver pancreas (Yepiz-Plascencia et al., 2000; Hamilton-Reeves et al., 2007). In the present trial, the levels of TC and TG in the hemolymph of *Litopenaeus vannamei* fed the LFLD diet were significantly reduced, and when the addition level of *S. Limacinum* increased to 0.6%, the levels of TC and TG increased significantly. Studies have shown that unsaturated fatty acids are effective in lowering hemolymph

cholesterol levels (Pentieva et al., 2003) and high levels of dietary unsaturated fatty acids prevent triglycerides from penetrating the lipoprotein particles of the liver, resulting in lower levels of TG secreted by hepatocytes into the hemolymph (Yu et al., 2012). According to (Zhang X. et al., 2021), diets supplemented with 0.5%-2.0% *S. Limacinum* significantly increased the hemolymph TG content of *Litopenaeus vannamei* and had no significant effect on the TC content, which was similar to the present study supplemented with 0.6% *S. Limacinum* but the TC content first decreased significantly and then increased. Hemolymph GLU is the most important energy substance in the hemolymph and is the direct source of energy required for all types of activities in aquatic animals (Boonanuntanasarn et al., 2016). Supplementation of *S. Limacinum* under the present experimental conditions decreased the hemolymph GLU content of shrimp, a result that is inconsistent with the results of studies on *Pelodiscus sinensis* (Zhang X. et al., 2021), possibly because of the species and possibly because of the enhanced energy consumption of the substance metabolism. HDL-C and LDL-C are able to be transported in shrimp for lipids (Yepiz-Plascencia et al., 2000). Whereas HDL-C removes TC from the blood and sedimentation in the liver, which is then excreted from the animal, LDL-C plays an important part in the immune system of shrimp (Yue et al., 2012; Chen et al., 2018). The current research showed a decrease in HDL-C and LDL-C levels and a significant decrease in HDL-C in the hemolymph of *Litopenaeeus vannamei* fed the LF diet, which is different from the results hemolymph in *Micropterus salmoides* fed low-level fish meal diet supplemented with 4% *S. Limacinum* (Liao et al., 2022). Reasons for this may be the differences in diet composition, species, and amount of *S. Limacinum* supplementation. AST and ALT activity are important health parameters of liver function in invertebrates (Song et al., 2018). AST and ALT are the two most important transaminases in the body and are generally present in the liver (Zhou et al., 2013; Liu et al., 2019). When the liver is damaged, the AST and ALT stored in the liver will be transferred to the blood (Barcellos et al., 2004). In the current research, the AST and ALT activities in the hemolymph of *Litopenaeus vannamei* fed the LFLD and LFHD diets were significantly reduced, a result consistent with the fact that dietary supplementation with 0.8%-1.2% *S. Limacinum* reduced the blood AST/ALT ratio of *Cyprinus carpio* var. Jian (Liu et al., 2016) and dietary supplementation with 1% *S. Limacinum* reduced the AST and ALT activities in the blood of *Labidochromis caeruleus* (Cui et al., 2018). These results indicated that the supplementation of *S. Limacinum* in the diet can decrease liver injury in aquatic animals.

Due to the altered hemolymph biochemical parameters in shrimp, we further investigated the effect of low fish meal diet supplementation with *S. Limacinum* on the expression of genes related to apoptosis, lipid metabolism, and autophagy in *Litopenaeus vannamei*. Lipid metabolism refers to the process of fat synthesis and catabolism, the digestion of fat, which is subject to the action of a variety of enzymes and bile (Serrano et al., 2021; Su et al., 2022). Lipid metabolism is mainly in the liver, and research has found that DHA can regulate the molecular mechanism of lipid metabolism and promote hepatocytes to stimulate the synthesis of lipoprotein lipase to further promote lipid metabolism (Morabito et al., 2019; Deragon et al., 2021). *Ampk* is a modulator of energy metabolism in cells. Under low energy regulation, *ampk* inhibits TG synthesis and activates the β-oxidation process of fatty acids to produce more ATP (Gaidhu et al., 2010; Wang et al., 2018; Xu et al., 2022b). The findings of this research revealed that the gene expression of *ampk* and *cpt-1* in shrimp fed the LF diet showed an increasing trend compared to those fed the FM diet but the gene expression of *ampk* and *cpt-1* increased and then decreased after supplementation with *S. Limacinum*, which indicated an increase in energy production from lipolysis. The gene expression of *fas* in shrimp fed the LFLD and LFHD diets was significantly lower than those fed the FM diet, and the gene expression of *acc1* and *cpt-1* in shrimp fed the LFLD and LFHD diets was significantly higher and then significantly lower than those fed the FM diet, suggesting a decrease in lipid synthesis after supplementation with *S. Limacinum*. The combined effect of lipid synthesis genes and lipolysis genes resulted in reduced lipid deposition. *Srebp* is a major regulator of cholesterol and fatty acids and a critical gene in lipid synthesis. (Eberle et al., 2004; Kamisuki et al., 2009). From the experimental results, shrimp fed the high SPC increased the expression of genes related to lipid synthesis, probably due to the ability of soy protein amphiphilic globulin to adsorb lipids (Lusas and Riaz, 1995). It may also be due to DHA's inherent property of lowering lipids (Horrocks and Yeo, 1999). Studies in human cardiovascular disease have found that DHA reduces total blood cholesterol and triglyceride levels (Mozaffarian and Wu, 2011), and in mice, DHA has been found to reduce the size of fat cells and lower body fat levels (Lu et al., 2015). Dietary supplementation with *S. Limacinum* decreased the expression of genes related to lipid synthesis, suggesting that *S. Limacinum* can reduce lipid synthesis. This is similar to the results of Zhu et al. (Zhu et al., 2013) which indicated that supplementation of *S. Limacinum* in the diet promoted lipid metabolism and inhibited fat deposition.

Apoptosis is divided into the endogenous mitochondrial pathway, the endoplasmic reticulum stress pathway, and the exogenous receptor apoptosis pathway (Sitarek et al., 2022). In the endogenous mitochondrial pathway, *bcl-2* family proteins (anti-apoptotic protein *bcl-2* and pro-apoptotic protein *bax*) control outer mitochondrial membrane permeability by regulating mitochondrial membrane potential (Green, 2022). *Bax* is normally found in the cytoplasm and when it receives an endogenous apoptotic signal, it relocates to the surface of mitochondria and constitutes a trans-mitochondrial membrane pore, which releases apoptotic factors. It has been suggested that the opening of the membrane pore causes a decrease in membrane potential and also leads to mitochondrial swelling and deformation (Chipuk et al., 2012).

Caspase 3 is also one of the downstream effectors of the endogenous pathway (Samali et al., 1999). It has been shown that a decrease in mitochondrial membrane potential leads to an upregulation of the *bax*/*bcl-2* ratio and promotes *caspase 3* activation (Zorova et al., 2018). In addition, *caspase 8* also activates *caspase 3* directly or indirectly in exogenous signal-regulated pathways (Bridgham et al., 2003). The results of the current research showed that supplementation with 0.6% *S. Limacinum* appeared to reduce the expression of *caspase 3* and thus inhibit the apoptosis in the hepatopancreas of shrimp; the expression of *caspase 8* was also significantly reduced and thus inhibit apoptosis. Dietary supplementation with 0.6% *S. Limacinum* activated the expression of *bcl-2* in the hepatopancreas of shrimp to further inhibit apoptosis. It has been suggested that autophagy eliminates damaged proteins and damaged organelles from the body (Tesseraud et al., 2021). Cellular autophagy is a highly conserved metabolic process that degrades its components through lysosomes to maintain cellular homeostasis and plays an important role in degrading damaged organelles, resisting pathogenic infections, and regulating inflammatory responses (Deretic, 2021). The process of cellular autophagy includes the formation of segmented membranes, the formation of autophagosomes, membrane fusion of autophagosomes with lysosomes, and the digestion of inclusions by autophagosomes (Cao et al., 2021). *Atg 13* and *beclin1* are participating in the startup of autophagic bubbles (Kabeya et al., 2005; Hosokawa et al., 2009), and *atg 12* is responsible for the formation of autophagosomes (Radoshevich et al., 2010). In this experiment, dietary supplementation of 0.6% *S. Limacinum* significantly increased hepatopancreas *atg 12* and *atg 13* gene expression in shrimp, and it is hypothesized that *S. Limacinum* has an effect of promoting autophagy. Researches have shown that hepatic autophagy promotes glycolipid metabolism and protein turnover (Song et al., 2010). Several studies have shown that autophagy is involved in intracellular nonspecific immune responses, that an appropriate numbers of autophagy is a self-protective function driving cell survival (Shi and Kehrl, 2008), and *S. Limacinum* could activate autophagy-related gene expression to improve the immune response in shrimp.

Intestinal morphology and structure are important for nutritional intake and sustain normal intestinal function (Gao et al., 2013; Vizcaíno et al., 2014), and intestinal morphology is associated with shrimp health (Tang et al., 2009). The height of the mucosal fold and the width of the mucosal folds can be used as a measure of the functional characteristics of the intestinal walls. (Emami et al., 2012). Muscle layer thickness also plays an essential role in the metabolic digestion and absorptivity of the intestine, and increasing the thickness of the muscles can increase the absorptivity and metabolic digestion of nutrients in the intestine (Chen et al., 2021b). The results of this research showed that *S. Limacinum* supplementation in low fish meal diets improved the intestinal morphology and structure of *Litopenaeus vannamei*, with an increase in mucosal folds length, mucosal folds width, and muscle layer thickness. Meanwhile, the TEM results showed that the intestinal microvilli height of shrimp fed the LFLD and LFHD diets were significantly increased compared to those fed the LF diet, indicating that dietary supplementation of *S. Limacinum* improved the intestinal microvilli structures. However, this result is contrary to previous studies, which showed that dietary supplementation with 1.2% *S. Limacinum* did not affect intestinal microvilli structure in Nile tilapia, possibly due to the different amounts of *S. Limacinum* supplementation and interspecific differences (Souza et al., 2020). The TEM results also showed that shrimp fed a LF diet showed endoplasmic reticulum stress, irregular mitochondrial ridges, and significant swelling in the intestine, which was improved when the diet was supplemented with *S. Limacinum*, and the intestinal mitochondria and endoplasmic reticulum recovered well when supplemented with 0.6% *S. Limacinum*. Earlier researches have demonstrated that reducing fish meal levels can damage the intestinal epithelial structures (Xie et al., 2018) and upregulate the gene expression associated with endoplasmic reticulum stress, which leads to the severity of endoplasmic reticulum stress in the intestine (Xie et al., 2020a). Some studies have reported that dietary supplementation with 3% and 6% defatted *S. Limacinum* can increase intestinal villi height, but excessive levels can impede nutrient absorption (Xiao et al., 2021). Supplementation with 0.75% *S. Limacinum* was found to improve intestinal health and enhance immunity in *Penaeus monodon* (Xie et al., 2020b). The low fish meal diet supplemented with 0.6% *S. Limacinum* in this study not only promoted the early development of the intestinal tract of *Litopenaeus vannamei* but also improved the digestive capacity of the intestine, improved the endoplasmic reticulum and mitochondrial structure, further improving the intestinal health and contributing to the healthy growth of shrimp.

Conclusion

The present study revealed that supplementation with 0.6% *S. Limacinum* in the low fish meal diet levels improved the growth performance, reduced hepatopancreatic cell apoptosis, promoted autophagy, and improved intestinal health in shrimp.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

XY and SX designed the experiments. XY carried out the experiments and drafted the manuscript. BT was accountable for

some aspects (such as ingredients and sites) of the work in ensuring that experiments can be carried out properly. YiL, MS, LC, KQ and YuL were analyzed for data. SX reviewed and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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A synthetic peptide based on large yellow croaker (*Larimichthys crocea*) IFNG1R protein sequence has potential antimicrobial activity against *Pseudomonas plecoglossicida*

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The overuse of antibiotics leads to the emergence of bacterial resistance, which poses a serious threat to aquaculture. Antimicrobial peptides (AMPs) display excellent antimicrobial activity and are thought to be the most feasible replacements for antibiotics. The usage of AMPs as feed additives has great application prospects in aquaculture. In this study, large yellow croaker interferon-γ related gene (IFNG1R) was cloned, and a 17-amino acids (aa) short peptide named SKL17-2 was synthesized based on its protein sequence. The synthesized SKL17-2 peptide possessed a strong antimicrobial activity against *Pseudomonas plecoglossicida*, which could cause visceral white nodules disease (VWND) in cultured marine fish, with a minimum inhibitory concentration (MIC) of 2 μM. SKL17-2 peptide also showed weak antimicrobial activities against other tested bacteria, indicating its narrow-spectrum antimicrobial activity. This suggested that SKL17-2 peptide may not kill probiotics in intestinal flora when used as the feed additive. Furthermore, SKL17-2 had broad temperature and pH stability, low cytotoxicity, and negligible hemolysis, indicating its good biosafety and stability. Mechanistically, the synthesized SKL17-2 peptide can form α-helical structure in a membrane environment and destroy *P. plecoglossicida* through membrane disruption. Thus, our data showed that SKL17-2 peptide may represent a potential feed additive used for prevention and treatment of VWND.

KEYWORDS

antimicrobial peptides, IFNG1R, visceral white nodules disease, large yellow croaker (*Larimichthys crocea*), aquaculture

Introduction

Aquaculture has made an important contribution to food security, but bacterial infections pose a serious threat to aquaculture, as they may result in high mortality and reduced productivity (Katzenback, 2015). Antibiotics can be used to prevent bacterial diseases. However, the overuse of antibiotics has hastened the evolution of multi-drug resistant bacteria (Roope et al., 2019). New antimicrobial drugs are urgently needed due to the high cost of conventional antibiotics used in aquaculture and the rise of drug-resistant bacteria.

Antimicrobial peptides (AMPs), also known as host defense peptides (HDPs) (Shafee et al., 2017), have the ability to kill tumor cells, parasites, viruses, fungi, bacteria, and other microorganisms (Zhong et al., 2017). Additionally, AMPs can mediate cell chemotaxis and apoptosis as well as increase cell immunity and promote wound repair (Zhong et al., 2017). AMPs are peptides that are amphiphilic (with more than 40% hydrophobic amino acids), mostly cationic (often between +2 and +10) even though some AMPs in fish shown to be anionic (Lai et al., 2002; Valero et al., 2020), and short (approximately 10 to 60 amino acids) (Rahman et al., 2018; Chaturvedi et al., 2020). Based on their structural characteristics, AMPs can be categorized into four groups, the α-helical, β-sheet, loop, and extended peptides. It is worth noting that most AMPs belong to α-helical and β-sheet peptides (Chaturvedi et al., 2020). The total net positive charge and hydrophobicity of AMPs are critical for their ability to kill bacteria. Although the compositions of gram-negative and gram-positive bacterial membranes differ, lipopolysaccharide (LPS) on the outer membrane of gram-negative bacteria and teichoic acid on the cell wall surface of gram-positive bacteria are anionic, and thus can interact with the net positive charge of AMPs (Strömstedt et al., 2009). It has been demonstrated that raising the overall net positive charge of AMPs improves their binding ability to the surface of bacterial membranes, hence boosting antimicrobial activity (Zhu et al., 2014). The hydrophobicity of AMPs can also alter their antimicrobial action. When AMPs attach to the surface of bacteria, the hydrophobic amino acids interact with the phospholipids in the bacterial cell membrane, thus damaging the cell membrane integrity (Schmidtchen et al., 2014). In general, high hydrophobicity will improve the antimicrobial ability of AMPs, but excessive hydrophobicity may damage the host cell membranes (Wood et al., 2014). AMPs can also kill bacteria *via* non-membrane targeting mechanisms, such as the suppression of nucleic acid and protein production and the reduction of enzymatic activity in bacteria (Brogden et al., 2005).

Based on the membrane and non-membrane targeting mechanisms, AMPs display broad-spectrum antimicrobial activity and low selection of resistance, and are considered as the most feasible replacements for antibiotics (Yasir et al., 2018; Mwangi et al., 2019). Nonetheless, endogenous AMPs have several drawbacks, such as high host toxicity and poor resistance to temperature, pH, and protease (Kim et al., 2014; Anunthawan et al., 2015). Furthermore, the expense of producing large amounts of amino acid residues restricts the therapeutic application of AMPs (Kim et al., 2014; Anunthawan et al., 2015; Huan et al., 2020). In order to overcome these drawbacks, researchers have recently concentrated on developing artificial AMPs with strong antimicrobial activity, low toxicity to host cells, high temperature and pH stability, and low production costs (Huan et al., 2020; Tan et al., 2021). The methods of AMP design include template-based design, site-directed mutation, *de novo* created peptides, computer design, and rational design (Huan et al., 2020). The template-based design technique uses natural protein sequence templates as a starting point, and then prefers and modifies peptide sequence according to the residue types, such as residue charge, polarity or hydrophobicity (Zelezetsky and Tossi, 2006; Pizzo et al., 2018; Yang et al., 2019). After alteration, peptide characteristics including cationicity, amphiphilicity, and hydrophobicity can be systematically adjusted to produce optimal AMPs (Huan et al., 2020). This method can lower the cost of design and synthesis while preserving natural peptide sequence information. For species whose genomes have been sequenced, for instance, the large yellow croaker (*Larimichthys crocea*) (Ao et al., 2015; Mu et al., 2018), novel AMPs may be generated based on the genomic sequences using the template-based design technique.

Pseudomonas plecoglossicida is a gram-negative bacterium that can cause visceral white nodules disease (VWND) in the large yellow croaker (*L. crocea*), orange-spotted grouper (*Epinephelus coioides*), and rainbow trout (*Oncorhynchus mykiss*) (Zhang et al., 2018). This illness, characterized by white nodules in the kidney, liver, and spleen of an infected fish, has resulted in massive economic losses in the cage-cultured large yellow croaker (Zhang et al., 2014). It has been demonstrated that an AMP named β-defensin has bactericidal effects against *P. plecoglossicida* (Li et al., 2021), suggesting that AMP could be used to prevent and cure VWND in fish. It is worth noting that most AMPs can indiscriminately kill pathogenic bacteria and probiotics, thereby disrupting gut flora and destroying the balance between healthy microbiota and the immune system (Eckert et al., 2012; Tan et al., 2021). As a result, AMPs with a narrow-spectrum antimicrobial activity against *P. plecoglossicida* would be ideal antimicrobial drugs against VWND.

In this study, large yellow croaker IFNG1R was cloned, and SKL17-2, a rationally designed peptide using the template-based design technique, was synthesized based on SKL17, a 17-aa peptide with total net positive charge and hydrophobicity existing in IFNG1R protein sequence. The synthesized SKL17-2 peptide possessed a strong antimicrobial activity against *P. plecoglossicida* with a MIC of 2 μM, but weak antimicrobial activity against other tested bacteria, indicating its narrow-

spectrum antimicrobial activity. SKL17-2 was found to have broad temperature and pH stability, low cytotoxicity, and negligible hemolysis. Further researches showed that the α-helical structure of SKL17-2 was crucial for cell membrane disruption and antimicrobial activity against *P. plecoglossicida*.

Materials and methods

Complete cDNA cloning of large yellow croaker IFNG1R

RNA isolation and cDNA synthesis were performed as previously described (Zhang et al., 2022). Based on the gene sequence of IFNG1R identified in the large yellow croaker genomic sequence (GenBank accession number: NC_040020.1), primers (F: CGTTTGTATCGAAGCGGTCC ATT, R: TGATTCATGATTTCTGTTTTTATTCG) were designed. And Eastep Super Total RNA Extraction Kit (Promega) was used to extrat total RNA, first-strand cDNA was synthesized by using Eastep RT Master Mix (Promega), and PCR was then performed to amplify the open reading frame (ORF) of large yellow croaker IFNG1R. PCR products were sequenced at Sangon Biotech Co., Ltd. (Shanghai, China). Subsequently, ClustalW software (Version 1.83) was used to perform the multiple sequence alignments. Protein identification was conducted using the Expert Protein Analysis System (http://www.expasy.org/tools/), and the signal peptide was predicted using the SignalP program (http://www.cbs.dtu.dk/services/SignalP/). Neighbor-joining (NJ) method of the MEGA program (version 11.0.11) was used to construct phylogenetic tree with 1000 bootstrap replicates.

Peptide synthesis

Peptides were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Crude peptide was purified by reverse phase-high performance liquid chromatography (RP-HPLC) to a final purity greater than 95%. The synthetic peptide was stored at −80°C in the form of dry lyophilized powder and resuspended in phosphate buffer saline (PBS) when used.

Bacterial strains

Escherichia coli (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella typhimurium* (ATCC 14028), *Vibrio parahaemolyticus* (ATCC 17802), *Staphylococcus aureus* (ATCC 25923), and *Streptococcus agalactiae* (ATCC 13813) were obtained from American Type Culture Collection (ATCC). *P. plecoglossicida* was isolated from diseased large yellow croaker and preserved in our laboratory (Li et al., 2020), and this strain was found to infect a variety of fish species, such as large yellow croaker, orange-spotted grouper, rainbow trout, ayu (*Plecoglossus altivelis*), pejerrey (*Odontesthes bonariensis*), spotted seabass (*Lateolabrax maculatus*), mandarinfish (*Siniperca chuatsi*), barramundi (*Lates calcarifer*), and zebrafish (*Danio rerio*) (Zhang et al., 2018; Sun et al., 2020). Bacteria were cultivated in tryptic soy broth (TSB), and the nutritional medium for *V. paraheamolyticus* was supplemented with 3% NaCl.

Antimicrobial activity assays

The antimicrobial activity of the peptide was determined using the microdilution method. Bacterial cells were grown to the mid-logarithmic phase before being diluted in TSB to a final concentration of 1×10^5 CFU/mL. Subsequently, 10 μL of synthetic peptide was combined with 90 μL of bacterial solution in a 96-well plate. After incubation for 18 h at 37°C (28°C for *P. plecoglossicida*), the minimum inhibitory concentrations (MICs) were determined at 600 nm using an Infinite M Nano spectrophotometer (Tecan, Switzerland). MICs were defined as the lowest peptide concentration that inhibited more than 95% of bacterial growth. Thereafter, 50 μL of each incubation mixture was transferred to tryptic soy agar (TSA) plates for overnight incubation to confirm the minimum bactericidal concentrations (MBCs). MBCs were defined as the lowest peptide concentration that killed more than 99.9% of the bacterial cells. Antimicrobial activity of SKL17-2 was also measured by a bacterial growth inhibition zone assay. In this assay, 60 μL of peptide with the concentration of 32 μM was spotted onto an TSA plate containing *P. plecoglossicida* (about 10^5 CFU/mL in TSA), and same volume of 10 mM PBS was set as a control. The plate was stored at 28°C for 16 h to observe the inhibition zone.

Kinetics of the peptide's bactericidal activity

P. plecoglossicida was exposed to the peptide at a concentration of 1×MBC, and the cell mixture was collected at various time points, serially diluted, and transferred to TSA plates to determine the survival rate. The PBS-treated *P. plecoglossicida* served as a control. The survival rate of bacteria (% survival) was calculated by dividing the number of peptide-treated cells by the number of PBS-treated cells.

Cytotoxicity and hemolysis of peptide

A CCK-8 assay was conducted to investigate the effect of the peptide on the viability of large yellow croaker macrophages. In

brief, 100 μL of LYC-FM cells (about 2×10^4 cells) were added to 96-well plates and cultivated at 28°C. Twelve hours later, the culture medium was replaced with 100 μL of fresh medium supplemented with a range of concentrations of the peptide. Cells were cultured for 48 h before adding CCK-8 solution (Biosharp, China) to the plates. After approximately 4 h of incubation, the absorbance was measured at 450 nm with the Infinite M Nano spectrophotometer (Tecan, Switzerland). Cells treated with 2% Triton X-100 served as the positive control (A_{100}), whereas untreated cells served as the negative control (A_0). The cell viability rate was calculated using the following formula: Cell viability rate (%) = $(A_{peptide}-A_{100})/(A_0-A_{100})\times100$. The LYC-FM cells were cultured in the same manner as previously described (Zhang et al., 2022).

To assess peptide hemolysis, the quantity of hemoglobin in large yellow croaker red blood cells (RBCs) following peptide treatment was evaluated. In brief, fresh blood cells from large yellow croaker were centrifuged at 500 × *g* for 10 minutes at 4°C, washed three times with PBS, and resuspended in PBS to a final concentration of 1.5×10^8 cells/mL. Then, 120 μL of RBCs were mixed with 80 μL of a series of peptide solutions and incubated for 1 h at 28°C. After centrifuging the cell mixtures at 500 × *g* for 10 minutes, the absorbance of the supernatants was measured at 405 nm with the Infinite M Nano spectrophotometer (Tecan, Switzerland). RBCs treated with 2% Triton X-100 served as the positive control (A_{100}), whereas untreated cells served as the negative control (A_0). The peptide hemolysis percentage was calculated using the following formula: Hemolysis (%) = $(A_{peptide}-A_0)/(A_{100}-A_0)\times100$.

Temperature and pH stability of the peptide

To determine the thermal stability, the peptide was incubated for 30 minutes at 20, 40, 60, 80, or 100°C. To evaluate the pH stability, the peptide was incubated for 4 h in 50 mM glycine-HCl buffer (pH 2.0), 50 mM sodium acetate buffer (pH 4.0), 50 mM 2-(N-morpholino) ethanesulfonic acid-NaOH buffer (pH 6.0), 50 mM Tris-HCl (pH 8.0), or 50 mM glycine-NaOH (pH 10.0). After treatment, 10 μL of the peptide solution was added to 90 μL of *P. plecoglossicida* solution in a 96-well plate, with a final concentration of peptide at 1×MBC. After 18 h of incubation at 28°C, bacterial cells were serially diluted and transferred to TSA plates to measure the survival rate. Bacterial cells treated with PBS served as a control. Bactericidal activity was calculated as 100% survival rate minus the survival rate of each treatment.

Structure measurement

A MOS-500 spectral polarizer (Bio-Logic, Grenoble, France) was used to measure the peptide's circular dichroism (CD) spectra between 190 and 250 nm. The spectra of samples containing 0.2 mg/mL peptide in PBS or membrane environments were examined, with the membrane environment including 60 mM SDS micelles and 100% 2,2,2-trifluoroethanol (TFE). Three independent spectra were scanned, and an average was calculated to obtain three technical replicates. The scanned spectra were then transformed to mean molar ellipticity using the formula: $\theta_M = mdeg{\cdot}M/(l{\cdot}c{\cdot}n)$, where θ_M is the molar ellipticity ($deg{\cdot}cm^2{\cdot}dmol^{-1}$); *mdeg* is the measured ellipticity corrected for the buffer at a given wavelength; *M* is the molar mass of the peptide; *l* is the path length (mm); *c* is the peptide concentration (mg/mL); and *n* is the number of amino acids. The HeliQuest analysis website (https://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParams.py) was used to display the helical wheel projection of SKL17-2.

Membrane integrity testing

To determine bacterial membrane integrity following peptide treatment, 180 μL of *P. plecoglossicida* cells (1×10^5 CFU/mL) of mid-logarithmic phase was combined with 20 μL of peptide at the final concentrations of 4, 8,16, or 32 μM and incubated for 2 h at 28°C. After incubation, a final concentration of 6 μg/mL propidium iodide (PI, Sigma-Aldrich) was added. The influx of PI into bacterial cells was investigated using an Accuri C6 Plus flow cytometer (BD Biosciences) with 10,000 events. The cell-penetrating efficiency was analyzed using the FlowJo software package (Tree Star).

Scanning electron microscopy imaging

The bacterial morphological alterations following peptide treatment were observed using a scanning electron microscopy. Ten microliters of peptide was added to 90 μL of *P. plecoglossicida* cells (1×10^8 CFU/mL in TSB) to a final peptide concentration of 4 μM. After incubation for 2 h at 28°C, the bacteria were fixed, dehydrated, vacuum dried, sputter coated with gold, and observed with a Sigma 300 field emission scanning electron microscopy (ZEISS) at an accelerating voltage of 5 kV. Bacteria treated with the same volume of PBS served as a control.

Statistical analysis

All experiments were carried out on three independent occasions unless otherwise stated. Data are presented as the mean ± standard error of mean (SEM). Statistical comparisons were carried out using one-way ANOVA or t-tests with SPSS software (version 19, IBM). Differences with a value of $P < 0.05$ were considered statistically significant.

Results

Complete cDNA sequence analysis of large yellow croaker IFNG1R

The complete cDNA sequence of large yellow croaker IFNG1R was cloned (GenBank accession number: ON997294) by PCR based on its genomic sequence (GenBank accession number: NC_040020.1). The ORF of IFNG1R consisted of 552 base pairs (bp), which encodes a protein of 183 amino acids (aa) (Figure 1A). According to the multiple sequence alignment results, the large yellow croaker IFNG1R shared the highest identity (about 54.2%) with mandarin fish IFNG1R and the lowest identity (about 5.4%) with common carp IFNG1R (Figure 1B). A phylogenetic tree was constructed to further confirm the identification of the large yellow croaker IFNG1R.

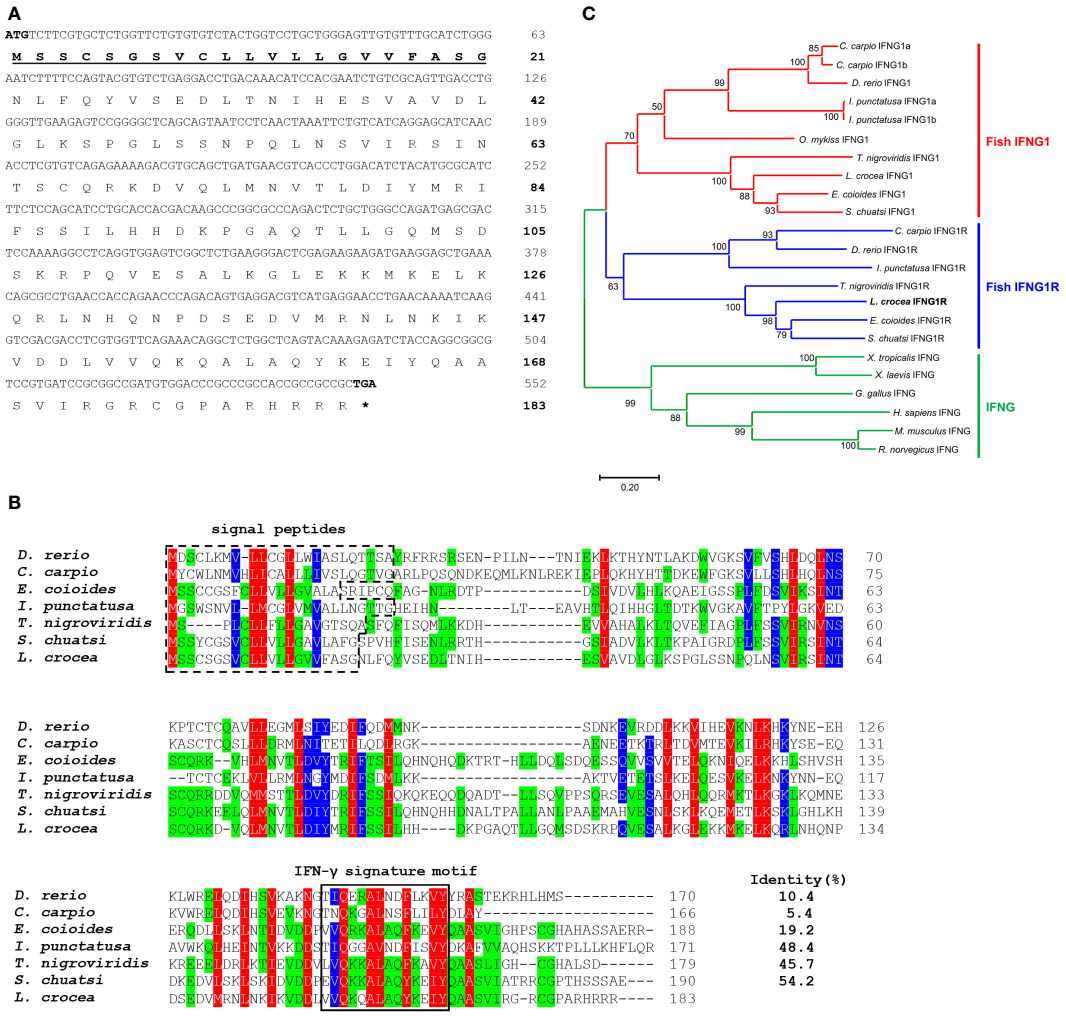

FIGURE 1
Sequence analysis of large yellow croaker IFNG1R molecule. **(A)** ORF and the deduced amino acid sequence of IFNG1R. The predicted signal peptides are in boldface and underlined. **(B)** Multiple sequence alignments of large yellow croaker IFNG1R with selected vertebrate IFNG1R. **(C)** An unrooted phylogenetic tree of vertebrate IFNG1R. Percentage values shown for each node represent 1000 bootstrap replications. In the multiple alignment and tree construction, GenBank accession numbers are as follows: human (*Homo sapiens*) IFNG, NP_000610.2; mouse (*Mus musculus*) IFNG, NP_032363.1; rat (*Rattus norvegicus*) IFNG, NP_620235.1; chicken (*Gallus gallus*) IFNG, NP_990480.1; African clawed frog (*Xenopus laevis*) IFNG, XP_018110090.1; tropical clawed frog (*Xenopus tropicalis*) IFNG, XP_002938555.1; zebrafish (*Danio rerio*) IFNG1, NP_998029.1; IFNG1R, BAD72865.1; common carp (*Cyprinus carpio*) IFNG1a, CAJ51088.1; IFNG1b, CAJ51089.1; IFNG1R, CAJ98867.1; orange-spotted grouper (*Epinephelus coioides*) IFNG1, AFM31242.1; IFNG1R, QEA72089.1; channel catfish (*Ictalurus punctatus*) IFNG1a, AAZ40505.1; IFNG1b, AAZ40506.1; IFNG1R, AAZ40504.1; spotted green pufferfish (*Tetraodon nigroviridis*) IFNG1, AHZ62714.1; IFNG1R, AHZ62713.1; mandarin fish (*Siniperca chuatsi*) IFNG1, QDO15115.1; IFNG1R, QDO15116.1; rainbow trout (*Oncorhynchus mykiss*) IFNG1, CAE82300.1; large yellow croaker (*Larimichthys crocea*) IFNG1, XP_010749999.2; IFNG1R, ON997294.

Large yellow croaker IFNG1R was grouped together with other fish IFNG1R sequence (Figure 1C), indicating that the gene cloned here was large yellow croaker IFNG1R.

SKL17-2 possesses a strong antimicrobial activity against *P. plecoglossicida*

Using large yellow croaker IFNG1R protein sequence as a template, a 17-aa peptide SKL17 was synthesized. Unfortunately, the SKL17 peptide showed no antimicrobial activity against *P. plecoglossicida* (data not shown). Thereafter, a novel peptide known as SKL17-2 was rationally developed by replacing the seventh and twelfth glutamic acids in the SKL17 peptide with arginine (Figure 2A). Interestingly, the resultant SKL17-2 peptide possessed antimicrobial activity against *P. plecoglossicida*, with MIC and MBC values of 2 and 4 μM, respectively (Figure 2B). To more intuitively demonstrate the antimicrobial activity of SKL17-2, the inhibition zone method was utilized. As shown in Figure 2C, a distinct inhibition zone appeared around SKL17-2 compared with the control in the TSA plate. Subsequently, the kinetics of bactericidal activity of SKL17-2 against *P. plecoglossicida* were examined. The results showed that SKL17-2 could kill 90% of *P. plecoglossicida* within 8 h, and all bacteria were eradicated by SKL17-2 after 14 h (Figure 2D).

SKL17-2 possesses weak antimicrobial activity against other tested bacteria

The above results demonstrated that SKL17-2 had a strong antimicrobial activity against *P. plecoglossicida*, and then its activity against other bacteria was further investigated. When the peptide concentration was within 32 μM, SKL17-2 showed negligible antimicrobial activities against *E. coli*, *P. aeruginosa*, *V. parahaemolyticus*, and *S. agalactiae*, and weak antimicrobial activities against *S. typhimurium* and *S. aureus* (Figure 3).

Low cytotoxicity and negligible hemolysis of SKL17-2

The CCK-8 assays revealed that the cell survival rate was greater than 70% following peptide administration, indicating that SKL17-2 was only weakly cytotoxic to LYC-FM cells (Figure 4A). The hemolysis of SKL17-2 towards large yellow croaker RBCs was less than 0.5% at the tested dose, indicating that SKL17-2 had negligible hemolytic activity (Figure 4B). Thus, SKL17-2 exhibited good biosafety, further assuring the security of its clinical application.

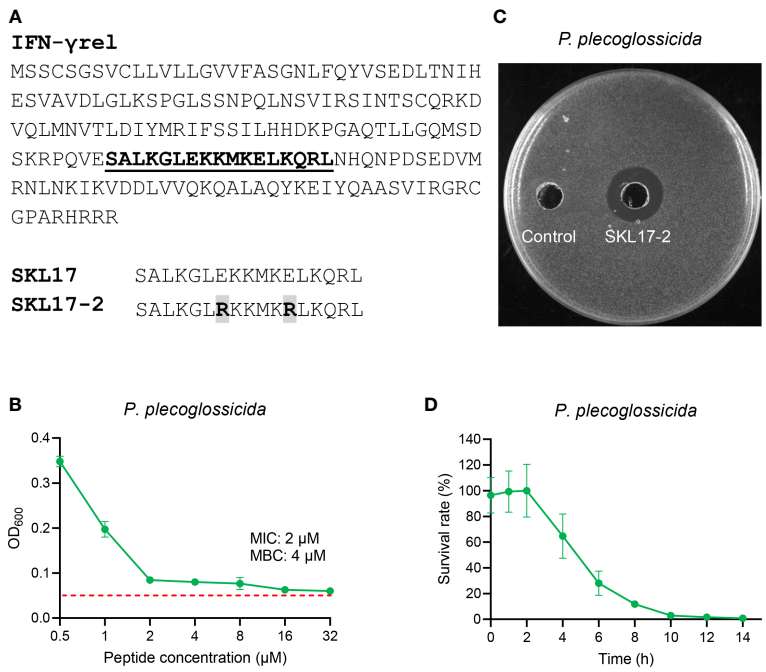

FIGURE 2
Antimicrobial activity of SKL17-2 against *P. plecoglossicida*. **(A)** Peptide sequence of SKL17-2. **(B)** Antimicrobial activity of SKL17-2 against *P. plecoglossicida*. The red dotted line represents the absorbance values of the medium. **(C)** SKL17-2 inhibition zone against *P. plecoglossicida*. **(D)** Time-kill kinetic curves of the SKL17-2 against *P. plecoglossicida* at 1×MBC. The kinetic graphs were the mean values of three independent experiments.

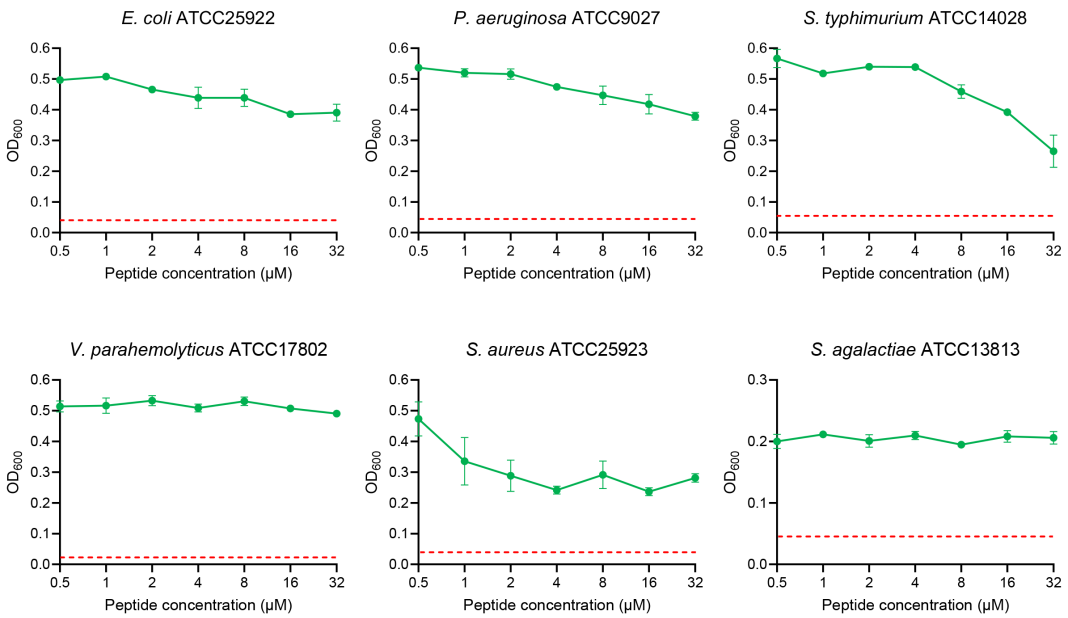

FIGURE 3
Antimicrobial activity of SKL17-2 against *E. coli*, *P. aeruginosa*, *S. typhimurium*, *V. parahemolyticus*, *S. aureus*, and *S. agalactiae*. The red dotted line represents the absorbance values of the medium.

High stability of SKL17-2 to temperature and pH

SKL17-2 displayed strong antimicrobial activity against *P. plecoglossicida*, a clinical pathogen that seriously affects fish aquaculture, thus suggesting its application potential in the treatment of VWND in fish. In practical applications, the stability of SKL17-2 in various conditions of temperature and pH must be considered. The antimicrobial activity of SKL17-2 was unaltered when exposed to 20, 40, 60, 80, or 100°C (Figure 5A). SKL17-2 exhibited robust antimicrobial activity at pH levels ranging from 2 to 10 (Figure 5B). These results indicated that SKL17-2 was well tolerant to different temperature and pH, even extreme conditions of temperature or pH.

Structure analysis of SKL17-2

The secondary structure in the membrane environment is essential for peptide antimicrobial activity, and the structure of SKL17-2 in different solutions was determined using CD spectroscopy. As shown in Figure 6A, the secondary structure of SKL17-2 in PBS was characterized by a coil, with a negative

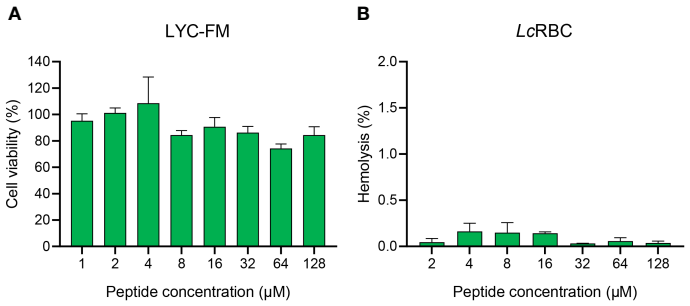

FIGURE 4
Cytotoxicity and hemolytic activity of SKL17-2. **(A)** Cytotoxicity of SKL17-2 against LYC-FM cells. **(B)** Hemolytic activity of SKL17-2 against large yellow croaker RBCs. The data are shown as the mean ± SEM of three independent experiments performed in triplicate.

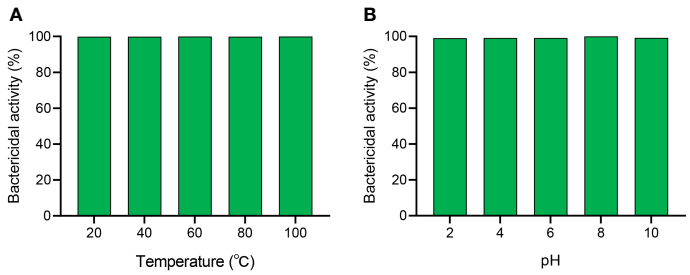

FIGURE 5
The effects of temperature **(A)** and pH **(B)** on the antimicrobial activity of SKL17-2. The final concentration of SKL17-2 was 4 μM (1×MBC). The data are presented as the mean ± SEM of three independent experiments performed in triplicate.

minimum at 200 nm. Interestingly, the CD spectrum of SKL17-2 in SDS and TFE, which mimicked the bacterial cell membrane environment, showed two negative minima at 208 and 222 nm, implying that α-helix was the primary structure of SKL17-2. Moreover, the wheel diagram (Figure 6B) revealed that SKL17-2 exhibited a hydrophobic face and a cationic face.

Bacterial membrane permeability of SKL17-2

PI staining of nucleic acid in cells can reflect a damaged cell membrane structure (Yang et al., 2019). This is a comprehensive method for determining the integrity of the cell membrane. According to the relative fluorescence intensity, the penetration efficiency of SKL17-2 to bacteria is shown in Figure 7. The results indicated that the control (no peptide) resulted in only 0.3% PI-positive cells, while SKL17-2 treatment resulted in positive nucleic acid staining of 14.1% (4 μM), 15.8% (8 μM), 41.1% (16 μM), and 50.9% (32 μM), suggesting that SKL17-2 damaged the bacterial membrane in a dose-dependent manner. These results revealed that SKL17-2 killed bacteria through membrane-permeabilizing action.

Observation of the bacteria morphology treated with SKL17-2

Scanning electron microscopy was used to observe the morphological changes in bacteria following treatment with SKL17-2. The membrane integrity of *P. plecoglossicida* was severely impaired by SKL17-2 treatment (Figure 8). The control bacterial cells exhibited smooth surface and normal morphologies, but *P. plecoglossicida* treated with SKL17-2 displayed clear morphological changes. The membrane surfaces of peptide-treated cells were extensively disrupted, becoming noticeably roughened and damaged.

Discussion

P. plecoglossicida is a lethal pathogen that can cause VWND and consequent high mortality in fish (Zhang et al., 2018; Li et al., 2020). In China, fluoroquinolone antibiotics are frequently used in the treatment of fish diseases (He et al., 2012). However, the rise of drug-resistant bacteria as a result of antibiotic overuse poses a substantial threat to aquaculture and has created

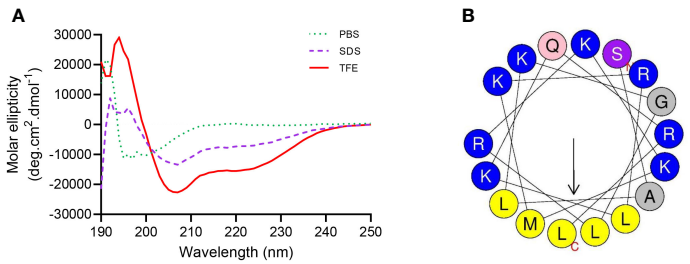

FIGURE 6
Structure analysis of SKL17-2. **(A)** Circular dichroism spectra of SKL17-2. The data from the three scans were averaged per sample, and the peptide concentration was set at 0.2 mg/mL. **(B)** Helical wheel projection of SKL17-2. The output of the helical wheel projection shows charged residues as blue, hydrophobicity residues as yellow, uncharged residues as light pink, alanine and glycine as gray, and serine as gray purple by default.

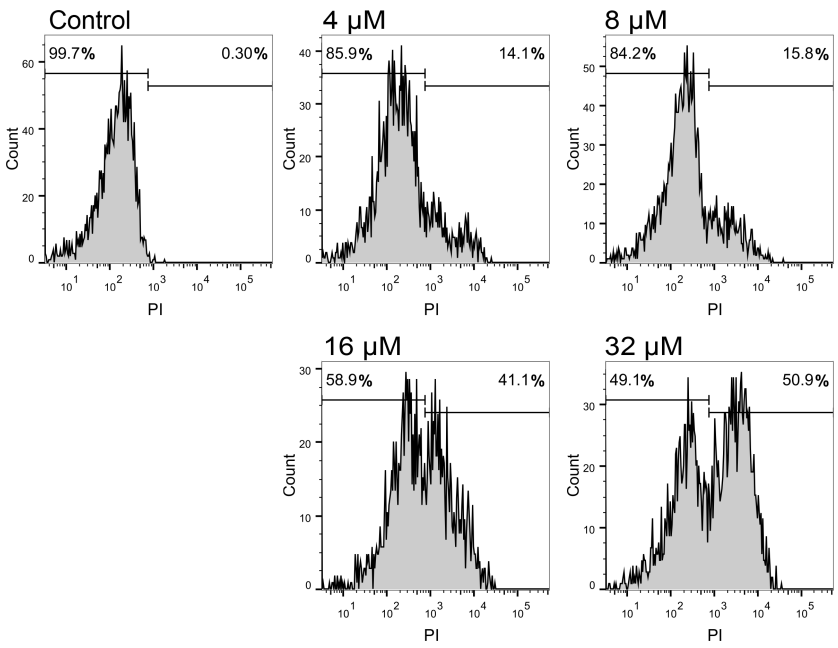

FIGURE 7
Flow cytometric analysis of *P. plecoglossicida* treated with SKL17-2 at different doses. *P. plecoglossicida* were incubated with SKL17-2 for 2 h at 28°C. The influx of PI into bacterial cells was then examined using flow cytometry. The cell-penetrating efficiency was shown as percentage. Data are representative of three independent experiments.

intractable clinical treatment bottlenecks (Katzenback, 2015). At present, developing novel antimicrobial agents is critical to combat antibiotic resistance in aquaculture. AMPs, which have unique antimicrobial activity that disrupts bacterial membranes, show the potential to replace traditional antibiotics as a novel alternative (Wang et al., 2018). However, the downsides of naturally occurring AMPs include weak efficacy, hemolytic or cytotoxic effects on host cells, and high synthetic cost. By comparison, *de novo* synthesized peptides may have the drawbacks of decreased bacteriostatic effectiveness and possible drug resistance (Campoccia et al., 2010). To circumvent the aforementioned drawbacks, short peptides generated artificially from natural peptide templates are thought to be an effective strategy (Luo et al., 2017).

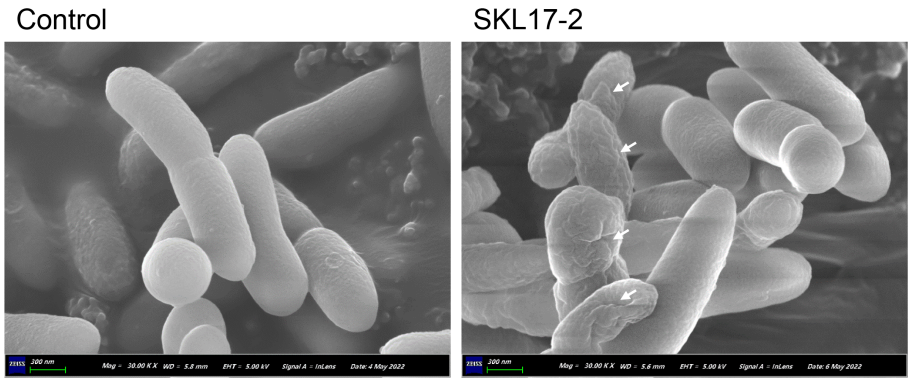

FIGURE 8
Scanning electron microscopy images of *P. plecoglossicida* treated for 2 h at 28°C with 4 μM SKL17-2 or 10 mM PBS (control). Following that, the bacteria were fixed and collected on polycarbonate filters. After dehydration and vacuum drying, filters with bacteria were placed onto aluminum stubs, coated with gold, and examined under scanning electron microscope. Scale bars, 300 nm.

Based on large yellow croaker IFNG1R protein sequence, a 17-aa short peptide named SKL17-2 that showed a strong antimicrobial activity was synthesized. Positive charges and overall net charges of AMPs have been shown to be crucial for electrostatic adsorption between peptides and anionic molecules on the bacterial membrane surface (Chou et al., 2016). It is worth noting that suitable positive charge of an AMP boosts its antimicrobial activity, whereas excessive positive charge may decrease its antimicrobial activity (Chou et al., 2016). Generally, a positive charge ranging from +4 to +6 is optimal for peptide's antimicrobial activity, and positive charges comprise contributions from positively charged residues such as lysine (K), arginine (R), and histidine (H) (Yang et al., 2019). Through the amine groups on its side chain, lysine can interact with the negatively charged phosphatidylglycerol, lipopolysaccharides, and lipoteichoic acid of bacterial membranes, and the lengthy aliphatic side chain helps to localize the peptide in the lipid bilayer of the bacterial plasma membrane (Bhat et al., 2022). With its side chain guanidinium groups, arginine can form strong bidentate hydrogen bonds with the phosphor-rich membrane surface of bacteria, promoting deeper membrane insertion and making AMPs more capable of membrane disruption (Yang et al., 2019). SKL17-2 contains eight positive charges, including five lysine and three arginine residues, compared to four positive charges in SKL17, and displays strong antimicrobial activity against *P. plecoglossicida*, while SKL17 has no antimicrobial activity (data not shown). This may be because SKL17 lacks sufficient positive charge to adsorb to bacterial membranes. Surprisingly, SKL17-2 showed strong antimicrobial activity against *P. Plecoglossicida*, while exhibiting weak or negligible antimicrobial activity against other bacteria. The possible reason is that the structure of LPS varies among different gram-negative bacteria and cell walls composition in gram-positive bacteria is different (Li et al., 2018). These results indicated that total net positive charge affected AMPs' antimicrobial activity.

Whether they are antibiotics or AMPs, ideal antimicrobial agents should target specific pathogens. If the antimicrobial agents have broad-spectrum antimicrobial activity, they may kill probiotics, causing intestinal flora diseases and disrupting the balance between healthy microbiota and the immune system (Tan et al., 2021). Interestingly, SKL17-2 peptide designed here could kill *P. plecoglossicida* but had a weak or negligible antimicrobial activity against other bacteria, indicating its narrow-spectrum antimicrobial activity. In contrast, β-defensin, a natural AMP, showed broad-spectrum antimicrobial activities against *P. plecoglossicida* as well as other bacteria (Li et al., 2021). The narrow-spectrum antimicrobial activity of SKL17-2 makes it an ideal therapeutic agent for combating *P. plecoglossicida* infection in aquaculture.

Many investigations have demonstrated that the formation of a stable spatial secondary structure is a significant element in AMP antimicrobial activity at the molecular level (Yang et al., 2019). In SDS and TFE solutions, our CD findings indicated that SKL17-2 had a distinct and stable α-helical secondary structure. Furthermore, SKL17-2 had an amphipathic α-helical structure with hydrophobic residues on one side and cationic residues on the other side. It has been demonstrated that the amphipathic character of AMPs is critical for their interaction with bacterial cell membranes and for their antimicrobial activity (Zelezetsky and Tossi, 2006). This effect was consistent with the membrane permeabilization and scanning electron microscopy data obtained here. *P. plecoglossicida* treated with SKL17-2 displayed positive nucleic acid staining, and the proportion of positive staining increased with peptide concentration, suggesting the membrane permeabilization of *P. plecoglossicida* following SKL17-2 treatment. Furthermore, the scanning electron microscopy data explicitly indicated that SKL17-2 caused morphological alterations of bacterial membranes such as roughening and corruption, suggesting that SKL17-2 may exert its strong antimicrobial effect primarily by destroying bacterial membrane structure.

Before clinical application, the toxicity of AMPs to eukaryotic cells should be evaluated. In our study, SKL17-2 had a low cytotoxicity to LYC-FM cells as well as a poor hemolytic reaction with red blood cells with a hemolytic rate less than 0.5%, suggesting that SKL17-2 may maintain a cell selectivity against *P. plecoglossicida* and would be safe for application in large yellow croaker. These findings further support the potential of SKL17-2 administration in the treatment of VWND. Our long-term goal is to use SKL17-2 as a feed additive in aquaculture, and thus antimicrobial activity variation in different temperature and pH should be evaluated due to high temperatures and complicated condition changes during feed procesing. In our investigation, SKL17-2 demonstrated good temperature and pH stability, hinting that it has an excellent therapeutic potential in clinical applications.

In summary, a peptide targeting *P. plecoglossicida* was developed and produced based on a large yellow croaker IFNG1R protein sequence. The peptide has low cytotoxicity and negligible hemolytic activity as well as high temperature and pH stability, indicating promising therapeutic potential in the treatment of VWND caused by *P. plecoglossicida*. As SKL17-2 demonstrated strong antimicrobial activity against *P. plecoglossicida in vitro*, the therapeutic potential of SKL17-2 as a feed additive will require further investigations in the future.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Author contributions

YL performed most of the experiments, analyzed the data, and wrote the manuscript. SY and XW contributed to antibacterial assays. RX, JC, and TH help with experimental operations. X-YZ and XC designed the research and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Dietary grape extract can, at an appropriate level, improve the growth performance and antioxidant activity of the white shrimp *Litopenaeus vannamei*

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This study evaluated the growth performance and *in vivo* antioxidant capacity of the white shrimp, *Litopenaeus vannamei*, fed diets containing the grape extract Nor-grape 80. A control diet containing 1000 ppm vitamin E without added grape extract and four other diets containing 250, 500, 750 and 1000 ppm grape extract were formulated. Shrimp, twenty 0.02 g of individuals in each aquarium, were randomly assigned to one of the above five treatment diets in triplicate and fed three times daily to satiation for six weeks. The final weight, weight gain percentage and specific growth rate of the shrimp larvae fed diets containing 250 and 500 ppm Nor-grape 80 were significantly higher than those fed the other treatment diets. The levels of superoxide dismutase activity of the shrimp fed diets containing 250 and 500 ppm Nor-grape 80 were significantly higher than those of shrimp fed the control diet. On the other hand, the thiobarbituric acid-reactive substance levels of the shrimp fed diets containing 250 and 500 ppm of Nor-grape 80 were significantly lower than those fed the other diets. Furthermore, the highest levels of dietary Nor-grape 80, namely 750 and 1000 ppm, gave rise to reduced growth performance, as well as lower levels of *in vivo* antioxidant activity. Therefore, for the best growth performance and highest level of *in vivo* antioxidant activity, it is suggested that the optimal level of Nor-grape 80 as part of a white shrimp diet falls between 250 and 500 ppm.

KEYWORDS

SOD, TBARS, weight gain percentage, white shrimp, grape extract

Introduction

The Pacific white shrimp *Litopenaeus vannamei*, a native species that is distributed along the East Pacific coasts of Mexico, Central America and Peru, has now become the most popular farmed penaeid shrimp species throughout the world (Dugassa and Gaetan, 2018; Lucas et al., 2019). In 2020, the global aquaculture production of white shrimp was

more than 5,400,000 tonnes and this represents approximately 80% of the total aquaculture production of crustaceans (FAO, 2022). This shrimp shows rapid growth, good survival under high density culture, and a high tolerance to a broad range of salinities and temperatures, and therefore it is highly suitable for intensive culture systems. The shrimp has widely been employed commercially in Southeast Asia, including in India, Thailand, Vietnam, the People's Republic of China and Taiwan (Briggs et al., 2004). Generally, the white shrimp require five to seven months of cultivation to reach a marketable size of 15 to 30 grams (Tacon et al., 2013). As part of feeding management practices, shrimp aquafeed includes costly fish meal that provides an excellent protein source within the formula; therefore, the cost of feeding these shrimp can reach as much as 40% to 60% of the total production costs when under semi-intensive and intensive systems (Hardy and Tacon, 2002).

In order to reduce dietary costs, several plant and animal protein sources have been successfully used as fish meal alternatives without compromising shrimp production (Davis and Arnold, 2000; Cummins et al., 2017). For white shrimp, it is known that the optimal dietary protein content ranges from 32% to 36% across the various growth stages (Lee and Lee, 2018). However, the effect of shrimp diets supplemented with phytochemicals, which are the bioactive components derived from plants and plant-based beverages, such as fruit, vegetables, grains, nuts, seeds, tea and wine (Chakraborty et al., 2013; Encarnação, 2016), has not yet been totally explored. Many of these phytogenics, which are classified as alkaloids, flavonoids, terpenoids, polyphenols, essential oils and pigments, have been exploited as feed additives in order to improve the growth performance of fish (Chakraborty et al., 2013; Encarnação, 2016; Artés-Hernández et al., 2021).

Grapes, rich in polyphenols, are characterized as having antioxidant, antimicrobial, anti-inflammatory and anti-carcinogenic properties (Shi et al., 2003; Xia et al., 2010; Doshi et al., 2013). The most abundant phenolic components of grapes are present in their seeds, which contain 74% to 78% proanthocyanidin oligomers and 6% free flavanols (Shi et al., 2003; Burdock, 2005), followed by grape skins and grape pulp (Shi et al., 2003). It is worth noting that grape polyphenols have been recognized as being able to improve nutrient absorption and biological growth of terrestrial animals (Viveros et al., 2011; Aditya et al., 2018). Broiler chickens fed diets supplemented with grape seeds, grape pomace and pomace extract containing polyphenols have been shown to have enhanced growth performance. These findings have been attributed to improved gut morphology and a better intestinal microbiota (Viveros et al., 2011; Abu Hafsa and Ibrahim, 2018; Aditya et al., 2018). Grape seed proanthocyanidin extract has also been reported to elevate intestinal release of both glucagon-like peptide-1 and peptide YY, two hormones that regulate appetite and glucose homeostasis (Casanova-Marti et al., 2020). With aquatic animals, grape seeds and pomace extracts have served as dietary additives to improve the growth of tilapia (*Oreochromis niloticus*) and of Pacific white shrimp (Zhai et al., 2014; Niyamosatha et al., 2015).

Grape seeds and pomace would seem to act as powerful antioxidants that scavenge the free radicals causing oxidative stress in cells, both *in vitro* and *in vivo* (Bagchi et al., 1998; Bouhamidi et al., 1998; Bagchi et al., 2000; Jayaprakasha et al., 2003; Shi et al., 2003). The antioxidant potential of grape-seed procyanidins, in particular, is twenty fold more than that of vitamin E (Uchida, 1980; Shi et al., 2003). In several terrestrial animals, diets including either grape seeds or grape pomace extract have been found to result in improved antioxidant capacity, namely an increase in superoxide dismutase (SOD) activity and a decrease in the level of thiobarbituric acid-reactive substances (TBARS) or malodialdehyde (MDA) (Garcia et al., 2002; Park et al., 2014; Guerra-Rivas et al., 2016; Zhao et al., 2017; Aditya et al., 2018). In addition to enhanced SOD activity, reduced levels of TBARS or MDA, reactive compounds, which are the result of lipid peroxidation of polyunsaturated fatty acids, has been observed in broilers fed diets containing increasing amounts of grape seeds and grape pomace (Abu Hafsa and Ibrahim, 2018; Aditya et al., 2018). Surprisingly, penned rams fed diets containing wine grape extract have even shown significantly higher levels of SOD activity, as well as a lower level of MDA, in their testes when compared to those fed diets without wine grape extract (Zhao et al., 2017).

For most animals, although dietary grape extract has been known to improve their growth performance and *in vivo* antioxidant capacity (Viveros et al., 2011; Chamorro et al., 2013; Park et al., 2014; Zhai et al., 2014; Niyamosatha et al., 2015; Zhao et al., 2017; Abu Hafsa and Ibrahim, 2018; Aditya et al., 2018), these also seems to be adverse effects on the growth performance, related to protein and amino acid digestibility, if the dietary grape products are provided at a level higher than their optimal requirements (Chamorro et al., 2013; Zhai et al., 2014; Abu Hafsa and Ibrahim, 2018). Broilers fed the highest dietary levels of grape seeds or seed extracts (Chamorro et al., 2013; Abu Hafsa and Ibrahim, 2018), as well as tilapia (*O. niloticus*) fed a diet containing the highest level of grape extract (Zhai et al., 2014), showed reduced growth performance. Previously, white shrimp fed diets containing 150 and 200 ppm Nor-grape 80, a commercial grape extract that consists of 80% polyphenols, were found to have a significantly higher weight gain percentage than those fed diets containing less than 150 ppm of Nor-grape 80 (Cheng et al., 2017). As the amount of Nor-grape 80 in the shrimp diet was increased to the highest level of 200 ppm, an increase in SOD and a decrease in TBARS levels were correspondingly detected (Cheng et al., 2017). However, the optimal dietary concentration of Nor-grape 80 for white shrimp, in terms of the growth performance and antioxidant capacity, has not yet been determined. The aim of the present study was to examine a suitable level of this grape extract that will give the best growth performance for white shrimp. *In vivo* antioxidant activity, specifically SOD activity and TBARS levels, of the white shrimp were also evaluated when the shrimp were fed diets supplemented with Nor-grape 80 at a level greater than 200 ppm.

Materials and methods

Five isonitrogenous (35% crude protein), isolipidic (9% crude lipid) and isoenergetic (300 Kcal/100 g) diets were formulated (Table 1). The control diet contained 1000 ppm vitamin E, while the four other diets contained 250 (N250), 500 (N500), 750 (N750) and 1000 ppm (N1000) of Nor-grape 80, an extract of whole *Vitis*

vinefera grapes that was provided by Kaye Bio-tech Co., Taiwan. Nor-grape 80 has been shown to comprise 80% polyphenols of which 60% are proanthocyanidins and 0.75% are anthocyanins. Fish meal was used as the protein source. A mixture of 2:1 fish oil and corn oil (w/w) was used as the source of lipid. Wheat flour and corn starch were used as sources of carbohydrate and also acted as binders. Cellulose was also included to balance out the composition of the diet. On preparing the experimental diets, all dietary ingredients were first ground into small particles using a hammer mill and then passed through a 250 µm mesh sieve. The dry ingredients were then thoroughly mechanically mixed to insure homogeneity before the addition of the oil. The mixture, after an addition of distilled water, was blended until a consistent dough was formed. Each of the diets was extruded through a chopper (3.0 mm die diameter) to produce pellets. The pellets were then ground into small particles (<500 µm) and dried in an air oven at 60 ^{0}C for 12 hours and finally the five experimental diets were stored separately at 4 ^{0}C in a refrigerator until use.

The post larvae shrimp of 20 days after hatch were obtained from a local aquafarm (Pintung, Taiwan), where the water temperature and salinity ranged from 28°C to 30°C and 30‰ to 33‰, respectively, and then acclimatized in a 2000 L fiberglass tank at National Taiwan Ocean University for one week. During the acclimatization phase, the shrimp were cultured under 26-28 °C and fed the control diet. At the beginning of the feeding trial, the shrimp, which had initial weights of 0.02 g, were starved for 24 h before they were randomly distributed into the 15 glass aquaria (57 x 35 x 30 cm), each of which finally contained twenty shrimp. Three replicate groups of shrimp were fed one of the five treatment diets three times a day. They were fed 30% of their body weight daily until satiation for six weeks. The experiment aquaria were closed systems with continuous aeration. Half of the water in each tank was renewed daily to maintain water quality. The water temperature, salinity, dissolved oxygen and pH ranged between 25 and 28°C, 31‰ and 33‰, 5.6 mg/L and 7.0 mg/L and 8.1 and 8.5, respectively. The concentration of total ammonia-nitrogen was maintained at less than 0.05 ppm during the entire feeding trial period. Uneaten feed and feces were siphoned off from each tank every day. At the end of the feeding trial, feeding was stopped for 24 h prior to weighing. Each shrimp was individually weighed, sacrificed by placing it in ice water and then carefully dissected. The muscle tissue of individual shrimp that had been cultivated in the same aquarium was pooled, dried and homogenized (n=3) in order to carry out the proximate analysis twice. In parallel, the hepatopancreas of the shrimp from each aquarium was isolated and pooled to measure SOD activity and the level of TBARS (n=3).

The growth parameters of the shrimp were calculated according to the following equations:

Weight gain (%) = 100 % x $(W_t - W_0)/W_0$, and specific growth rate (SGR) (% day^{-1}) = 100% x $[(\ln W_t - \ln W_0)/t]$, where W_0 is the initial mean body weight (g), W_t is the final mean body weight (g).

The compositions of the experimental diets and the shrimp muscle samples were analyzed according to the method of AOAC (1984). Crude protein was determined using the Kjeldahl system (Kjeldahl system 1002, Tecator, Sweden) after acid digestion. Crude lipid was measured by the chloroform and methanol (2:1, v/v) extraction method (Folch et al., 1957). Crude fiber was determined by acid and alkaline digestion using the Fibertec M 1020 system (Foss Tecator, Sweden). Moisture and ash were determined by conventional methods using an oven at 105 ^{0}C and a muffle furnace at 540 ^{0}C, respectively. Nitrogen-free extract (NFE) was calculated as follows: NFE= [100 – (crude protein + crude lipid + crude fiber + ash)] %. A proximate analysis of the experimental diets is presented in Table 2. The crude protein, crude lipid, ash, crude fiber and NFE of the treatment diets ranged from 37.85% to 38.03%, 8.86% to 9.03%, 14.11% to 14.23%, 11.90% to 12.29% and 26.59% to 27.77%, respectively.

The SOD activity of each pooled hepatopancreas sample was measured using SOD assay kits (19160 SOD determination kit, Sigma, USA). In brief, a total of 0.5 g of hepatopancreas tissue was homogenized in 0.1 M phosphate buffer using a T25 homogenizer (IKA, Germany). The homogenate was then centrifuged at 3000 rpm for 10 min and the supernatant was collected. From this point onwards, the procedure described in the instruction manual of the SOD assay kit was followed. The working solution in the kit, namely, WST-1 (2- (4-iodopheny) - 3- (4-nitophenyl) -5- (2, 4-disulfophenyl) -2 H tetrazolium), reacts with the superoxide radicals generated in the presence of oxygen and the enzyme, xanthine oxidase. The superoxide radicals would be reduced and this results in the formation of a yellowish water-soluble formazan dye. However, the superoxide anions are also reduced by SOD and such reaction will decrease the amount of formazan formed. Thus, after the optical density has been measured at 450 nm using a spectrophotometer (Synergy HT, Biotek, USA), the SOD activity is able to be expressed as a percentage inhibition rate.

TBARS was measured using the method of Kornbrust and Mavis (1980). A total of 1 g hepatopancreas tissue was homogenized in a buffer solution (0.15M KCL and 5 mM Tris-maleate, pH 7.4) using a T25 homogenizer (IKA, Germany). Next, 1 ml of ascorbic acid (2 mM) was added and the mixture was incubated at 37 ^{0}C for 30 min. After the addition of 5 ml HCL (0.7 M) and 5 ml thiobarbituric acid (0.05 M), the samples were boiled for 25 min and then placed on ice. Next, 5 ml of trichloroacetic acid (200 g/L) was added and then the samples were centrifuged at 495 g for 5 min. The supernatant was collected to measure the amount of TBARS present using a U-1800 spectrophotometer (Hitachi, Japan) at 530 nm. The concentration of TBARS was expressed as micrograms of malondialdehyde equivalent per milligram of tissues of the hepatopancreas. Standard MDA solutions (Sigma T-1642) were used to create a calibration curve in order to measure the concentration of MDA in each pooled shrimp hepatopancreas sample.

Significant differences among treatments were analyzed by analysis of variance (ANOVA) after confirming the homogeneity of variance using the Levene's test (Snedecor and Cochran, 1980). A one-way analysis of variance was performed to examine differences in weight gain percentage, SGR, and survival in the five treatments. When a significant difference was observed, Tukey's range test was used to compare differences among treatments. Weight gain

TABLE 1 Ingredient composition of dietary treatments for white shrimp.

Ingredients	Diets				
	Control	N250	N500	N750	N1000
Fish meal[1]	50	50	50	50	50
Shrimp meal[2]	6	6	6	6	6
Oil[3]	1.5	1.5	1.5	1.5	1.5
α-starch	15	15	15	15	15
Choline chloride	0.5	0.5	0.5	0.5	0.5
Cholesterol	0.5	0.5	0.5	0.5	0.5
Mineral mix[4]	4	4	4	4	4
Vitamin mix[5]	4	4	4	4	4
Vitamin A	0.1	0.1	0.1	0.1	0.1
Vitamin D_3	0.1	0.1	0.1	0.1	0.1
Vitamin E	0.1	0	0	0	0
Cellulose	12.700	12.775	12.750	12.725	12.700
Yeast	5	5	5	5	5
Lecithin	0.5	0.5	0.5	0.5	0.5
Nor-grape 80	0	0.025	0.050	0.075	0.100

[1]Fish meal: CP 67.42%, CL 12.53%.
[2]Shrimp meal: CP 49.60%, CL 6.29%.
[3]Fish oil: Corn oil = 2: 1.
[4]Calcium carbonate 2.1%, Calcium phosphate dibasic 73.5%, Citric acid 0.227%, Cupric acid 0.046%, Ferric acid (16-17% Fe) 0.558%, Magnesium oxide 2.5%, Magnesium citrate 0.835, Potassium sulfate 6.8%, Sodium chloride 3.06%, Sodium phosphate 2.14%, Zinc citrate 0.133%, Potassium iodine 0.001%, Potassium phosphate dibasic 8.1% (Bernhart and Tomarelli, 1966)
[5]Thiamin HCl 0.5%, Riboflavin 0.8%, Niacinamide 2.6%, D-biotin 0.1%, Ca-pantothenate 1.5%, Pyridoxine HCl 0.3%, Folic acid 0.5%, Inositol 18.1%, Ascorbic acid 12.1%, Para-aminobenzoic acid 3%, Cyanocobalamin 0.1%, BHT 0.1%, alpha-cellulose 60.3%.

percentage data were log-transformed while others were arcsine-transformed before statistical analysis. Polynomial contrast procedures were applied to detect linear and quadratic trends between the growth parameters of the shrimp and the dietary levels of Nor-grape 80. The significant level was set at $p< 0.05$ and all statistical analyses were conducted using a SAS software program for Windows (V.9.3., SAS Institute, Cary, NC, U.S.A.).

Results

The growth performance and survival of the white shrimp fed the experimental diets for six weeks are shown in Table 3. The survival of white shrimp fed the treatment diets was in all cases more than 90%. The shrimp fed diets containing different levels of Nor-grape 80 showed better growth performance than those fed the control diet

TABLE 2 Proximate composition of diets for white shrimp.

Composition (%)	Diets					Statistical test
	Control	N250	N500	N750	N1000	
Moisture[a]	4.85 ± 0.02	4.81 ± 0.05	4.90 ± 0.12	4.84 ± 0.19	4.90 ± 0.34	NS
Crude protein[a]	37.97 ± 0.05	38.03 ± 0.12	38.00 ± 0.42	37.85 ± 0.32	37.97 ± 0.23	NS
Crude lipid[a]	9.03 ± 0.15	8.88 ± 0.07	8.88 ± 0.08	8.86 ± 1.08	8.89 ± 0.05	NS
Ash[a]	14.12 ± 0.08	14.23 ± 0.09	14.13 ± 0.05	14.23 ± 0.04	14.11 ± 0.02	NS
Crude fiber[a]	12.29 ± 0.09	11.90 ± 0.14	12.14 ± 0.29	12.19 ± 1.02	12.06 ± 0.76	NS
NFE[b]	26.59 ± 0.14	27.77 ± 0.10	26.85 ± 0.08	26.87 ± 0.11	26.97 ± 0.52	NS
Calculated energy (kcal/100g)[c]	339.51	343.12	339.32	338.62	339.77	–

[a]Expressed as percent of dry weights.
Values are presented as mean ± SD (n=3).
[b]Nitrogen-free extract (NFE): [100 - (crude protein + crude lipid + crude fiber + ash)] %.
[c]Calculated digestible energy (kcal/g) was calculated based on protein 4 kcal/g, lipid 9 kcal/g and carbohydrate 4 kcal/g.
NS in statistical test means not significant at p<0.05.

that contained 1000 ppm vitamin E without Nor-grape 80. The final weight, weight gain percentage and SGR of the white shrimp fed diets containing 250 and 500 ppm Nor-grape 80 were significantly higher than those of shrimp fed the other diets. However, no significant differences were found in the final weight, weight gain percentage and SGR of white shrimp fed diets containing 250 and 500 ppm Nor-grape 80. Only the quadratic trends analyzed using polynomial contrast procedures between the growth parameters and Nor-grape 80 levels showed a significant difference.

The proximate analysis of the muscle of white shrimp fed the treatment diets for six weeks is shown in Table 4. The crude protein content of the muscle tissue from shrimp fed with the treatment diets was all above 80%. The crude lipid of muscle from shrimp fed the treatment diets ranged from 6.81 to 7.38%, The ash of the muscle tissue from shrimp fed the treatment diets ranged from 6.61 to 6.91%.

The levels of SOD activity and the amounts of TBARSs present in the hepatopancreas samples from white shrimp fed the experimental diets for six weeks are shown in Table 5. The highest SOD activity and the lowest TBARS level were found in the hepatopancreas of shrimp fed the diet containing 250 ppm of Nor-grape 80. Both the SOD value and the TBARS level in the hepatopancreas of shrimp fed the diet containing 250 ppm Nor-grape 80 are significantly different from those of shrimp fed the control diet containing 1000 ppm vitamin E. However, the hepatopancreatic SOD values of shrimp fed the diets containing 250 and 500 ppm of Nor-grape 80 were not significantly different. By way of contrast, the hepatopancreatic TBARS level of shrimp fed the diet containing 250 ppm of Nor-grape 80 was significantly lower than that of shrimp fed the other diets. A decrease in SOD activity and an increase in TBARS level in the hepatopancreas of shrimp fed the treatment diets, without the control diet being compared, was found to be proportional to the increase in the amount of Nor-grape 80 in the shrimp diets.

Discussion

Positive and negative effects of grape extract were observed in relation to the growth performance of the white shrimp. In this study, white shrimp fed the diets supplemented with 250 and 500 ppm Nor-grape 80 showed an equivalent best growth performance, and a decrease in growth performance was observed among the white shrimp fed diets containing 750 and 1000 ppm Nor-grape 80. Both tilapia and shrimp fed diets supplemented with grape by-products, depending on the dosage used, showed either an improved or a reduced growth performance (Zhai et al., 2014;

TABLE 3 Initial weight, final weight, weight gain percentage, SGR and survival of white shrimp fed dietary treatments for 6 weeks.

Diets	Initial weight (g)	Final weight (g)	Weight gain (%)	SGR (% day^{-1})	Survival (%)
Control	0.019 ± 0.000	0.406 ± 0.031^{c}	1985.81 ± 160.32^{c}	7.23 ± 0.21^{c}	92.78 ± 2.55
N250	0.020 ± 0.000	0.968 ± 0.227^{a}	4863.47 ± 1154.99^{a}	9.25 ± 0.53^{a}	97.78 ± 1.92
N500	0.020 ± 0.000	0.968 ± 0.107^{a}	4856.86 ± 504.29^{a}	9.29 ± 0.25^{a}	92.22 ± 0.96
N750	0.020 ± 0.000	0.681 ± 0.093^{b}	3394.93 ± 484.14^{b}	8.45 ± 0.32^{b}	97.78 ± 0.96
N1000	0.019 ± 0.000	0.642 ± 0.021^{b}	3200.73 ± 144.43^{b}	8.32 ± 0.10^{b}	95.00 ± 3.33
ANOVA					
Pr > F	–	0.0009	<0.0001	<0.0001	0.3336
Linear Trend					
Pr > F	–	0.6926	0.4096	0.0530	–
Quadratic Trend					
Pr > F	–	0.0052	0.0002	<0.0001	–

Values are presented as mean ± SD (n=3). Different superscript letters in the same column denote difference between diets (p<0.05).

TABLE 4 Proximate analysis of muscle samples from white shrimp fed dietary treatments for 6 weeks.

Composition (%)	Diets					Statistical test
	Control	N250	N500	N750	N1000	
Moisture	76.49 ± 0.04	76.22 ± 0.05	76.66 ± 0.15	76.89 ± 0.12	76.69 ± 0.03	NS
Crude protein[a]	80.18 ± 0.63	80.06 ± 0.35	80.10 ± 0.03	80.05 ± 0.91	80.13 ± 0.02	NS
Crude lipid[a]	6.81 ± 0.08	7.05 ± 0.13	7.09 ± 0.05	7.25 ± 0.03	7.38 ± 0.06	NS
Ash[a]	6.91 ± 0.01	6.78 ± 0.03	6.62 ± 0.08	6.68 ± 0.01	6.61 ± 0.04	NS

[a]Expressed as percent of dry weights.
Values are presented as mean ± SD (n=3).
NS in statistical test means not significant at p<0.05.

TABLE 5 SOD activity and TBARS levels in the hepatopancreas of white shrimp fed dietary treatments for 6 weeks.

Treatments	SOD (U/ml)	TBARS (μg MDA mg^{-1} hepatopancreas)
Control	$34.394.99^{c}$	$0.210.01^{b}$
N250	$73.372.14^{a}$	$0.110.01^{d}$
N500	$65.169.46^{ab}$	$0.160.01^{c}$
N750	$46.6313.41^{bc}$	$0.240.01^{a}$
N1000	$47.090.69^{bc}$	$0.230.01^{a}$
ANOVA		
Pr>F	0.020	0.0001
Linear Trend		
Pr>F	0.973	0.202
Quadratic Trend		
Pr>F	0.101	0.079

Values are presented as mean ± SD (n=3). Different superscript letters in the same column denote difference between diets ($p<0.05$).

Rosas et al., 2022). In tilapia, *O. niloticus*, the weight gain increased as the diets contained increasing amounts (0, 200 and 400 ppm) of grape seed proanthocyanidins and this was followed by a decrease as the dietary grape seed proanthocyanidins were increased to 600 and 800 ppm (Zhai et al., 2014). Similarly, white shrimp fed a diet containing 2.5 x 10^4 ppm grape bagasse had the highest growth parameters, while shrimp fed a diet containing the highest 1.5 x 10^5 ppm grape bagasse showed the lowest growth performance (Rosas et al., 2022). Although grape polyphenols, when included in diets at a lower dose, have previously been described as benefiting the growth performance of a wide range of animals (Dolara et al., 2005; Rhodes et al., 2006; Kao et al., 2010; Tucsek et al., 2011; Viveros et al., 2011; Fiesel et al., 2014; Zhai et al., 2014; Shimizu, 2017; Abu Hafsa and Ibrahim, 2018; Ao and Kim, 2020), it is also obvious that there are detrimental effects on the growth performance of animals fed diets containing high levels of grape by-products. This could be due to an increase in the antinutritional factors present in grapes, such as alkaloids, saponins and tannins (Shi et al., 2003; Iriti and Faoro, 2006), as well as intensified grape polyphenol-protein interactions that are likely to reduce the nutritional value of the protein present in food matrix (Shi et al., 2003). As a result, we suggested that the dietary level of Nor-grape 80 fed to 0.02 g white shrimp should not be greater than 500 ppm in order to prevent the above adverse effects on the shrimp growth performance.

In this study, it is hypothesized that dietary grape extract enhances the growth performance of animals and that this might be due to the phenolic components present in the supplement, which appear to promote intestinal health. Several phenolic components in grape extract are known to possess antibacterial activity that can alter the intestinal microbiota (Dolara et al., 2005; Rhodes et al., 2006; Kao et al., 2010; Viveros et al., 2011; Zhai et al., 2014; Abu Hafsa and Ibrahim, 2018; Ao and Kim, 2020), as well as being able to attenuate intestinal inflammation (Kao et al., 2010; Tucsek et al., 2011; Fiesel et al., 2014; Shimizu, 2017). Anthocyanins and their metabolites when interacting with the microbiota have been reported to enhance the growth of *Bifidobacteria* spp. and *Lactobacillus-Enterococcus* spp. (Hidalgo et al., 2012). In rats, broilers and Pekin ducks, ingestion of red wine polyphenols and grape seeds has been shown, not only to increase the ileal and colonic numbers of probiotic bacteria such as *Lactobacilli* and *Bifidobacteria* spp., but also to inhibit the intestinal growth of detrimental bacteria such as *Escherichia coli*, *Clostridia* and *Streptococcus* (Dolara et al., 2005; Abu Hafsa and Ibrahim, 2018; Ao and Kim, 2020). Additionally, grape phenolic compounds have been found to be capable of modulating the intestinal morphology in poultry (Viveros et al., 2011; Ao and Kim, 2020). In post larvae stage of the freshwater prawn, *Macrobrachium rosenbergii*, the activity of digestive enzymes has been observed to increase whengrape waste is included in the diets (Bhavan et al., 2013). The dominant intestinal microbiota of white shrimp fed diets containing tannins, which are part of the polyphenols present in grapes, have recently been characterized and found to include Proteobacteria and Bacteroidota (Bolívar-Ramírez et al., 2022); these are groups of bacteria that are normally present in the intestinal tracts of heathy shrimps (Xiong et al., 2017; Ayiku et al., 2020; Schleder et al., 2020). Taking these findings together, we suggest that grape polyphenols improve nutrient absorption by directly affecting the digestive tracts of animals and thus enhance the growth performance of these animals, including the white shrimp in our case.

Previously, white shrimp fed the diets containing 50 to 200 ppm of Nor-grape 80 have been shown to exhibit an increase in SOD activity and a decrease in TBARS levels (Cheng et al., 2017). In the present study, the *in vivo* antioxidant capacity of the hepatopancreas of white shrimp fed diets containing 250 to 1000 ppm of Nor-grape 80 also showed trends involving a decrease in SOD activity and an increase in TBARS levels as the level of Nor-grape 80 increased. To date, the adverse effects of dietary grape by-products on *in vivo* antioxidant activity have not been investigated in terrestrial animals (Abu Hafsa and Ibrahim, 2018; Aditya et al., 2018; Ao and Kim, 2020). Pekin ducks fed diets containing 0, 0.01 and 0.02% grape seed extract showed an increase in growth parameters and serum SOD levels, and a decrease in serum TBARS values (Ao and Kim, 2020). Similar increases in SOD activity and the reduction in TBARS levels were also found in the serum and the meat of the broilers fed diets supplemented with increasing amounts of grape pomace and grape seeds (Abu Hafsa and Ibrahim, 2018; Aditya et al., 2018). On the other hand, Rosas et al. (2022) have shown a decrease in the hepatopancreatic TBARS level of white shrimp fed the diets containing 0, 2.5 and 5% grape bagasse; this was accompanied by a slight increase in the hepatopancreatic TBARS level of the white shrimp, as the dietary grape bagasse was increased from 5% to 15%. Therefore, it is possible that animals fed diets containing grape extract have an optimal level of such supplementation and going above this optimal level will result in both a reduced growth performance and a detrimental effect on *in vivo* antioxidant capacity.

Feed manufacturers have used vitamin E as an antioxidant in aquafeeds for many decades. In terms of highest weight gain, optimal SOD activity and lowest TBARS levels, the findings of the present study indicated that 250 ppm of Nor-grape 80 is more suitable than 1000 ppm of vitamin E when producing an aquafeed for white

shrimp. In the present study, the weight gain of the shrimp fed a diet supplemented with 250 ppm Nor-grape 80 was almost 2.5 times higher than that of shrimp fed a diet containing 1000 ppm vitamin E. As a powerful antioxidant, the grape extract Nor-grape 80, at an optimal level, is not only able to promote the growth performance and *in vivo* antioxidant capacity of white shrimp, but using Nor-grape 80 will also lower the dietary cost of feeding white shrimp in an aquaculture system. In 2022, the prices for Nor-grape 80 and vitamin E in the Taiwan market were US $ 10/kg and US $ 30/kg, respectively. If 1000 ppm of vitamin E is completely replaced with a minimum of 250 ppm of Nor-grape 80, one that allows the white shrimp to reach their highest yield, the dietary cost of the aquafeed would be reduced by US $ 27.5 per tonne. Notwithstanding the above, the optimal dietary level of Nor-grape 80 for white shrimp that will give the maximum growth performance appears to be in the range between 250 and 500 ppm. This is a wide range and further large feeding trials of the white shrimp are needed to pinpoint exactly the optimal dietary level of Nor-grape 80 that gives the best weight gain. This would need to include a cost-benefit analysis and the optimal diet might change depending on the market price of Nor-grape 80, the total feed needed to reach a marketable size for white shrimp and the price that can be obtained for such white shrimp in the market place.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, upon request by any qualified researcher.

Ethics statement

Ethical review and approval were not required for the animal study because the research utilized the invertebrate *Litopenaeus vannamei*.

Author contributions

AC conceptualized, wrote the manuscript and designed the study. SS secured funding and supervised the study. YC performed the experiments, data collection. RK wrote and revised sections of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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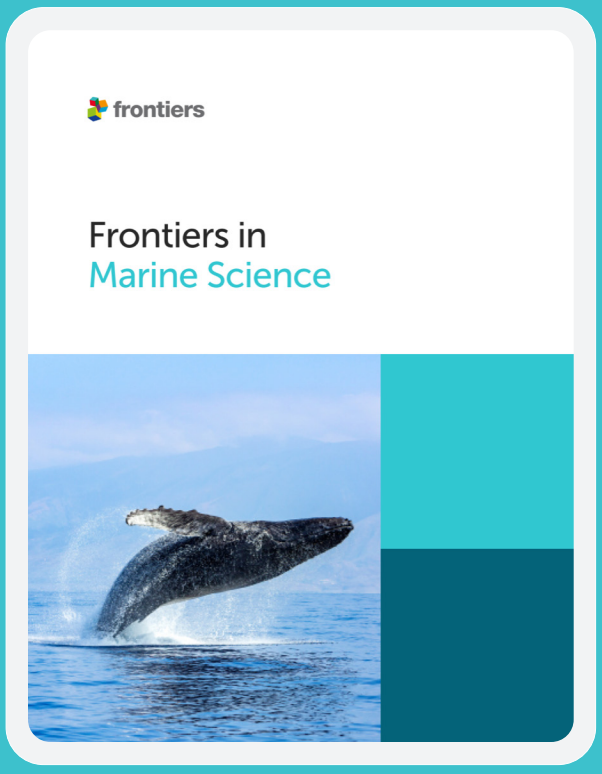

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