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New progresses and effects of functional feed additives on marine aquatic animals

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New progresses and effects of functional feed additives on marine aquatic animals

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Table of contents

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Replacement of Astaxanthin With Lutein in Diets of Juvenile *Litopenaeus vannamei*: Effects on Growth Performance, Antioxidant Capacity, and Immune Response

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An 8-week feeding experiment was conducted to investigate whether diet supplementation of lutein could result in similar growth performance, antioxidant capacity, and immunity of *Litopenaeus vannamei* when compared to dietary astaxanthin. Juvenile *L. vannamei* (initial body weight: 0.64 ± 0.04 g) were fed with one of five isonitrogen and isolipids diets with/without lutein or astaxanthin [control group (C); the lutein (L) groups contained 0, 62.5, 75, 87.5 ppm lutein, respectively, the astaxanthin (A) group contained 50 ppm astaxanthin]. Results showed that dietary supplementation of lutein ranging from 62.5 to 75 ppm resulted in similar growth performance (WGR, SGR, FCR, and SR) of *L. vannamei* compared with the A group ($P > 0.05$). Apart from that, no statistical difference was observed in antioxidant parameters (hemolymph T-AOC, hemolymph MDA, and RNA expression level of GSH-PX, CAT), anti-inflammatory ability (Relish, Rho, and HSP70) and apoptosis-related gene expression (Caspase3) among lutein treatments ranging from 62.5 to 87.5 ppm and the A group ($P > 0.05$). These results indicate that a dose of 62.5–75 ppm of lutein was suitable in the diet of *L. vannamei* for substituting dietary astaxanthin.

Keywords: lutein, *L. vannamei*, astaxanthin, growth performance, antioxidant capacity, immunity

INTRODUCTION

In recent years, there have been two significant factors existing in the shrimp farming industry that may affect the profit of shrimp. Firstly, water environments were deteriorated by plenty of pollutants (such as heavy metals, pesticides, bacteria, and viruses) due to industrialization's development and human activity's involvement, resulting in seriously high stress-induced diseases and low survival rate in the shrimp culture industry (Zhang et al., 2013). In addition, maintaining bright and appropriate body pigmentation is also a significant factor closely related to customers'

preference and shrimp's price because it means freshness and high quality of the merchandise (Shahidi and Brown, 1998; Seidgar, 2015). In fact, shrimp and other crustaceans are incapable of bio-synthesize carotenoids *de novo*, which makes it essential for them to obtain carotenoids from the daily diet for body pigmentation (Boonyaratpalin et al., 2001). Previous studies paid more attention to diet supplementation with astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) on crustaceans as it helps to enhance the stress resistance of crustaceans by acting as an effective antioxidant against ROS as well as being a green additive for improving growth performance and skin pigmentation of aquatic animals (Latscha, 1989; Petit et al., 1997; Chien et al., 2003; Seabra and Pedrosa, 2010). However, diet supplementation with astaxanthin seems to considerably increase the shrimp feed cost because of the expensive synthetic process of this carotenoid pigment (Boonyaratpalin et al., 2001). Therefore, other cost-effective additives need to be identified for substituting the astaxanthin in the shrimp diets.

Crustaceans could convert many carotenoids like β-carotene, which are easy to be acquired from vegetable ingredients of the feed, into astaxanthin (Giuliano et al., 2000; Niu et al., 2014). Therefore, these green additives are of high interest in the aquaculture industry (da Costa and Miranda-Filho, 2020). For example, lutein, one of the significantly cheaper natural carotenoid pigments compared to astaxanthin, can be extracted from some vegetables like marigolds (da Costa and Miranda-Filho, 2020). Ettefaghdoost and Haghighi (2021) reported that dietary supplementation with 200 mg/kg lutein could enhance the growth performance, immune capacity and total carotenoid pigment content in different tissues of *Macrobrachium nipponense*.

Litopenaeus vannamei, one of the most profitable shrimp species in south China, is very popular with customers because of its delicious flesh and high nutritional value (Sirirustananun et al., 2011). Previous studies demonstrated that both lutein and astaxanthin play essential roles in growth performance, anti-resistance as well as pigmentation of aquatic animals (Kalinowski et al., 2005; Meilisza et al., 2017). However, knowledge about whether lutein can become a substitution for astaxanthin remained little understood. In our present study, diet supplementation with/without lutein or astaxanthin was compared regarding the effects of the two carotenoids on growth performance, anti-resistance and metabolism of *L. vannamei*. These results may provide a reference for the efficient diet formulation of *L. vannamei*.

MATERIALS AND METHODS

Experiment Diet

In the current study, the commercial lutein (5% lutein) and astaxanthin (10% astaxanthin) were kindly provided by Guangzhou Leader Bio-Technology Co., Ltd., China. Other ingredients like fish meal were all purchased from Guangzhou Chengyi Co., Ltd., China. Five isonitrogen and isolipids experiment diets were formulated with or without lutein or astaxanthin [control group (C); the lutein (L) groups, L1-L3, contained 0, 62.5 ppm, 75 ppm, 87.5 ppm lutein, respectively, the astaxanthin (A) group contained 50 ppm astaxanthin] (**Table 1**). All feeds contained 405 g kg^{-1} crude protein and 71 g kg^{-1} crude lipid approximately. The diets were made according to the methods reported by Niu et al. (2012). Briefly, all ingredients presented in the **Table 1** and deionized water (250 ml/kg dry ingredients mixture) were thoroughly mixed in the Hobart-type mixer, and then the diets (1.2 mm diameter) were extruded using the pelletizer (South China University of Technology, Guangdong, China). Then, diets were heated for 60 min in a ventilated oven (70°C). Afterward, the diets were stored at −20°C and were kept in dark prior to the feeding trail.

Shrimp and Experimental Conditions

Juvenile *L. vannamei* were obtained and cultured at the Chinese Academy of Fishery Sciences (Lingshui, China). Before the feeding experiment, shrimps were fed with the C group diet for 2 weeks to acclimate to the experiment conditions. At the beginning of the feeding trial, 800 lively shrimps (initial body weight: 0.64 ± 0.04 g) with similar size were distributed randomly into recirculating water systems with 20 cylindrical fiber tanks (300 L). Each diet was randomly assigned to quadruplicate tanks.

TABLE 1 | Ingredients and proximate composition of five experiment diets (g kg^{-1} diets).

Ingredients	C	L1	L2	L3	A
Fish meal	250	250	250	250	250
Soybean meal	270	270	270	270	270
Peanut meal	120	120	120	120	120
Wheat flour	232.2	232.2	232.2	232.2	232.2
Beer yeast	30	30	30	30	30
Shrimp meal	30	30	30	30	30
Fish oil	10	10	10	10	10
Soybean lecithin	10	10	10	10	10
Soybean oil	10	10	10	10	10
Choline chloride (50Vitamin C phosphate	1	1	1	1	1
Vitamin and mineral premix[a]	20	20	20	20	20
Calcium dihydrogen phosphate	10	10	10	10	10
Astaxanthin[b]	0	0	0	0	0.5
Lutein[b]	0	1.25	1.5	1.75	0
Cellulose	1.75	0.5	0.25	0	1.25
Sum	1000.00	1000.00	1000.00	1000.00	1000.00
Nutrient levels (g kg^{-1})					
Moisture	74.5	79.2	76.6	73.7	75.8
Crude lipid	71.2	70.3	69.7	72.8	70.8
Crude protein	405.2	408.3	406.9	404.1	404.8

[a]*Composition of vitamin and mineral mixture (kg-1 of mixture): vitamin A, 250,000 IU; riboflavin, 750 mg; pyridoxine HCL, 500 mg; cyanocobalamin, 1 mg; thiamin, 500 mg; menadione, 250 mg; folic acid, 125 mg; biotin, 10 mg; a-tocopherol, 3,750 mg; myo-inositol, 2,500 mg; calcium pantothenate, 1,250 mg; nicotinic acid, 2,000 mg; vitamin D3, 45,000 IU; vitamin C, 7,000 mg; Zn, 4,000 mg; K, 22.500 mg; I, 200 mg; NaCl, 2.6 g; Cu, 500 mg; Co, 50 mg; $FeSO_4$, 200 mg; Mg, 3,000 mg; Se, 10 mg.*

[b]*Supplied by Guangzhou Leader Bio-Technology Co., Ltd., China.*

Shrimps were fed to apparent satiation three times (at 6:00, 12:00, and 18:00; with 5–8% body weight) daily for 8 weeks.

During the feeding trial, the water temperature was maintained 26.9–28.2°C, pH 7.6–7.8, salinity 30–32‰, dissolved oxygen > 7 mg/L, total ammonia nitrogen < 0.1 mg/L, and sulfide < 0.05 mg/L.

Sample Collection

At the end of the feeding trial, shrimps were starved for 24 h and then weighed. The total number of shrimps in each tank was counted at the same time. Afterward, eight shrimps from each tank were randomly collected and anesthetized (MS-222, Sigma, St. Louis, MO, United States) to collect the hemolymph samples. Subsequently, shrimps were dissected and hepatopancreas samples were removed for enzyme and RNA expression analysis. The hemolymph sample was centrifuged (7,100 g, 10 min, 4°C) and the hemolymph was separated for antioxidant parameter analysis. All samples were collected rapidly and frozen in liquid nitrogen until analysis.

Proximate Composition Analysis

Proximate compositions of diets were assayed using the standard methods of AOAC (Horwitz et al., 2010). Briefly, diets crude protein content (N × 6.25) was detected using the Kjeldahl method (1030- Autoanalyzer; Tecator, Höganäs, Sweden). Diet crude lipid was detected by the Soxhlet extractor method (Soxtec System HT6, Tecator, Sweden). Diets moisture was analyzed by drying the diets in oven at 105°C to constant weight.

Detection of Hepatopancreas and Hemolymph Antioxidant Parameters

Hepatopancreas samples were homogenized and centrifuged according to the methods of Fang et al., 2021b). Briefly, hepatopancreas samples and phosphate buffer (1:10) were mixed and homogenized, and then centrifuged for 10 min (4°C, 1,200 g), and the supernatants were collected. Supernatants, as well as hemolymph samples, were determined for antioxidant parameters, including superoxide dismutase (SOD) (A001–1), total antioxidant capacity (T-AOC) (A015–2-1) and malondialdehyde (MDA) (A003–1). The analysis of antioxidant parameters was carried out following the kits' instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Hepatopancreas RNA Extraction and Expression Analysis

RNA extraction and Real-time PCR were following the methods reported in our previous articles (Fang et al., 2019). Briefly, total RNA was extracted from the hepatopancreas using Trizol® reagent (Invitrogen, United States) following the manufacturer's instructions. 1% agarose gel electrophoresis and spectrophotometer (NanoDrop 2,000, Thermo Fisher, United States) were used to determine RNA quality and concentration, respectively. Afterward, cDNA was synthesized using the PrimeScript™ RT reagent kit (Takara, Japan), following the manufacturer's instructions. Real-time PCR for the target genes was performed using an SYBR® Premix Ex Taq™ II (Takara, Japan) and quantified on the LightCycler 480 (Roche Applied Science, Basel, Switzerland).

The primers used in the current study are presented in **Table 2**.

Statistical Analysis

All data in the current study are presented as means ± standard error (SE). Data were analyzed by SPSS 22.0 (SPSS, Chicago, IL, United States) and followed by one-way analysis of variance (ANOVA) and Duncan's test. $P < 0.05$ was regarded as significant difference among groups.

RESULTS

Growth Performance

As shown in **Table 3**, diet supplementation with a suitable level of lutein or astaxanthin significantly altered the growth performance of *L. vannamei*. Significantly higher weight gain rate (WGR) and specific growth rate (SGR) of *L. vannamei* were obtained in the L1, L2, and A groups compared to the C group ($P < 0.05$), while no statistical difference was found between the C and L3 groups ($P > 0.05$). At the same time, data in the A group showed no significant difference compared with the L1 and L2 groups ($P > 0.05$). Feed conversion ratio (FCR) of *L. vannamei* in the A group was significantly lower than that in the C group ($P < 0.05$) and no statistical difference was observed compared

TABLE 2 | Sequences of primers used for real-time quantitative PCR.

Gene	Primer sequence (5′-3′)
EF1a-F	TGGCTGTGAACAAGATGGAC
EF1a-R	AGATGGGGATGATTGGGACC
SOD-F	CCGTGCAGATTACGTGAAGG
SOD-R	GTCGCCACGAGAAGTCAATG
GSH-PX F	GGCACCAGGAGAACACTAC
GSH-PX R	CGACTTTGCCGAACATAAC
CAT-F	TACTGCAAGTTCCATTACAAGACG
CAT-R	GTAATTCTTTGGATTGCGGTCA
HSP70-F	CAACGATTCTCAGCGTCAGG
HSP70-R	ACCTTCTTGTCGAGGCCGTA
Caspase 2-F	ATGGCTCGTGGTTCATTCAG
Caspase 2-R	CATCAGGGTTGAGACAATACAGG
Caspase 3-F	AGTTAGTACAAACAGATTGGAGCG
Caspase 3-R	TTGTGGACAGACAGTATGAGGC
Relish-F	CTACATTCTGCCCTTGACTCTGG
Relish-R	GGCTGGCAAGTCGTTCTCG
Rho-F	GTGATGGTGCCTGTGGTAAA
Rho-R	GCCTCAATCTGTCATAGTCCTC
Chymotrypsin-F	GGCTCTCTTCATCGACG
Chymotrypsin-R	CGTGAGTGAAGAAGTCGG
Trypsin-F	TCCAAGATCATCCAACACGA
Trypsin-R	GACCCTGAGCGGGAATATC
HK-F	AGTCGCAGCAACAGGAAGTT
HK-R	CGCTCTTCTGGCACATGATA
FAS-F	GCGTGATAACTGGGTGTCCT
FAS-R	ACGTGTGGGTTATGGTGGAT

TABLE 3 | Growth performance of *L. vannamei* fed diet supplementation with/without lutein or astaxanthin for 56 days.

	C	L1	L2	L3	A
IBW[a]	0.64 ± 0.01	0.61 ± 0	0.64 ± 0.01	0.66 ± 0.01	0.63 ± 0.01
FBW[b]	5.98 ± 0.03^{a}	6.44 ± 0.08^{c}	6.34 ± 0.06^{bc}	6.14 ± 0.09^{ab}	6.5 ± 0.08^{c}
WGR (%)[c]	828.31 ± 15.07^{a}	950.96 ± 10.36^{c}	890.33 ± 2.3^{b}	832.82 ± 3.01^{a}	926.69 ± 17.54^{bc}
SGR (%)[d]	3.98 ± 0.03^{a}	4.2 ± 0.02^{c}	4.09 ± 0^{b}	3.99 ± 0.01^{a}	4.16 ± 0.03^{bc}
FCR[e]	1.24 ± 0.03^{a}	1.19 ± 0.02^{ab}	1.18 ± 0.05^{ab}	1.18 ± 0.07^{ab}	1.12 ± 0.02^{b}
SR (%)[f]	96.25 ± 1.25	94.38 ± 2.13	96.67 ± 2.2	98.13 ± 2.77	96.88 ± 0.63

[a] *IBW (g per shrimp), initial body weight.*
[b] *FBW (g per shrimp), final body weight.*
[c] *Weight gain rate (WGR,%) = 100 * (final body weight–initial body weight)/initial body weight.*
[d] *Specific growth rate (SGR,% day-1): 100 × (Ln final shrimp weight–Ln initial shrimp weight)/the experimental duration in days.*
[e] *Feed conversion ratio (FCR) = dry diet fed/wet weight gain.*
[f] *Survival rate (SR) (%) = 100 * (final number of shrimp)/(initial number of shrimp).*
Values are mean ± SE (n = 4). Means in the same row with different superscripts are significantly different ($P < 0.05$).

with the other three lutein-supplemented groups ($P > 0.05$). The survival rate (SR) of *L. vannamei* ranged from 94.38 to 98.13% after 8 weeks of feeding treatment ($P > 0.05$).

Antioxidant Capacity

The hepatopancreas and hemolymph antioxidant parameters of *L. vannamei* fed with/without dietary lutein or astaxanthin are shown in **Table 4**. Four carotenoid pigments-supplemented groups (L1-L3, A) all had significantly lower enzyme activity of hepatopancreas T-SOD, lower level of hemolymph T-AOC and lower concentration of hemolymph MDA than the C group ($P < 0.05$). Meanwhile, no significant difference in hemolymph T-AOC and MDA of *L. vannamei* was found among the L1-3 and A groups ($P > 0.05$). Although there was no statistical difference in hepatopancreas MDA among five experiment groups ($P > 0.05$), the MDA value in four carotenoid pigment-supplied groups was still relatively lower than the control group.

RNA Expression of Genes Related to Immunity

RNA expression levels of antioxidative genes (SOD, GSH-PX, and CAT) in the hepatopancreas of *L. vannamei* are presented in **Figure 1**. The RNA expression levels of hepatopancreas GSH-PX and CAT in four carotenoid pigments-supplemented groups showed significantly lower values compared to the C group ($P < 0.05$), and data were not statistically different among these four groups ($P > 0.05$). Compared to the C group, the RNA expression level of SOD was no statistically different in *L. vannamei* fed with/without dietary lutein or astaxanthin ($P > 0.05$).

Hepatopancreas RNA expression of anti-inflammatory genes (Relish, Rho, HSP70) of *L. vannamei* exposed to different diet treatments was shown in **Figure 2**. The RNA expression level of HSP70 in the A group was significantly lower than that in the C group ($P < 0.05$), and no statistical difference was observed compared with the other three lutein groups ($P > 0.05$). Significantly lower data of Relish has been observed in four carotenoid pigments-supplemented groups compared to the C group ($P < 0.05$). However, regarding the RNA expression level of Rho, no statistical difference was found among all the groups ($P > 0.05$).

As shown in **Figure 3**, the hepatopancreas RNA expression level of Caspase 3 in four carotenoid pigments groups were significantly lower compare to the C group ($P < 0.05$), and the data were not statistically different among these four groups ($P > 0.05$). Apart from that, significantly lower RNA expression level of Caspase 2 was found in the L3 and A groups than in C, L1 and L2 groups ($P < 0.05$).

RNA Expression of Genes Related to Digestive and Metabolic Enzymes

As shown in **Figure 4**, diet supplementation with lutein or astaxanthin was unable to alter the hepatopancreas RNA expression level of digestive enzyme genes (chymotrypsin and trypsin) of *L. vannamei* ($P > 0.05$).

Hepatopancreas RNA expression levels of metabolic enzyme genes [Hexokinase (HK) and Fatty acid synthase (FAS)] of *L. vannamei* fed diet supplemented with/without lutein or astaxanthin were shown in **Figure 5**. Regarding the RNA expression level of HK, the data in the L1 and L2 groups were significantly higher than that of the C and A groups ($P < 0.05$). In addition, the RNA expression level of FAS in the L2 and A groups was higher than that in the C group ($P < 0.05$), and statistical difference was not found between the L2 and A group ($P > 0.05$).

DISCUSSION

Diet supplementation with carotenoid pigment could improve the growth performance of the aquatic animals. For example, dietary astaxanthin supplementation could optimize the growth performance of *Marsupenaeus japonicus* (Wang et al., 2018), *Paralithodes camtschaticus* (Daly et al., 2013), *Trachinotus ovatus* (Fang et al., 2021a) and *Micropterus salmoides* (Xie S. et al., 2020). In addition, diet supplementation with β-carotene also enhanced the growth performance of *Penaeus monodon* (Niu et al., 2014), *Piaractus mesopotamicus* (Bacchetta et al., 2019) and *Oreochromis niloticus* × *O. aureus* (Hu et al., 2006). Similar results were shown in the present study. Carotenoid

TABLE 4 | Hepatopancreas and hemolymph antioxidant parameter of *L. vannamei* fed diet supplementation with/without lutein or astaxanthin for 56 days.

	C	L1	L2	L3	A
Hepatopancreas					
T-SOD (U/mgprot)	10.4 ± 0.88^{a}	7.79 ± 0.34^{b}	6.3 ± 0.4^{bc}	7.52 ± 0.71^{b}	5.3 ± 0.19^{c}
T-AOC (mgprot/ml)	0.27 ± 0.01	0.17 ± 0.03	0.24 ± 0.09	0.22 ± 0.02	0.16 ± 0.02
MDA (nmol/mgprot)	1.26 ± 0.03	1.13 ± 0.09	0.89 ± 0.24	1.03 ± 0.13	0.81 ± 0.07
Hemolymph					
T-SOD (U/mgprot)	273.75 ± 6.08^{a}	228.35 ± 8.56^{c}	251.38 ± 2.01^{abc}	241.72 ± 6.46^{bc}	257.08 ± 10.09^{ab}
T-AOC (mgprot/ml)	3.7 ± 0.12^{a}	2.59 ± 0.19^{b}	3 ± 0.15^{b}	2.59 ± 0.19^{b}	2.84 ± 0.25^{b}
MDA (nmol/mgprot)	8.27 ± 1.04^{a}	3.92 ± 0.28^{b}	4.69 ± 0.74^{b}	4.15 ± 0.41^{b}	3.54 ± 0.13^{b}

Values are mean ± SE (n = 4). Means in the same row with different superscripts are significantly different (P < 0.05).

pigment-supplemented groups except for the L3 group all showed significantly higher growth performance (WGR and SGR) of *L. vannamei* than the C group. There were two main reasons for why carotenoid pigments could improve the growth performance of crustaceans. Firstly, the carotenoid pigment could regulate the metabolism of aquatic animals and thus promote the digestion of nutrients (Baron et al., 2008; Zhang et al., 2013). In addition, the carotenoid pigment could shorten the molt cycle interval of crustaceans and regulate the NADPH metabolism for reducing energy consumption, resulting in enhanced growth performance (Hertrampf and Piedad-Pascual, 2012; Mao et al., 2017). Meanwhile, in the current study, similar growth performances (WGR and SGR), FCR, and SR of *L. vannamei* were found in the L1-2 groups and A group, indicating that diet supplementation with lutein ranging from 62.5 to 75 ppm could substitute astaxanthin in the diets of *L. vannamei*. However, previous studies also showed that carotenoid pigment was unable to alter the growth performance of aquatic animals. For example, dietary astaxanthin could not improve the growth performance of *Portunus trituberculatus* (Han et al., 2018), *Eriocheir sinensis* (Jiang et al., 2020), *Symphysodon* spp. (Song et al., 2017), and *Pagrus pagrus* (Nogueira et al., 2021). These results might be attributed to the culture environment, aquatic animal species and carotenoid pigment doses.

Nutrient digestion and metabolism capacity in aquatic animals are also crucial parameters for evaluating a diet additive. In the present study, no significant differences in RNA expression level of Chymotrypsin and Trypsin were found between the four carotenoid pigment treatment groups and the C group, indicating that dietary carotenoid pigment was unable to alter the protein digestive capacity of *L. vannamei*. In terms of

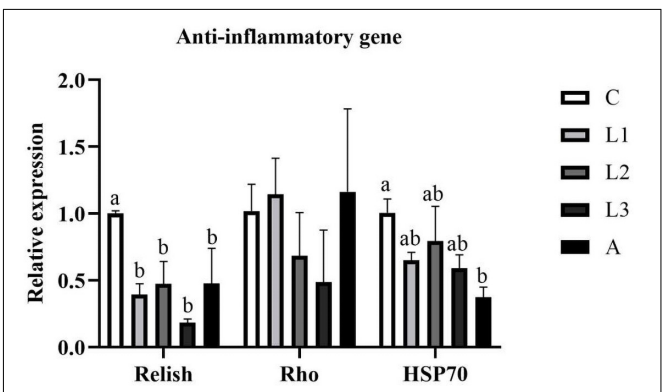

FIGURE 2 | Hepatopancreas RNA expression level of anti-inflammatory genes of *L. vannamei* fed diet supplementation with/without lutein or astaxanthin for 56 days.

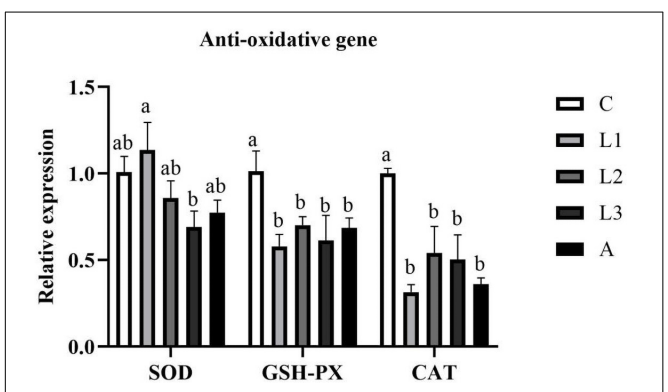

FIGURE 1 | Hepatopancreas RNA expression level of antioxidative genes of *L. vannamei* fed diet supplementation with/without lutein or astaxanthin for 56 days.

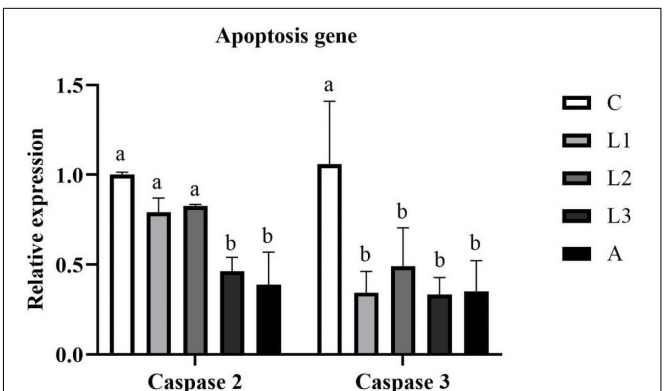

FIGURE 3 | Hepatopancreas RNA expression level of apoptosis genes of *L. vannamei* fed diet supplementation with/without lutein or astaxanthin for 56 days.

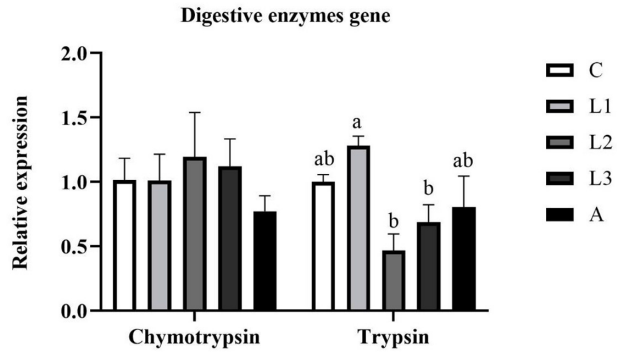

FIGURE 4 | Hepatopancreas RNA expression level of digestive enzyme genes of *L. vannamei* fed diet supplementation with/without lutein or astaxanthin for 56 days.

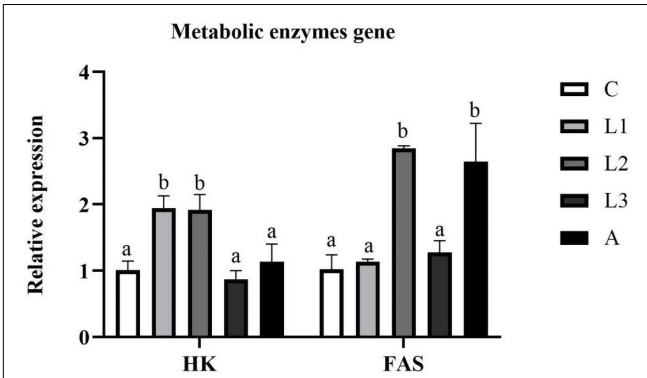

FIGURE 5 | Hepatopancreas RNA expression level of metabolic enzymes genes of *L. vannamei* fed diet supplementation with/without lutein or astaxanthin for 56 days.

metabolic enzyme genes, HK is a critical rate-limiting enzyme in regulating the glycolysis cycle; FAS plays a significant role in catalyzing the synthesis of the fatty acids (Chirala and Wakil, 2004; Guo et al., 2008). In the present study, RNA expression level of HK in the L1 and L2 groups was higher than in other groups, indicating that diet supplementation of lutein ranging from 62.5 to 75 ppm could improve the capacity of carbohydrate metabolism. In addition, RNA expression level of FAS in the L2 and A groups was significantly higher than in other groups, meaning that lutein and astaxanthin could improve the fat synthesis ability of *L. vannamei*.

During the feeding trial, the shrimp might be affected by several uncontrollable external factors, such as water temperature change, bacterial contaminations in aquatic water and feed, as well as stress caused by the feeding process. In this situation, oxidative burst would occur in cells to produce numerous reactive oxygen species (ROS) to protect the organism from oxidative stress (Wilhelm Filho, 2007; Banh et al., 2016). However, ROS overproduction may cause DNA damage and other severe detriments in cells (Piconi et al., 2006; de Souza Grinevicius et al., 2016). If cells were exposed to excess ROS, they would activate the antioxidant system and upregulate the expression of antioxidant genes and enzymes, like SOD, GSH-PX, and CAT (Ighodaro and Akinloye, 2018). In addition, T-AOC was also a significant parameter to reflect the antioxidant capacity of crustaceans (Dai et al., 2018). Since carotenoid pigment contains ionone rings with hydroxyl and keto, they could remove ROS and protect cells from various environmental stress (Chien et al., 2003). In the current study, compared to the control group, significantly lower levels of hepatopancreas T-SOD activity, hemolymph T-AOC and RNA expression of GSH-Px and CAT were observed in four carotenoids pigments-supplemented groups, indicating lutein and astaxanthin could act as an antioxidant for scavenging ROS and thus protecting cells from oxidative stress. As a result, the cells unnecessarily stimulate the antioxidant system and produce more antioxidant enzymes. These results were similar to the previous results reproted by Zhang et al. (2013), Xie J. et al. (2020), and Ettefaghdoost and Haghighi (2021). MDA is a product of lipid peroxidation, which can be used to evaluate the damage degree of cell structure and function (Çilingir Yeltekin and Oğuz, 2018). The present study demonstrated that significantly lower hemolymph MDA was obtained in four carotenoid pigment treatment groups than the C group, indicating lutein and astaxanthin could improve the antioxidant capacity of *L. vannamei*. No significant statistical difference in hemolymph T-AOC, MDA, and hepatopancreas RNA expression level of GSH-PX, CAT between dietary lutein treatment groups (L1-3) and the astaxanthin group was found in the present study, indicating that dietary supplementation with lutein ranging from 62.5 to 87.5 ppm could obtain similar antioxidant effects *in L. vannamei* compared to astaxanthin.

The antioxidant property of lutein and astaxanthin had been widely reported (Zhang et al., 2013; Rahman et al., 2016; Song et al., 2017). However, few studies focused on anti-inflammation and other immune functions of these carotenoid pigments. The NF-κB pathway is an essential moderator related to inflammatory responses, and Relish as well as Rho are two essential NF-κB family proteins (Ko et al., 2017; Xie et al., 2018). In addition, HSP70 could also exert the anti-inflammatory property by stimulating its IL-10 producing T cells (Wendling et al., 2000; Tanaka et al., 2007). Lower expression of inflammatory cytokines or higher expression of anti-inflammatory cytokines can suppress the inflammatory response (Zhao et al., 2020). In the present study, significantly lower RNA expression level of Relish was found in four carotenoid pigment treatment groups than in the C group, indicating lutein and astaxanthin could improve the anti-inflammation capacity of *L. vannamei* by regulating the NF-κB pathway. Caspases, which belong to the cysteine proteases family, are essential regulators of programmed cell death (apoptosis) (Taylor et al., 2008). In general, Caspase 2 was a factor which mainly responded to stress-induced apoptosis, while Caspase 3 frequently acted as a proenzyme and was activated to destroy various specific cellular structures and proteins (Dorstyn et al., 2012; Gao et al., 2013). The present study demonstrated significantly lower RNA expression level of Caspase 3 in four carotenoid pigment treatment groups than in the control group, indicating diet supplementation of lutein or astaxanthin could

inhibit the apoptosis of *L. vannamei*. In addition, no statistical differences in Relish, HSP70 and Caspase 3 were observed among L1-L3 and A groups, meaning that diet supplementation of lutein ranging from 62.5 to 87.5 ppm could stimulate similar immune response in *L. vannamei* compared to diet supplemented with astaxanthin.

CONCLUSION

Overall, diet supplementation of lutein ranging from 62.5 to 75 ppm could result in similar growth performance, antioxidant capacity and immune response of *L. vannamei* compared with diet supplemented with 50 ppm astaxanthin. Therefore, dietary lutein was suggested to be suitable in the diet of *L. vannamei* for substituting astaxanthin.

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/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Experimental Animal Ethics Committee of Sun Yat-sen University.

AUTHOR CONTRIBUTIONS

HF, YL, and LT designed the study. HZ provided relative experiment material. HF, XH, and JN analyzed data. HF carried out the experiment and wrote this manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Marine Science

REVIEW
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Tracing the Trophic Fate of Aquafeed Macronutrients With Carbon Isotope Ratios of Amino Acids

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To meet future seafood demands, ingredients derived from algae and other novel and sustainable sources are increasingly being tested and used as replacers to traditional aquafeed ingredients. Algal ingredients in particular are being promoted for their sustainability and their additional functional attributes in farmed aquatic animals. Test on algal supplemented aquafeeds typically focus on a suite of immunological and physiological indicators along with fish growth performance or muscle quality. However, to optimize the replacement of fish meal with algal derived ingredients, it is crucial to understand the metabolic fate in the algal macronutrients (carbohydrates, fats, and proteins), and their nutritional interactions with other ingredients after ingestion. Here, we assess the potential of using the emerging technology- stable carbon isotope ($\delta^{13}C$) analysis of single amino acids (AAs) as a nutritional biomarker in aquaculture. Applications of $\delta^{13}C_{AA}$-based approaches in feeding trials show promise in closing the knowledge gap in terms of understanding how fish and other aquaculture taxa assimilate and metabolize algal derived macronutrients. Source diagnostic $\delta^{13}C$ fingerprints among the essential AAs can trace the protein origins to broad phylogenetic groups such as red macroalgae, brown macroalgae, bacteria, and terrestrial plants. Among the non-essential AAs, $\delta^{13}C$ patterns have the potential to inform about metabolic routing and utilization of dietary lipids and carbohydrates. Despite the potential of $\delta^{13}C_{AA}$ as a nutritional biomarker, the few applications to date in fish feeding trials warrant further development and implementation of $\delta^{13}C_{AA}$-based approaches to improve understanding of protein origins and macronutrient metabolic routing.

Keywords: macroalgae, aquafeed, metabolism, salmon, isotope fingerprinting, nutrition, essential and non-essential amino acids

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INTRODUCTION

Novel macro aquafeed ingredients are continually being developed and validated to lower the demand of marine-derived proteins, i.e., fish meal and oil, but also to allow the sustainable increase in fish production. For instance, marine macroalgae, or seaweeds, possess a number of positive nutritional and functional attributes that could be exploited in aquafeeds (Xu et al., 2017; Gomez-Zavaglia et al., 2019; Thépot et al., 2021). Seaweeds can be classified into three major phylogenetic

groups that each have unique pigmentation and biochemical characteristics: (1) brown seaweed (Phaeophyceae); (2) red seaweed (Rhodophyceae); and (3) green seaweed (Chlorophyceae). These multicellular macroscopic algae are considered to have a smaller environmental footprint compared to fish meal and terrestrial plants crops (e.g., soybean, cereals, and legumes) that are typically used in aquafeeds. This is because algae can be grown at scale without the need for arable land, freshwater, and substantial amounts of non-renewable resources (e.g., fertilizers and pesticides). Furthermore, the use of seaweeds in aquafeed can play a role in mitigating the effects of eutrophication and climate impact through the bioremediation of nitrogen and phosphorous, and carbon capture (Duarte et al., 2017; Xiao et al., 2017).

One of the key attractiveness of using macroalgae is that they possess bioactive compounds and functional properties that can be conferred to the aquafeeds and the farmed aquatic animal, e.g., as immunomodulation, maintaining fish growth, and feed physical-chemical integrity (**Figure 1**; Gomez-Zavaglia et al., 2019). Inclusions of macroalgal ingredients have also been tested for crustaceans and echinoderms (Schleder et al., 2017; Omont et al., 2021; Xu et al., 2021). Besides supplying a source of dietary protein in aquafeeds, the proteins and peptides found in seaweeds have shown an extensive range of properties that include antihypertensive, antioxidative, and immune-supportive activities in fish (Harnedy and Fitzgerald, 2011). These bioactives could potentially be further augmented and enhanced by biorefinery techniques, such as hydrolyzing the seaweed proteins, optimizing the amino acid (AA) composition, and releasing previously bound bioactive sites and/or compounds (Choi et al., 2015; Yan and Wang, 2019; Thépot et al., 2021). Furthermore, some polysaccharides found in macroalgae such as those classified as phycocolloid and their derivatives can modify the gut microbiome (prebiotic) and possess immunostimulatory effects (Roberfroid, 2007; Xu et al., 2017). Similarly, many secondary metabolites produced by the algae such as mycosporine-like amino acids (Carreto and Carignan, 2011), carotenoids, and phenolics (Dethier et al., 2005) can also confer significant health benefits to farmed aquatic animals.

Feeding trials that test dietary seaweed inclusions typically focus on a suite of immunological parameters in concert with fish growth performance or muscle quality (Wan et al., 2018; Hua et al., 2019; Thépot et al., 2021). Moreover, the inclusion of seaweeds in aquafeeds can lead to several nutritional benefits: Their AA composition is relatively complete and although commercially important seaweeds can be limited in their lipid content, the quality of these lipids are typically higher than those derived from terrestrial plants (Wan et al., 2018). Many macroalgal species possess a high proportion of polyunsaturated fatty acids (PUFA) compared to, e.g., terrestrial ingredients. In particular, long-chain ω-3 highly unsaturated fatty acids (HUFA, e.g., eicosapentaenoic acid, 20:5nω3), which can increase mitochondrial β-oxidation activity leading to leaner farmed fish (Todorčević et al., 2009). The levels and types of complex polysaccharides found in different macroalgae species are major factors in influencing nutrient digestibility (Gyurcsik and Nagy, 2000; Marrion et al., 2003). In addition, the natural feeding strategy and genotype in the farmed fish are important variables that determine the efficiency to which seaweeds are utilized as a nutrient source (Krogdahl et al., 2005; Kamalam et al., 2017). Even though carnivorous and omnivorous species have reduced capacity to digest and utilize complex polysaccharides, feeding trials have demonstrated that in addition to enhancing innate immune responses, seaweed inclusions in aquafeed can enhance growth and feed intake (Wan et al., 2016; Thépot et al., 2022).

Despite a suite of beneficial functional properties, the metabolic fate of algal macronutrients are generally poorly understood. This knowledge gap makes it more challenging to optimize the use of macroalgae as an aquafeed ingredient – especially for carnivorous species. Isotopic evidence shows it is unlikely that algal polysaccharides function as energy sources for Atlantic salmon (*Salmo salar*) (Wang et al., 2019). This is probably due to a lack or low presence of complex carbohydrate degrading enzymes, e.g., cellulase, and hemicellulose (Hidalgo et al., 1999). This lack of assimilation of macroalgal ingredients should in theory decrease the feed conversion ratio, at least for carnivorous species. Hence, to strike an optimal balance between the health benefits of seaweeds and feed utilization, it is crucial to trace the metabolic fate of seaweed ingredients in aquaculture animals. While there has been only a limited number of seaweeds tested in feeding trials to date (Wan et al., 2018; Hua et al., 2019; Thépot et al., 2021), it is also important to be mindful that seaweeds are a highly diverse group of organisms.

While molecular methods (e.g., DNA quantification and targeted metabolite profiling) are suited for characterizing aquafeed ingredients before or right after ingestion, stable isotope analyses of animal tissues are usually employed to characterize ingredients after their nutrients have been absorbed (Belghit et al., 2021; Gamboa-Delgado, 2022). The stable isotope values of total organic carbon (bulk $\delta^{13}C$), the most commonly analyzed element, have been used successfully for food authentication and tracing aquaculture ingredients (Molkentin et al., 2007; Anderson et al., 2010; Hassoun et al., 2020). Isotopes can be analyzed on different tissues. For example, blood and splanchnic tissues (e.g., intestine and spleen) are used to infer more recent diets because they have a higher turnover rate than structural tissues (e.g., muscle fiber and tendon) (Tieszen et al., 1983; Buchheister and Latour, 2010). It can, however, be challenging interpreting bulk isotope results because $\delta^{13}C$ values are affected by a host of physical, chemical and physiological variables that can confound isotope values among different sources and impart variable and relatively poorly constrained isotope discrimination during trophic transfer (Casey and Post, 2011; Robinson et al., 2021). Among marine-derived ingredients, a common challenge with bulk $\delta^{13}C$ tracers is that they lack the ability in distinguishing between, e.g., marine fish and macroalgae because of the equifinality of those sources.

Recently, stable isotope analysis in single AAs has shown considerable more precision in discerning among algae and other novel functional feed additives within test aquafeed composites (Wang et al., 2018b, 2019; Belghit et al., 2021; Xu et al., 2021). In terms of nutritional requirements, the 20 proteogenic AAs can be divided into two functional classes: the essential and the

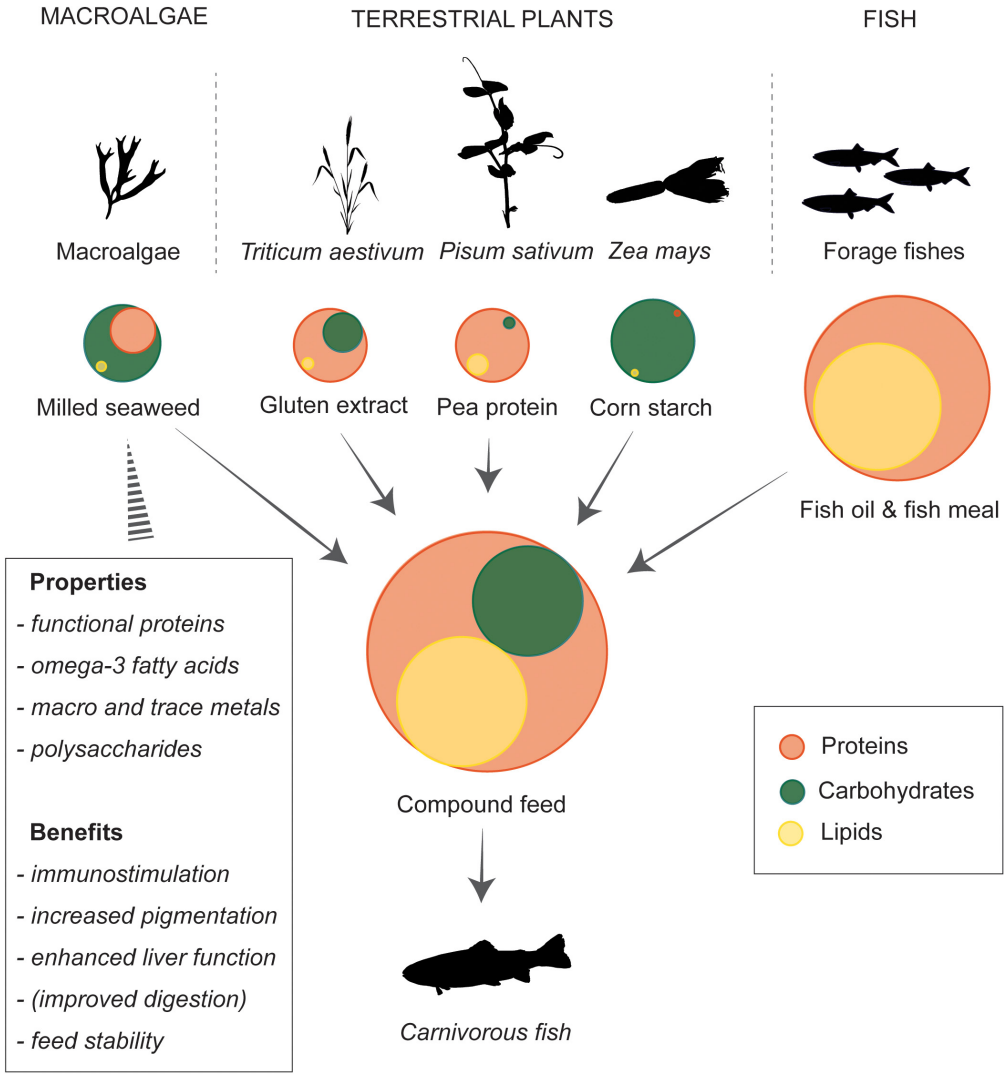

FIGURE 1 | Schematic presentation of seaweed inclusion in compound feeds containing plant and fish derived ingredients for a carnivorous fish. The filled circles represent the relative proportion of macronutrients in each ingredient in the P10 experimental diet by Wan et al. (2016), the arrows pointing to the compound feed denote its ingredients, the arrow to the carnivorous fish indicates it feeds on the compound diet, and the striped triangle links to the box outlining key properties and benefits of milled seaweed ingredients in aquafeed. Silhouettes from PhyloPic (http://phylopic.org) under Creative Commons license.

non-essential. Like many other animals, the AAs that finfish cannot synthesize *de novo* are referred to as essential amino acids (EAAs) and are usually passed on from one trophic level to the next with no or minor alterations of their carbon skeletons, i.e., the carboxyl group and the α-carbon to which a variety of functional groups are attached. Regarding the non-essential amino acids (NEAAs), animals can incorporate them directly from the diet or synthesize them *de novo* from both glycolytic and tricarboxylic acid (TCA) cycle intermediates (see **Figure 2**). Therefore, the differences in isotopic composition between a consumer and its diet, which is denoted $\Delta\delta^{13}C$, is greater for the NEAAs than the EAAs (McMahon et al., 2010; Barreto-Curiel et al., 2017; Liu et al., 2018; Wang et al., 2018b; Xu et al., 2021).

This review will examine the analytical and theoretical underpinnings behind $\delta^{13}C_{AA}$ analysis, and evaluate how $\delta^{13}C_{AA}$ based methods can be used to understand macronutrient metabolism and trace back dietary protein origins. While this review will focus on the potential of tracing seaweed ingredients in aquafeed fed to farmed finfish, it will also evaluate the use of $\delta^{13}C_{AA}$ analysis in other animal species and dietary sources. Furthermore, the review will discuss the current barriers for using $\delta^{13}C_{AA}$ as a nutritional biomarker in farmed aquaculture animals, and the possible solutions to overcome these obstacles.

AMINO ACID ABBREVIATIONS

Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Asx, asparagine/aspartic acid; Cys, cysteine; His, histidine; Gln, glutamine; Glu, glutamic acid; Glx, glutamine/glutamic acid; Gly,

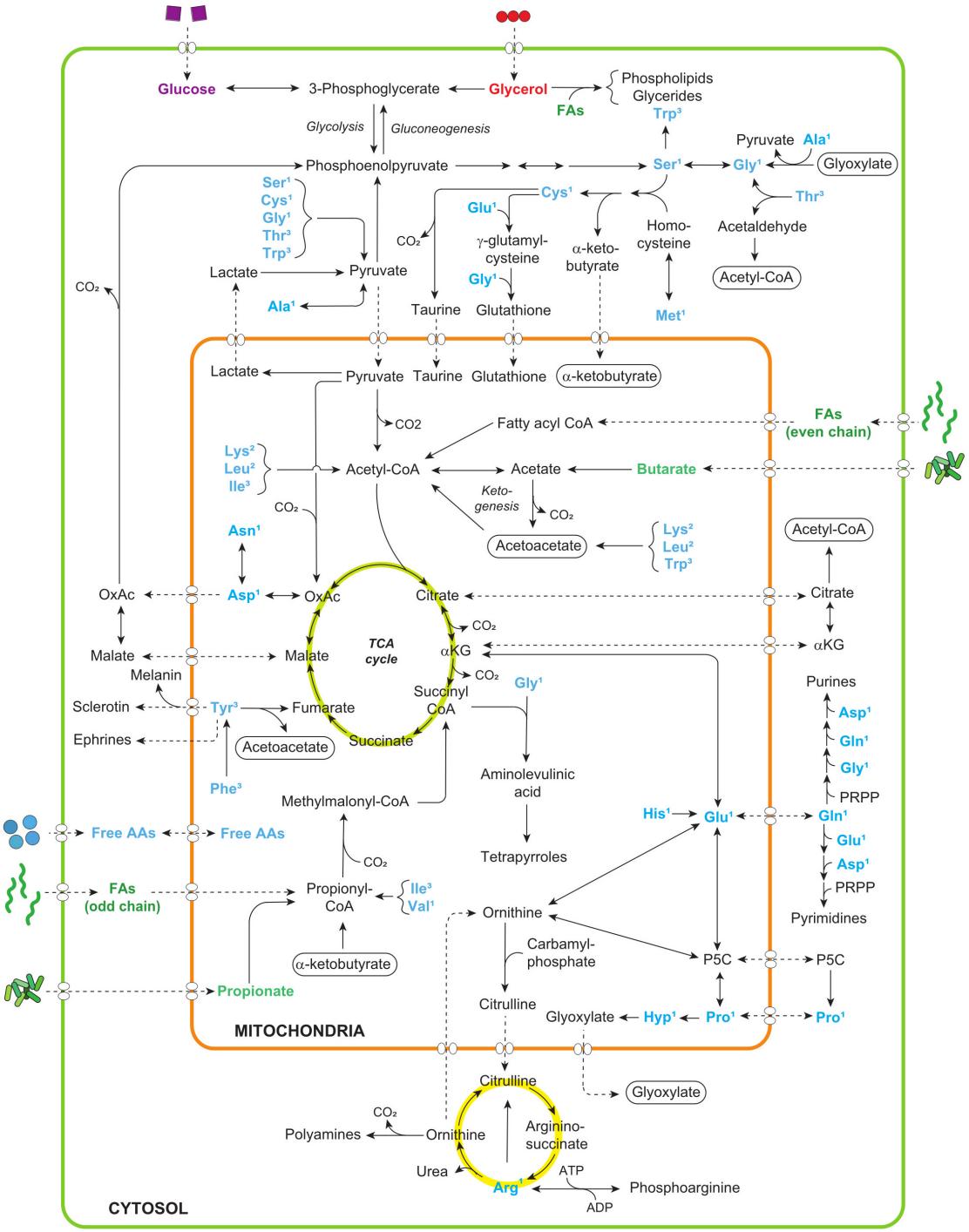

FIGURE 2 | The chart depicts the main anabolic and catabolic amino acid (AA) pathways in vertebrates according to Berg et al. (2015) and Caspi et al. (2020) (all the proteogenic amino acids are assigned three-letter symbols according to IUPAC nomenclature). The non-essential AAs can be grouped according to their association with their main biosynthesis pathways: The glycolytic AAs are synthesized from metabolic intermediates (3-PGA, 3-phosphoglyceric acid; PEP, phosphoenolpyruvic acid) of the glycolytic pathway (in the cytosol) and the Krebs NEAAs are synthesized from intermediates of the or tricarboxylic acid cycle (TCA; αKG, α-Ketoglutaric acid; OAA, oxaloacetate) (in the mitochondria). Glucose and glycerol are sourced to the glycolytic pathway, and fatty acids (FAs) and short chain fatty acids are sourced to the TCA cycle. TCA products can also be function as intermediates for Ala *via* phosphoenolpyruvate and pyruvate. The catabolism of excess AAs either occurs *via* gluconeogenesis or ketogenesis. Gluconeogenesis is the synthesis of glucose from non-carbohydrate precursors such as the glucogenic amino acids (marked with 1) and ketogenesis is the metabolic pathway for producing ketone bodies by breaking down fatty acids and ketogenic amino acids (marked with 2). A large group of AAs can be catabolized by both processes (marked 3). PRPP and P5C signify phosphoribosyl pyrophosphate and 1-pyrroline-5-carboxylic acid, respectively.

glycine; Hyp, hydroxyproline; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Tyr, tyrosine; Thr, threonine; Val, valine.

ANALYTICAL CONSIDERATIONS

Gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) is the most common methodological approach for determining $\delta^{13}C_{AA}$ values. The methodology encompasses three core steps:

(1) sample cleaning and protein isolation (if needed) followed by AA extraction and purification;

(2) derivatization of the AAs to increase their volatility; and

(3) analysis with isotope ratio mass spectrometry where the analytes are separated on a GC column, combusted to CO_2 and other gases before determining the isotopic composition of CO_2 molecules with different molecular weights (e.g., $^{12}C^{16}O^{16}O$, $^{13}C^{16}O^{16}O$, $^{12}C^{16}O^{18}O$).

The most commonly used derivatization approaches for GC-C-IRMS involve esterification of the carboxylic acid group with an acidified alcohol and acylation of the amine, hydroxyl and thiol groups (Corr et al., 2007b). To account for the kinetic isotope effect and added carbon from the reagents, AA mixtures with known $\delta^{13}C$ values are treated in parallel with the collected samples. The resulting $\delta^{13}C$ values of these reference mixtures can then be used to correct for the isotope effect of the samples. The shift in isotope values can be reduced by using a derivatization method that adds as few carbon atoms as possible such as *N*-acetylmethyl esters (NACME) and selecting AA references, alcohols, and acylation agents with $\delta^{13}C$ values that approximate those of the samples of interest (Corr et al., 2007a).

The core tenants underlying $\delta^{13}C_{AA}$ applications are consistency in preparation, measurement, and as mentioned above, correction for added carbon during derivatization and the use of reference standards and samples (Meier-Augenstein and Schimmelmann, 2019). The accuracy and precision of the data depend on the quality of GC separation, interface design and isotopic calibration. In short, the isotopic drift of analytical standards and reference materials should be monitored to ensure data accuracy and precision, and scale normalization should be based on two or more reference analytes (Paul et al., 2007). Operators must also ensure that the GC-C-IRMS system is regularly serviced by changing inlet liners, maintaining the integrity of the guard/main columns, and checking the combustion reactors and leakages when needed. To date, the accuracy of GC-C-IRMS produced $\delta^{13}C_{EAA}$ data has been somewhat inconsistent across laboratories (Arthur et al., 2014; Jarman et al., 2017; Stücheli et al., 2021). Hence, for furthering $\delta^{13}C_{AA}$ applications in aquaculture, it will be essential to carry out inter-laboratory comparison exercises, sharing of standardized protocols, and globally available standard reference materials, e.g., reference centers (Yarnes and Herszage, 2017). Accuracy is less of an issue for liquid chromatography–isotope ratio mass spectrometry (LC-C-IRMS) produced $\delta^{13}C_{AA}$ data since AAs do not need to be derivatized, but they come with the limitation that most studies report a low number of AAs (McCullagh et al., 2008; Smith et al., 2009; Dunn et al., 2011). A further consideration is that GC-C-IRMS based methods require approximately 0.3 μg of total protein per injection as opposed to 6 μg for LC-C-IRMS based methods (Smith et al., 2009; Dunn et al., 2011). Finally, the use of $\delta^{13}C_{AA}$ analyses as a biomarker method should be weighed against the fact that these measurements remain relatively expensive and time-consuming compared to, e.g., bulk isotope analysis. However, ongoing advances in analytical approaches and an expansion of laboratories with the capacity to measure $\delta^{13}C_{AA}$ are likely to increase accessibility and affordability. In-depth discussions of these topics can be found in publications authored by van Leeuwen et al. (2014), Jochmann and Schmidt (2015), and Meier-Augenstein (2018).

DIGESTIVE PHYSIOLOGY AND ISOTOPE EFFECTS

To infer dietary information from $\delta^{13}C_{AA}$ values, it is important to consider the role of digestive and metabolic processes on trophic discrimination. The isotopic composition of a whole organism is the result of a dynamic equilibrium between nutrient assimilation and discharge of excreta (e.g., indigestible molecules) and colonic fermentation products (e.g., carbon dioxide, methane and indoles) (Butt and Volkoff, 2019). For example, a study found that the gut contents of the sand goby (*Pomatoschistus minutus*) were more ^{13}C enriched in the hindgut than in the foregut, a result that in part can be ascribed to gut microbial activities (Guelinckx et al., 2008). Gut bacterial diversity and activity is generally lower in carnivores, and progressively increase from omnivores to herbivores (Wang et al., 2018a). Furthermore, carnivorous and omnivorous species usually rely far more on acid digestion than herbivorous species (Egerton et al., 2018). The intestines of carnivorous fish have evolved for processing a highly digestible, energy and nutrient-dense diet that is high in protein and low in carbohydrates. For this reason, their gut to body length is far shorter than that of detritivores and herbivores. Carnivorous fish also lacks defined gastrointestinal structures typically found in herbivorous species, e.g., gizzard, and compartmentalization of the stomachs (De Silva and Anderson, 1994), and carnivores are poor at utilizing dietary carbohydrates owing to low intestinal glucose uptake rates and limited ability to digest complex polymers (Kamalam et al., 2017). In herbivorous and detritivorous fish species, the hindgut microbiome plays a particularly important role in digesting complex polymers and providing the host with short-chain fatty acids and in some cases, *de novo* synthesized EAAs (Newsome et al., 2011; Clements et al., 2014). These activities incurred by the gut microbiome (Larsen et al., 2016a) and the mucous membrane of the intestinal tract (Burrin and Stoll, 2009) can lead to an increase in metabolic costs. NEAAs such as Gln, Glu, and Asp may be catabolized extensively for oxidative fuel (**Box 1**), and lack of these NEAAs in the diet can lead to increased catabolism of certain EAAs.

Once the macronutrients are digested into smaller molecules and absorbed into somatic cells, they are broken down for

BOX 1 | Overview of main anabolic and catabolic pathways of the non-essential amino acids in finfish. See **Figure 2** for the visualization of the biochemical pathways and definition of key biochemical terms.

Alanine (**Ala**) Is the predominant amino acid (AA) catabolized by the liver where it is a main contributor to gluconeogenesis. The carbon of its precursor pyruvate derives from glucose, lactate, and other AAs. **Ala** and **Asp** are major glucogenic precursors. **Ala** and **Gln** are the main carriers of fish muscle derived nitrogen that is transported to
the liver *via* the blood (Felig, 1973; Okun et al., 2021).

Arginine (**Arg**) In fish, Arg is key for modulation of ureagenesis and ammonia detoxification, and like **Pro**, it is often rate limiting for growth and metabolic functions if the quantities are insufficient in the diet (Hoseini et al., 2020). It is abundant in tissue fluid as phosphoarginine, a major reservoir of ATP (Li et al., 2009).

Asparagine (**Asn**) and aspartate (**Asp**) Are major metabolic energy source for intestinal epithelial cells and have also been shown to regulate intestinal and neurological development and function (Wu, 2014). Together with **Gly** and **Gln**, **Asp** act as a precursor for purines and pyrimidines, which form part of the structural subunits of nucleic acids.

Cysteine (**Cys**) Is synthesized from methionine **(Met)** and serine **(Ser)**. This semi-essential AA is a precursor for the two antioxidants, taurine and glutathione, which are key metabolites for mitochondrial functioning (Prabhu et al., 2014).

Glutamate (**Glu**) and glutamine (**Gln**) **Glu** is involved in diverse processes such as nitrogen assimilation and cofactor for biosynthesis (increase the rate of a chemical reaction), as well as a building for the construction of complex molecules beyond proteins (Walker and Van Der Donk, 2016). **Glu** and its decarboxylation product function as neurotransmitter (Li et al., 2009). **Gln** is one of the most abundant free α-AA in fish plasma and muscle. A large fraction of dietary **Glu** and **Gln** carbon skeletons may be degraded in the gut (Wu, 1998).

Glycine (**Gly**) and serine (**Ser**) **Gly** and **Ser** participate in fat digestion and one-carbon unit metabolism (Fang et al., 2002), and can also stimulate feed intake (Shamushaki et al., 2007). Gly is likely to regulate gene expression in fish (RileyJr., Higgs et al., 1996), and it plays critical role in the osmoregulatory responses of fishes (Powell et al., 2007). **Ser** is essential to maintain mitochondrial respiration (Lucas et al., 2018).

Proline (**Pro**) and hydroxyproline (**Hyp**) **Pro** fulfills a unique biological role in stress adaptation. The requirements of **Pro** for whole-body protein synthesis are the greatest among all AAs. The rates of endogenous **Pro** synthesis are inadequate in fish and can as such be regarded as a semi-essential AA, especially for juvenile fish (Dabrowski et al., 2005; Wu et al., 2011). **Hyp** is a main constituent of collagen. Although **Hyp** is considered a NEAA, it is a potent growth promoters for fish (Aksnes et al., 2008).

Tyrosine (**Tyr**) Is synthesized from phenylalanine **(Phe)**, and is the main precursor for melanin synthesis and sclerotization (Vavricka et al., 2014). Adding **Tyr** to aquafeed can reduce fish requirement for **Phe**.

producing energy and synthesizing metabolic intermediates needed for synthesizing new molecular structures. The liver functions as the main hub for AA metabolism catabolizing most AAs, synthesizing NEAAs, and providing AAs for protein synthesis in the liver and other tissues (**Figure 2**). The availability of AAs at the moment of protein synthesis is critical for protein retention efficiency and protein turnover (Brezas and Hardy, 2020): If one AA is not present in sufficient amounts, the remaining AAs are alternatively catabolized for energy. Since the capacity of the organism to store free AAs is very low, it is critical that EAAs are released and assimilated approximately simultaneously to maximize the incorporation of dietary AAs into somatic tissues (Rungruangsak-Torrissen et al., 2009). Both catabolism and anabolism lead to kinetic isotope effects. This occurs when the reaction rates are affected by the isotopic composition of molecules (the isotopologues). For example, enzymatic decarboxylation reactions in the tricarboxylic acid cycle produce ^{13}C-depleted CO_2 and ^{13}C-enriched residual acids (Takizawa et al., 2020). Thus, greater rates of biochemical reactions usually lead to greater trophic discrimination (Hayes, 2001). Sourcing of metabolic intermediates derived from macronutrients also affects δ^{13}C values of *de novo* synthesized NEAAs because lipid moieties and short-chain fatty acids are ^{13}C depleted relative to proteins and carbohydrates (Deniro and Epstein, 1977; Melzer and Schmidt, 1987; Weber et al., 1997). While not always the case for the EAAs (Newsome et al., 2020), most feeding trials show that $\Delta\delta^{13}C_{EAA}$ values usually fall within 1‰ for healthy animals feeding on nutritionally adequate diets (McMahon et al., 2010; Barreto-Curiel et al., 2017; Liu et al., 2018; Wang et al., 2018b, 2019; Takizawa et al., 2020; Xu et al., 2021). Nevertheless, some animals specialized in feeding detrital or poorly digestible diets can make up for nutritional insufficiencies by assimilating EAAs synthesized by their gut microbiomes (Newsome et al., 2011; Arthur et al., 2014; Ayayee et al., 2015; Larsen et al., 2016b). Excess of dietary proteins may cause trophic discrimination. A feeding trial with the totoaba (*Totoaba macdonaldi*) found that $\Delta\delta^{13}C_{EAA}$ for Ile and Leu increased linearly with higher protein levels (Ile: from 0.1‰ $\Delta\delta^{13}$C at 38% protein level to 0.5‰ at 49%; Leu: 0.1‰ at 38% to 1.1‰ at 49%) probably because of isotope effects associated with EAA catabolism for energy (Barreto-Curiel et al., 2019). The study found no clear correlations between $\Delta\delta^{13}$C and dietary protein levels for the remaining EAAs (Lys, Met, Phe, and Val). Starvation, at least over a relatively short time span, appears to have little effect on $\Delta\delta^{13}C_{EAA}$ values in muscle tissues according to a study of the yellowtail (*Seriola lalandi*) that were starved for 35 day (Barreto-Curiel et al., 2017).

INFERRING DIET AND NUTRITION FROM AMINO ACID δ^{13}C VALUES

Essential Amino Acids

A key source diagnostic feature of the EAAs is that algae, bacteria, fungi, and terrestrial plants have distinct $\delta^{13}C_{EAA}$ patterns in which the relative differences among EAAs are consistent, regardless of the actual source bulk δ^{13}C value (Scott et al., 2006; Larsen et al., 2009a, 2013). These $\delta^{13}C_{EAA}$ patterns are termed fingerprints when they are unique and source characteristic. The $\delta^{13}C_{EAA}$ patterns of both micro- and macroalgae are diverse and appear to be more diverse compared to terrestrial vascular plants (Larsen et al., 2013; McMahon et al., 2015a; Elliott Smith et al., 2018). Brown macroalgae differ from red macroalgae (**Figure 3**; Larsen et al., 2013), and within the monophyletic group of

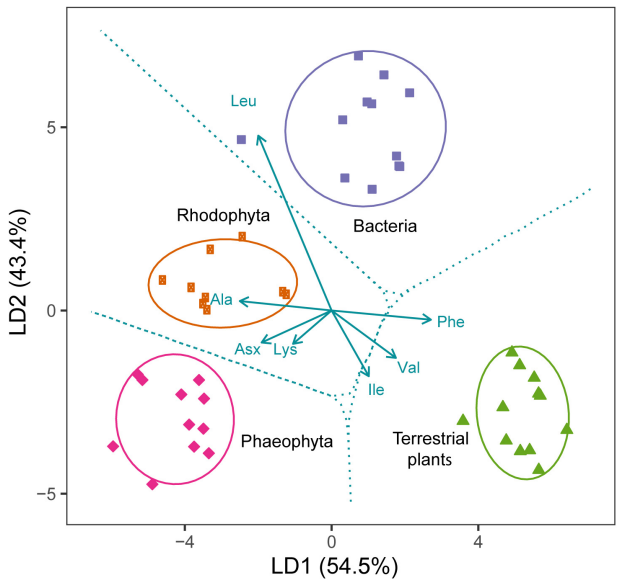

FIGURE 3 | Linear function discriminant analysis (LDA) based on the seven most discriminate amino acid δ^{13}C variables for separating bacteria (n = 12), Phaeophyta (n = 11), Rhodophyta (n = 9) and terrestrial plants (n = 12). Insufficient data available for Chlorophyta. Values in parentheses are the percentage variations accounted by each LD axis (54.5 and 43.4% for LD1 and LD2, respectively), the ellipses represent the 95% confidence intervals of each group, and the broken lines represent the decision boundaries. The median values of the four groups are significantly different (Pillai's Trace = 1.90, $F_{6,80}$ = 247.1; p < 0.001). The data were compiled from Larsen et al. (2013).

cyanobacteria, diazotrophic vs. non-diazotrophic cyanobacteria appear to have distinct fingerprints (McMahon et al., 2015b). Likewise, regional differences in microalgal assemblages result in distinct $\delta^{13}C_{EAA}$ fingerprints in trophic chains fueled by these basal resources (Wang et al., 2018b; Larsen et al., 2020).

Non-essential Amino Acids

In contrast to the EAAs, NEAAs are less suited as source tracers because metazoans either incorporate dietary NEAAs directly into their tissue or synthesize them from metabolic intermediates sourced from lipids, carbohydrates, and proteins (Berg et al., 2015). Furthermore, dietary NEAAs are more likely to be catabolized for energy, act as metabolic precursors or be a source of nitrogen than the EAAs (Wu, 2014; McMahon et al., 2015c). While the term non-essential implies that animals can synthesize them at a rate that meets the cellular demand for protein synthesis, it is well documented that adequate amounts of dietary NEAA are required for maximum growth and optimum health (Peres and Oliva-Teles, 2006; Gaye-Siessegger et al., 2007). Hence, the rate by which the NEAAs are incorporated directly into proteinogenic tissue or synthesized *de novo* from metabolic intermediates varies according to physiological demands, and the quality and supply of dietary proteins. It is important to note that the term non-essential is misleading because NEAA synthesis is energetically expensive and animals have limited capacity to maintain physiological functions if their diets are NEAA limited (Borman et al., 1946; Womack and Rose, 1947; Reeds, 2000). Instead, synthesizable, and non-synthesizable AAs would be more accurate but unconventional terms for the NEAAs and EAAs (Wu, 2014; Hou and Wu, 2017). Despite the metabolic complexity of NEAA routing and synthesis (Hayes, 2001) and the variable factors affecting fractionation, controlled feeding trials have demonstrated the potential of $\delta^{13}C_{NEAA}$ patterns to inform about dietary ingredients and macronutrients (Newsome et al., 2014; McMahon et al., 2015c; Wang et al., 2018b, 2019; Whiteman et al., 2018).

TRACING AQUAFEED MACRONUTRIENTS WITH $\delta^{13}C_{AA}$

Commercial aquafeeds have in recent years gone from a primary protein source, fishmeal, and a singular lipid, fish oil, to more than several dozen ingredients such as soy, cereals, legumes, insects, crustaceans, yeast, and algae. This development has made it more challenging to trace the origins and metabolic fate of this diverse array of ingredients and their interactions. Despite the limited development and applications of $\delta^{13}C_{AA}$–based approaches in aquaculture, the few studies published to date have shown considerable promise for tracing new ingredients in aquafeed.

Wang et al. (2018b) studied Atlantic salmon to test whether $\delta^{13}C_{AA}$ could differentiate among fish fed aquafeed with and without seaweed inclusion. These aquafeeds contained macronutrients from up to five different marine and terrestrial sources (see **Figure 1**). The alternative diet substituted 15% of macroalgae, the green (*Ulva* sp.) or the red (*Palmaria* sp.), into the aquafeed by decreasing fishmeal by 5% and corn starch by 10%, while the compositions of other ingredients remain unchanged (Moroney et al., 2015; Wan et al., 2018). The three

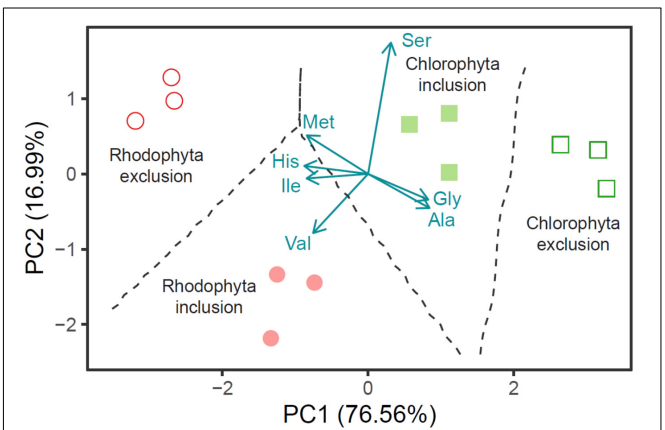

FIGURE 4 | Principal component analyses (PCA) based on the seven most discriminate amino acid δ^{13}C variables for separating Atlantic salmon (n = 3 in each group) fed composite diets with (15%) and without Rhodophyta and Chlorophyta ingredients. Values in parentheses are the percentage variations accounted by each PC axis (76.6 and 17.0% for PC1 and PC2, respectively), and the broken lines represent the decision boundaries. The data were compiled from Wang et al. (2019).

glycolytic AAs (Ala, Gly, and Ser) against four EAAs (His, Ile, Met, and Val) separated the control groups from their respective algal inclusion groups with high certainty (**Figure 4**). The two control groups cluster separately in part because their respective fishmeal ingredients originated from different locations and marine fish species, and in part due to the different nutritional and functional profiles of the two macroalgal species. To understand why the three glycolytic AAs could function as biomarkers of macroalgal inclusions, Wang et al. (2019) analyzed $\delta^{13}C_{AA}$ of salmon muscle tissue and the various protein sources in the aquafeed (fishmeal, pea protein, wheat gluten and *Palmaria palmata*). Macroalgal inclusion resulted in more positive $\Delta\delta^{13}C$ values of the three glycolytic AAs ($P < 0.01$). Two factors can explain the $\Delta\delta^{13}C$ shift. First, fish oil, the major source of lipids in the aquafeed, is more ^{13}C depleted than the other macronutrients. Second, the replacement of high with low digestible carbohydrates (corn starch vs. algal carbohydrates) lowered the energy content of the aquafeed due to Atlantic salmon's inability to digest complex carbohydrates. For this reason, we posit that salmon fed diets with macroalgal ingredients allocated a relatively higher proportion of lipids for energy than the control group. Since these catabolized lipids could not be sourced as metabolic intermediates for synthesizing NEAAs, macroalgal inclusion resulted in more positive $\delta^{13}C_{NEAA}$ values. This case study exemplifies the usefulness of knowing the $\delta^{13}C$ values of individual ingredients and/or macronutrients for inferring metabolic routing from $\delta^{13}C_{NEAA}$ values.

For algivorous aquaculture species, aquafeed diets can comprise completely of seaweeds. In a feeding trial, the sea cucumber *Apostichopus japonicus* was either fed the microalga *Cylindrotheca fusiformis* (CF; 21.1% protein, 10.8% lipid, 19.1% carbohydrate) or the brown macroalga *Sargassum thunbergii* (ST; 17.1% protein, 3.9% lipid, 50.0% carbohydrate) (Xu et al., 2021). CF was tested as an alternative feed source to ST because in China it is becoming increasingly difficult to meet the demands of brown macroalgae for the rapidly growing sea cucumber industry. The results showed that the growth rates of sea cucumbers were highest on the CF diet despite the relatively lower feed conversion efficiency compared to the ST diet. Between the two treatments, the $\Delta\delta^{13}C$ values of Pro, Asp, and Ala were consistently more negative (from −5.0 to −2.5‰) in sea cucumbers reared on CF than ST diets. Xu et al. (2021) proposed that these differences in part can be explained by the relatively lower NEAA abundance in the CF diet. However, the abundance was only lower for some and not all NEAAs (such as Ala), and the absolute AA concentration was higher in the CF than ST diet. It is also possible that the higher lipid content of CF than ST led to more negative $\Delta\delta^{13}C$ values of Pro and Asp in the CF treatment. This hypothesis is, however, at odds with the $\Delta\delta^{13}C$ values of Glx being similar between the two treatments. Since Glu acts as a precursor for Pro *via* pyrroline-5-carboxylate (see **Figure 2**). We propose a third hypothesis for the $\Delta\delta^{13}C$ differences, namely that the different macronutrient profiles of the two algal diets affected gut metabolic activities and therefore catabolic demands for certain NEAAs. For example, diets that cause increased osmotic stress of the intestinal microbiome is likely to surge the demand for polyamines, not only for gut bacteria but also for the enterocytes (the cells of the intestinal lining) (Wu et al., 2000; Rothe and Blaut, 2013). Although three NEAAs, Glu, Pro, and Arg, can act as precursors for polyamine synthesis *via* ornithine (see **Figure 2**), Pro appears to be the major source of ornithine (Wu et al., 2000). It is also worth noting that the $\Delta\delta^{13}C$ values of Asx were more negative in the CF treatment. Asp is one of the major oxidative fuels for intestinal epithelial cells (Wu, 2014). If Asp served as an energy source, it could have decreased incorporation of dietary Asp into the body walls of the sea cucumbers (the tissue being analyzed). Whether the two diets affected gut metabolic activities differently remains speculative on our part, but the sea cucumber study illustrates the need to employ additional biomarkers of enzymatic activities and tracers such as radiocarbon to better understand $\Delta\delta^{13}C_{NEAA}$ controls.

Feeding trials can also comprise a trophic chain encompassing primary producers and primary and secondary consumers. In an elegantly designed feeding trial where the microalga *Chlorella* spp. (18.5% protein, 11.1% lipid, 62.7% carbohydrate) was fed to the copepod *Calanus sinicus* (74.3% protein, 20.4% lipid, 2.8% carbohydrate), and the copepods to the European anchovy, *Engraulis encrasicolus* (50.5% protein, 41.8% lipid, 1.1% carbohydrate), Liu et al. (2018) determined the $\Delta\delta^{13}C$ values of the two consumers. In summary, the copepod $\Delta\delta^{13}C$ values were positive for Pro, Ser, Glx, and Gly (from 6.5 to 10.3‰), but not for Ala and Asx (−4.9 and −2.3‰). The anchovy $\Delta\delta^{13}C$ values were positive for Pro and Asx (2.2 and 5.4‰) but not for Gly, Ser, Ala, and Glx (from −9.7 to −3.8‰). The mostly contrasting $\Delta\delta^{13}C$ results of the two consumers can in large part be ascribed to the different macromolecular compositions of their diets. The copepods fed on a low protein but high carbohydrate content diet, which would increase metabolic sourcing from carbohydrates rather than proteins (and lipids) to *de novo* synthesized NEAAs. The reason that the pyruvate group (Ala and Asx; see **Figure 2**) has more negative $\Delta\delta^{13}C$ values than the α-ketoglutarate group (Glx and Pro) group is probably that the pyruvate to α-ketoglutarate pathway losses two CO_2 molecules (see **Figure 2**). The anchovies fed on a diet almost completely devoid of carbohydrates. Like the study's authors (Liu et al., 2018), we cannot completely explain the contrasting $\Delta\delta^{13}C$ results of Asx and Pro vs. Gly, Ser, Ala, and Glx. We suppose these results are a mismatch of some NEAAs being redundantly ingested or catabolized during digestive processes (i.e., Asx and Pro), which would leave the remaining pool ^{13}C enriched. For Gly, Ser and Ala, the negative $\Delta\delta^{13}C$ values might be explained by carbon sourcing from ^{13}C depleted glycerol *via* 3-phospoglycerate to *de novo* synthesized carbon skeletons (see **Figure 2**).

The feeding trials reviewed above with sea cucumbers, copepods, and anchovies show it can be complex making inferences about macronutrient routing with just one aquafeed ingredient. A study by McMahon et al. (2010) illustrates that the complexity increases when consumers are fed compound feeds with varying nutritional compositions. Juvenile mummichogs (*Fundulus heteroclitus*) were fed high carbohydrate Vegi-Pro (9.0% protein, 6.7% lipid, 82.0% carbohydrate partially from corn meal, 2.3% fiber) or high protein Bio-Vita (63.8% protein: 28.6 lipid: 7.2% carbohydrate, 0.4% fiber) aquafeeds. Interestingly, the two glycolytic AAs Gly and Ser had contrasting responses:

$\Delta\delta^{13}C_{Gly}$ values were quite positive (∼3‰) in the Vegi-Pro fish indicating carbohydrate sourcing and the very negative (∼−8‰) in the Bio-Vita fish indicating sourcing of lipid moieties. Conversely, $\Delta\delta^{13}C_{Ser}$ values were slightly negative (∼−1‰) in the Vegi-Pro fish and quite positive (∼3‰) in the Bio-Vita fish indicating that metabolic routing of lipid moieties to Ser synthesis was minimal. It is possible that carbohydrates were sourced to Ser synthesis in both treatments, which leaves the question of why it was not the case for Gly in the Bio-Vita treatment. In comparison, in a more recent feeding study where hatchery-reared Chinook salmon (*Oncorhynchus tshawytscha*) were fed Bio-Vita, the $\Delta\delta^{13}C_{Gly}$ values were positive (2.6‰; $\Delta\delta^{13}C_{Ser}$ was not reported) (Rogers et al., 2019), which raises the possibility of mummichogs and salmon having species-specific metabolic responses. In the mummichog study, the $\Delta\delta^{13}C$ values of the third glycolytic AA, Ala, was negative in both treatments indicating sourcing from lipids. We also want to highlight that $\delta^{13}C_{Glu}$ values in fish mirrored those of the Bio-Vita diets, but not among the Vegi-Pro fish ($\Delta\delta^{13}C$: ∼5‰). This might indicate that Glu was synthesized to a lesser degree in fish feeding on high than low protein diets. Moreover, gut microbial activity may have been higher for fish feeding on Vegi-Pro owing to its comparatively higher carbohydrate and fiber content, which in turn could have increased Glu and Gln degradation in the gut (Wu, 1998). Despite the multiple nutritional variables between the two aquafeeds, the study sheds light on important controls underlying macronutrient routing.

$\delta^{13}C_{AA}$-based approaches can also be used to investigate the contributions of gut microbial AAs to protein synthesis. This would be particularly relevant for understanding the effects of seaweed dietary supplements in herbivorous or omnivorous fish species. For example, a feeding trial with Nile Tilapia (*Oreochromis niloticus*) found that gut symbiotic microbes supplemented *de novo* synthesized EAAs to host when dietary proteins were replaced by hardly digestible fibers (Newsome et al., 2011). While $\delta^{13}C_{EAA}$ fingerprinting is suited for assessing gut microbial supplementation of EAAs to host, it is much more complex understanding the controls underlying $\delta^{13}C_{NEAA}$ patterns. The reason is that the host also synthesize NEAAs, which means that it is necessary to consider three rather than two NEAA sources, namely the food, gut microbes and host synthesized NEAAs. In such cases, we see little scope for inferring macromolecular metabolism from $\delta^{13}C_{NEAA}$ patterns.

The rapid expansion of alternative ingredients in aquafeeds makes it more pressing to develop new authentication and traceability methods. In this regard, $\delta^{13}C_{AA}$ analysis is emerging as a complementary approach to already established tracer methods. As reviewed above, $\delta^{13}C_{AA}$ values could detect whether salmon fed on aquafeed containing milled seaweed ingredients (15% seaweed substituted the caloric equivalent of fishmeal and corn starch) (Wang et al., 2018b). Likewise, in a feeding trial with black soldier flies reared for aquafeed, the $\delta^{13}C_{AA}$ method could detect whether the flies had fed on diets spiked with non-permitted bovine blood residues (Belghit et al., 2021). This is a remarkable result because the flies were fed a control diet for 7 days after initially being fed a diet with 10% (w/w) bovine blood residues. The study also assessed the suitability of legacy molecular analysis tools such as qPCR and LC-MS/MS, but only the $\delta^{13}C_{AA}$ method could detect whether the flies had ingested bovine blood 1 week prior to being fed the control diets. However, both legacy and novel authentication methods displayed shortcomings. Hence, to detect particular contaminants or ingredients across two trophic levels Belghit et al. (2021) recommended a tiered combined use of complementary approaches.

OUTLOOK AND PERSPECTIVES

Given the rapid expansion of aquaculture and the need to find more sustainable and low environmental impact proteinaceous ingredients (i.e., alternatives to fish meal and soybean) for aquafeed production, macroalgae stand out as an important alternative that has the potential to improve feed efficiency and fish health (Hua et al., 2019). Globally, more than 11,500 seaweed species have been identified (Guiry and Guiry, 2022). Their early divergence and genetic diversity have led to a range of different bioactive and nutritional compounds to be evolved. Yet, only 34 different seaweed species are represented in the 142 studies reviewed for their immunological properties by Thépot et al. (2021). Probably due to low commercial interests, only two studies out of the 142 studies evaluated the effects of seaweed dietary supplements in marine herbivorous fish, i.e., species that would naturally forage on seaweed in the wild. For both carnivorous and herbivorous fish species, $\delta^{13}C_{AA}$ based approaches can provide further insight into how fish utilize macroalgal macronutrients. As with any other novel and relatively untested aquafeed ingredients, it is important to rely on complementary approaches to holistically assess the suitability of macroalgae through their nutritional value and potential hazards such as potential toxic metals (National Food Institute, Technical University of Denmark, Denmark, Monteiro et al., 2019).

The case studies presented in this paper have primarily focused on using $\delta^{13}C_{AA}$ to trace macroalgal ingredients and detect gut microbial supplementation of AAs to host. Another promising $\delta^{13}C_{AA}$ application would be the use of marine protist and heterotrophic microalgae as an aquafeed ingredient (Klamczynska and Mooney, 2017; Fossier Marchan et al., 2018). These promising alternative aquafeed ingredient can be cultured without light and in higher densities compared to photoautotrophic algae. Depending on the culture strain and conditions during cultivation, it is possible within a short time span to produce mixotrophic heterokonts such as *Schizochytrium* sp. under heterotrophic conditions that contain >60% proteins or >70% lipids. According to a cradle-to-gate assessment of whole algal protein products, heterotrophically grown microalgae (i.e., through bioreactors and fermenters) also leave a smaller CO_2 footprint compared to terrestrial protein sources such as soy byproducts (Thinkstep, 2015). However, whole algae proteins contain very high levels of NEAAs compared to other proteinaceous aquafeed ingredients, e.g., animal-based proteins (Klamczynska and Mooney, 2017). For this reason, it will be important to

develop biorefinery methods for augmenting the AA profiles of heterotrophically grown microalgae for aquafeed, such as altering their nutrient growing substrate (Nham Tran et al., 2020). Another possibility to augment the nutritional profile of aquafeed containing whole algae protein sources would be to mix them with other functional feed additives such as macroalgae and spent yeasts (Rakowska et al., 2017). From an analytical perspective, the addition of multiple feed additives to aquafeed increases the complexity of tracing the trophic fate of their macronutrients.

While the $\delta^{13}C_{EAA}$ fingerprinting method holds considerable promise for investigating the biosynthetic of EAAs from a diverse set of feed additives, the phylogenetic resolution of fingerprints beyond algae, bacteria, fungi and plants is still unknown and needs further exploration. The $\delta^{13}C_{EAA}$ variability within each of the broad taxonomic groups can be large, which may lead to unspecific results. Also, the ability of fingerprints to detect microbial fermentation of feed additives remains unknown. To overcome these constraints and explore the phylogenetic specificity and robustness of the fingerprinting method, a concerted transdisciplinary effort is needed to expand $\delta^{13}C_{AA}$ reference libraries of primary production sources. It will be key to investigate how environmental conditions and nutritional composition of growth media affect algal $\delta^{13}C_{EAA}$ fingerprints (Larsen et al., 2015). For example, crude protein, total lipid, and secondary metabolite (e.g., fucoxanthin and polyphenols) concentrations of algae have been found to vary considerably across seasons and geographic locations (Steinberg, 1989; Fleurence, 1999; Nomura et al., 2013). It is likely that algal internal organs with high concentrations of storage lipids and secondary metabolites can affect $\delta^{13}C_{EAA}$ profile due to the upregulation of these compounds may affect ^{13}C fractionation of upstream EAA precursors (Hayes, 2001). Related to growth conditions and substrates, litter-using and humus-using ectomycorrhizal fungi appear to have distinctive $\delta^{13}C_{EAA}$ profiles. This could be due to the conversion of source carbon to metabolic AA precursors affecting isotopic discrimination (Pollierer et al., 2019). In microbial mats dominated by anaerobic methanotrophic archaea, $\delta^{13}C_{EAA}$ patterns are variable and not fixed as is the case with photoautotrophic microalgae (Takano et al., 2018; Stücheli et al., 2021). During biosynthesis of the pyruvate family AAs, ^{13}C fractionation widens between short- and long-chain carbon AAs with increasing ^{13}C depletion of the substrate methane (Takano et al., 2018). While microbial mats are an unlikely aquafeed ingredient, the examples above serve to show the limitations and possibilities of $\delta^{13}C_{EAA}$ fingerprints for tracing protein sources.

To deepen our understanding of the metabolic routing of macronutrients and their constituent molecules with $\delta^{13}C_{AA}$-based methods, further, carefully designed feeding trials are warranted. In terms of assessing trophic EAA discrimination and inferring gut microbial EAA supplementation to the host, it is key that the metabolically active tissues being analyzed are in full equilibrium with the diet. Feeding trials where animals are switched to a diet with distinct isotope values consistently show memory effects of the former diet (Tieszen et al., 1983; Bauchinger and Mcwilliams, 2009; Larsen et al., 2009b; Buchheister and Latour, 2010). It is also key that feeding trials strive to vary only one nutritional or isotopic parameter at a time. The aforementioned totoaba fish feed trial kept dietary levels of aquafeed lipids and carbohydrates fixed while varying protein levels by changing the ratio of digestible to non-digestible proteins (Barreto-Curiel et al., 2019). This was done by crosslinking the proteins by exposing them to formaldehyde and heat. Another approach that simplifies the nutritional interpretation of $\delta^{13}C_{AA}$ data is maintaining a fixed macromolecular composition between the control and the experimental diets as demonstrated in the aforementioned salmon feeding trials with macroalgal ingredients (Wang et al., 2018b, 2019). It is also possible to keep the nutritional composition fixed and instead change the isotopic values of the constituent ingredient and/or macronutrients being used in the feed formulation. For example, substituting C_3 plant ingredients (e.g., soy) with C_4 plants (e.g., maize), or freshwater with marine algae. This approach based on naturally occurring isotope variability would in most cases be sufficient to trace metabolic routing of macronutrients in question, and it is less costly and laborious than using isotope labeled ingredients.

Disentangling the direct and indirect interactions between seaweed and their digested derivatives during the metabolic pathways in the animals presents a significant challenge. While $\delta^{13}C_{AA}$ is an emerging and promising tool in understanding metabolic routing of aquafeed ingredients and macronutrients, it cannot stand alone. Interpretations of $\delta^{13}C_{AA}$ data can be enhanced by merging with other biomarkers as demonstrated in a comparison of traditional molecular biomarkers (e.g., qPCR and LC-MS/MS) and $\delta^{13}C_{AA}$ for their ability to trace adulterated ingredients in the aquafeed chain (Belghit et al., 2021). Overall, the use of $\delta^{13}C_{AA}$ as a biomarker will allow feed formulations to be more nutritionally optimized and refined to specific farmed fish species. The data collected can also be used in further downstream assessments, such as life cycle analysis on fish production impact and sustainability through more accurate data in dietary amino acid contribution (Cooney et al., 2021). In addition, $\delta^{13}C_{AA}$ can advance our understanding of the dietary requirements of new farmed fish species and continue our exploitation of novel aquafeed ingredients, i.e., algae, bacteria, protists, fungi, annelids, and insects.

MATERIALS AND METHODS

Statistical Methods

To compare $\delta^{13}C_{AA}$ patterns among phylogenetic groups and treatments, we applied principal component analysis (PCA) (R: *prcomp*) and linear discriminant function analysis (LDA) (R: *MASS*). PCA is commonly used for exploring $\delta^{13}C_{AA}$ variability and patterns because it is an unsupervised technique that seeks to maximize variability among samples while reducing the number of dimensions. LDA is a supervised technique that

seeks to maximize variability among the predefined groups or classes with the goal of predicting specific protein sources with the $\delta^{13}C_{AA}$ fingerprinting approach. For the PCA, we used the covariance matrix approach that preserves variance as the range and scale of variables are in the same units of measure. Based on the first and second LD scores, we used 95% prediction ellipses to visualize variability relative to the group centroid. We applied Multivariate Analysis of Variance (MANOVA, R: *manova*) in conjunction with Pillai's trace to test the null hypothesis that groups have a common centroid in a dependent variable vector space. A rejection of this hypothesis entails that the groups have significantly different $\delta^{13}C_{EAA}$ patterns or fingerprints. All data for multivariate comparisons were first assessed for homogeneity of variance by using Fligner-Killeen tests (R: *fligner.test*) and visually checked for departures from normality on Q-Q plots. R version 3.6.3 was used for statistical analyses (R-Development-Core-Team 2020) and ggplot2 for figure production (Wickham, 2016).

AUTHOR CONTRIBUTIONS

TL and AW: conceptualization. TL: methodology, statistics, and visualization. TL and AW: writing and original draft preparation. TL, AW, and YW: review and editing. All authors contributed to the article and approved the submitted version.

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Effects of Dietary *Rhodobacter sphaeroides* Protein Substitution of Fishmeal and Coenzyme Q10 Supplementation on Growth Performance, Intestinal Microbiota and Stress Tolerance of *Litopenaeus vannamei* in Acute Low Salinity

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A 56-day culture experiment followed by an acute low salinity challenge was conducted to evaluate the effects of *Rhodobacter sphaeroides* protein (RSP) substitution of fishmeal and Coenzyme Q10 (CoQ10) supplementation on growth performance, intestinal microbiota and compressive capacity of *Litopenaeus vannamei* (*L. vannamei*). Four experimental diets were formulated: FM diet (20% fishmeal), RSP diet (20% RSP instead of 20% fishmeal), CoQ10 diet (20% fishmeal supplemented with CoQ10 at 0.08 g kg^{-1}), RSP + CoQ10 diet (20% RSP supplemented with CoQ10 at 0.08 g kg^{-1}). The obtained results were denoted that *L. vannamei* fed CoQ10 diet could improve growth performance (weigh gain and specific growth rate), condition factor and crude lipid, and decrease hepatosomatic index, but no differences were found in survival rates. High throughput sequencing on intestinal microbiota indicated that intestinal microbiota of *L. vannamei* consisted mainly of *Proteobacteria* and *Firmicutes*. The species richness of the RSP diet was remarkably higher than that of the other diets. Moreover, the presence of RSP and CoQ10 may improve intestinal homeostasis by inhibiting the propagation of *Vibrio*. Compared to FM diet and RSP diet, supplementation of CoQ10 significantly improved the compressive capacity of *L. vannamei* against the acute low salinity challenge, as indicated by higher survival rates as well as higher activities of T-AOC and higher transcript levels of *SOD*, *HSP70*, and *Relish* gene. Our findings demonstrated that RSP could serve as a novel FM and CoQ10 could serve as a prospective feed additive to help *L. vannamei* to overcome environmental stresses.

Keywords: *Litopenaeus vannamei*, nutrition, *Rhodobacter sphaeroides* protein, CoQ10, intestinal microbiota, low salt stress

INTRODUCTION

Although as a highly efficient osmoregulator, the salinity of *L. vannamei*' live was wide-ranging, from 0.5 to 50 ppt (Xu et al., 2018). More and more studies have shown that low salinity can increase ammonia excretion and oxygen consumption, and decrease ammonia or nitrite tolerant in *L. vannamei* (Jiang et al., 2000; Shinji et al., 2012; Wang et al., 2015). Besides, 5 and 54 ppt salinities increase the susceptibility of *L. vannamei* to White Spot Syndrome Virus (WSSV) (Ramos-Carreno et al., 2014). Meanwhile, *L. vannamei* juveniles exhibited an impaired resistance to pathogens due to long-term low salinity (2.5–5 ppt) (Lin et al., 2012). On the other hand, the growth performance of juvenile *L. vannamei* would be significantly suppressed when exposure to salinity 3 ppt (Huang et al., 2019). All these data indicate that the tolerance to stress, the resistance to pathogens and the poor survival rates have become restrictive factors for inland low salinity *L. vannamei* farming (Li et al., 2008). And so far, a few studies have started to address the effects of low salinity on survival and growth of *L. vannamei* (Zheng et al., 2017; Li et al., 2019). In the long-term feeding experiment, there is a need to further explore some new fishmeal substitutions and dietary supplementations to enhance nutrient uptake and increase host defense against acute salinity challenge.

Coenzyme Q10 (CoQ10; also called ubiquinone) is naturally occurring endogenous lipophilic antioxidant, the main function of which was to produce the ATP by the mitochondrial Electron Transport Chain (ETC). CoQ10 has been reported to contribute a significantly improvement to human and animal health in multiple diseases, like cancer, obesity, diabetes, etc. (Roffe et al., 2004; Adarsh et al., 2008; Sohet et al., 2009; Prakash et al., 2010; Varela-López et al., 2016). In addition, CoQ10 has an anti-inflammatory potency in THP-1 cells (Schmelzer et al., 2008, 2009). It has been suggested that there was a markedly higher weigh gain when CoQ10 was supplemented at 0.04 g kg^{-1} in broiler diet (Geng et al., 2007), a few studies also using CoQ10 to reducing the mortality of broiler in a stress environment (Gopi et al., 2014). El et al. observed that inclusion of CoQ10 has improved the growth performance, health being and antioxidant capacity in *Nile tilapia* (El et al., 2020). Therefore, CoQ10 was regarded as an ideal supplement to increase the antioxidative capacity to combat oxidative stress, while related studies on its effect on farmed shrimp were lacked. As known as a high potential as nutritious feed, *Rhodobacter sphaeroides* is a purple non-sulfur bacterium that contains essential vitamins and carotenoid pigments (Sasaki et al., 1998). Supplements of *Rhodobacter sphaeroides* improved water quality, reduced the mortality rates, and stimulated immune system and the growth of seawater red *tilapia* (Chiu and Liu, 2014). *L. vannamei* fed with *Rhodopseudomonas* addition diets were more resistant against ammonia stress and *Vibrio* pathogens (Alloula et al., 2020). Studies have found the protein content of *Rhodobacter sphaeroides* protein was up to 63%, which can replace FM up to 100% with no negative effects on survival rate, feed intake, growth performance, antioxidant and intestinal tissue health (Liao et al., 2021). On the other hand, *Rhodobacter sphaeroides* had been established as a strong candidate for CoQ10 production (Choi et al., 2005). Although *Rhodobacter sphaeroides* and CoQ10 have been researched widely in aquaculture, the resistance to acute low salinity of which remained to be further investigated.

As a burgeoning area, the metagenomics approach was extensively used in the research of mammalian intestinal microbiota (Maccaferri et al., 2011; Zhu et al., 2011; Song et al., 2017). By contrast, metagenomics is seldom used in aquaculture (Martínez-Porchas and Vargas-Albores, 2017). Recently, researches on the intestinal microbiota in *L. vannamei* have been carried out successively (Chen et al., 2017; Gainza et al., 2018; Hou et al., 2018; Huang et al., 2018). As the main constituents of the shrimp digestive system, it has been recognized now that intestine was an extremely intricate ecosystem, which contains a variety of active microorganisms (Fan and Pedersen, 2021). What's more, regarded as the "second genome" or "additional organ" (Bäckhed et al., 2007), intestinal microbiota has significantly influenced host metabolism, body composition and immune capacity (Clarke et al., 2014). Recent study has indicated that the composition of intestinal microbiota was more influenced by host diets than the water environment (Li et al., 2017). Interestingly, the addition of probiotics in shrimp commercial dietary could remarkably modify the intestinal microflora (Vargas-Albores et al., 2017). To further evaluate whether CoQ10 can be safe additives modulating microbial community structure in *L. vannamei*, the intestines of shrimp fed with different diets were chosen for 16S rRNA gene sequencing analysis, which may provide a solid theoretical basis for fishmeal substitutions or dietary supplementations. In a word, the experiment was aimed at elucidation of the impacts of RSP substitution of fishmeal and CoQ10 supplementation on growth performance, intestinal microbiota and stress tolerance against acute low salinity challenge.

MATERIALS AND METHODS

Cultured Environmental Conditions

For this research, the cultured *Pacific White Shrimp* (*L. vannamei*) were afforded by Evergreen South Ocean Tech Co., Ltd, Zhanjiang, China. 16 aquaria (300 L, 0.6 m^2 bottom, 4 aquaria per dietary treatment) with 40 shrimp/aquaria were used for four experimental diets. The shrimp weight 0.85 g were selected and adapted for a week with basic feed prior to the experiments. For the evaluation of the impacts of RSP (All the main raw materials (include: *Rhodobacter sphaeroides* and glucose, corn steep liquor, inorganic salts, etc.) were fermented, then inactivated and dried to gain the *Rhodobacter sphaeroides* protein product; Zhejiang NHU Company Ltd., Xinchang, Zhejiang) and CoQ10 (Zhejiang NHU Company Ltd., Xinchang, Zhejiang) on *L. vannamei*, four experimental diets were designed as follows: FM diet contains 20% fishmeal; RSP diet contains 20% RSP instead of 20% fishmeal; CoQ10 diet contains 20% fishmeal supplemented with CoQ10 at 0.08 g kg^{-1}; RSP + CoQ10 diet contains 20% RSP supplemented with CoQ10 at 0.08 g kg^{-1} (**Table 1**). The composition of basal feed was as follows: crude protein 38%, crude fat 6% and moisture 9%. The method of diet preparation

TABLE 1 | Ingredients and proximate composition of four experimental diets (g kg^{-1}).

Ingredients	FM	RSP	CoQ10	RSP + CoQ10
Peruvian fishmeal	200	0	200	0
Rhodobacter sphaeroides protein	0	200	0	200
CoQ10	0	0	0.08	0.08
Dehulled soybean meal	270	270	270	270
Peanut meal	120	120	120	120
Wheat flour	258	249	258	249
Beer yeast	30	30	30	30
Shrimp head noodles	40	40	40	40
Fish oil	10	16.6	10	16.6
Soybean oil	10	10	10	10
Choline	2.0	2.0	2.0	2.0
Vc phosphate	1.0	1.0	1.0	1.0
Soybean lecithin	10	10	10	10
Vitamin premix[a]	10	10	10	10
Mineral premix[b]	10	10	10	10
Ca $(H_2PO_4)_2$	10	10	10	10
Methionine	2.4	2.0	2.4	2.0
Lysine	3.2	7.1	3.2	7.1
Threonine	3.3	2.2	3.3	2.2
Sodium alginate	10	10	10	10
Proximate composition (%)				
Moisture	9.25	9.34	9.28	9.38
Crude lipid	6.31	6.31	6.31	6.31
Crude protein	38.10	38.22	38.22	38.22

[a] *Vitamin 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; a-tocopherol, 2,500 mg; myo-inositol, 8,000 mg; calcium pantothenate, 1,250 mg; nicotinic acid, 2,000 mg; vitamin D3, 45,000 IU; vitamin C, 7,000 mg. Guangzhou Chengyi Company Ltd., Guangzhou, China.*

[b] *Mineral premix (kg^{-1} of diet): $ZnSO_4 \cdot 7H_2O$, 4 g; $CaCO_3$, 37.9 g; KCl, 53 g; KI, 0.04 g; NaCl, 26 g; $CuSO_4 \cdot 5H_2O$, 2 g; $CoSO_4 \cdot 7H_2O$, 0.02 g; $FeSO_4 \cdot 7H_2O$, 9 g; $MnSO_4 \cdot H_2O$, 3 g; $MgSO_4 \cdot 7H_2O$, 35 g. Guangzhou Chengyi Company Ltd., Guangzhou, China.*

was the same as described by Niu et al. (2010). In short, all dry ingredients of four diets were weighed and mixed fully to homogeneity in a Hobart-type mixer (A-200T Mixer Bench Model unit, Resell Food Equipment Ltd., Ottawa, Canada). After that soy oil, fish oil and soybean lecithin were then added and mixed well for 5 min, and the mixed components were added and mixed to deionized water for 10 min. Post conditioning, the wet mixture were randomly extruded using monoscrew extruder (Institute of Chemical Engineering, South China University of Technology, Guangzhou, P.R. China) having barrel inner diameter of 1.2 mm. The four diets were stored at −20°C before use. All shrimp were fed to apparent satiation three cycles per day at 8:00, 12:00, and 18:00 by hand with a total amount of approximately 6% of its body weight, and maintained in fresh seawater with the temperature ranged from 27 to 29℃, *pH* was 8.0–8.2, salinity was approximately 30 ppt. The aquaria conditions were kept identical during feeding experiment.

Sampling

The feeding experiment was carried out for 8 weeks. At the start and the end of the feeding experiment, all shrimp were weighed. The routine nutritional compositions of the body samples were then analyzed ($n = 5$), meanwhile, intestine samples were obtained for the analysis of intestinal microbiota ($n = 5$). All samples were frozen in liquid nitrogen immediately and then stored in −80°C until measurement.

Survival Rates and Growth Performance

During the feeding experiment (8 weeks), the number of dead individuals were counted from beginning to end for measurement of the survival rates. In this study, weigh gain (WG), feed conversion ratio (FCR), specific growth rate (SGR), condition factor (CF), and hepatosomatic index (HSI) were used to evaluate the growth performance in *L. vannamei*. The parameters were calculated as follows: WG = 100 × (final body weight - initial body weight)/initial body weight; SGR = 100 × (Ln final mean weight—Ln initial mean weight)/number of days; FCR = dry diet fed/wet weight gain; $CF = 100 \times (\text{body weight})/(\text{body length})^3$; HSI = 100 × hepatopancreas weight/whole body weight.

16S rRNA Gene Sequence Analysis

Genome DNA of the intestinal contents was extracted with a DNA microbiome kit (Qiagen, Germany). The integrality of total DNA was assessed with 1% agarose gel electrophoresis, DNA purity and concentration were quantified using a spectrophotometer (Thermo Fisher Scientific Inc., United States). Purified genomic DNA was then sent for sequencing at the Novogene Biological Information Technology Co. (Tianjin, China). Quality filtering and splicing on raw data were performed using QIIME procedures (Caporaso et al., 2010). The retained clean data was performed Operational Taxonomic Unit (OTU) cluster analysis. Clustering of OTUs (at 97% similarity) were performed by VSEARCH for further annotation. The further diversity difference analysis was conducted according to the results of the relative OTU abundance. Alpha diversity measurements were evaluated with ACE, Chao1 and Rank-Abundance curves (richness), Shannon and Simpson (diversity) (Schloss et al., 2011). Beta diversity measurements were manifested as principal component analysis (PCA) and principal coordinates analysis (PCoA). Finally, the microbial communities' differences between four diet treatments were evaluated using Linear Discriminant Analysis Effect Size (LEfSe) method.

Challenge Tests

At the end of the 8-week feeding trial, 10 shrimp from each experimental aquarium were transferred randomly into the desalinated seawater (salinity, 2 ppt). The mortality was monitored for 4.5 h post- attack, and the relative percentage survival rates were calculated. In addition, hepatopancreas samples were collected for further analysis.

Biochemical Analysis

The frozen hepatopancreas were weighed and homogenized by adding ice-cold PBS (1:10 dilution) until the hepatopancreas were completely broken. The homogenate tissues were centrifuged for 20 min (3,000 × g at 4°C). T-AOC, GSH-PX and MDA were detected by commercial assay kits (cat. nos. A015, A005, and

TABLE 2 | Sequences of primers used in this study.

Genes	Forward (5′–3′)	Reverse (5′–3′)	GenBank no.
HSP70	CTCCTGCGTGGGTGTGTT	GCGGCGTCACCAATCAGA	XM-027369405.1
Relish	ATTCTTCTGCGTTTCAAGGTGT	GAGGTATGGTCAGGGTATGGTG	KM204120.1
Caspase-3	AGTTAGTACAAACAGATTGGAGCG	TTGTGGACAGACAGTATGAGGC	DQ846887.1
SOD	TGCCACCTCTCAAGTATGATTTC	TCAACCAACTTCTTCGTAGCG	KU958381.1
β-*actin*	CGAGGTATCCTCACCCTGA	CGGAGCTCGTTGTAGAAGG	AF300705.2

A003, respectively; Nanjing Jiancheng Bioengineering Institute, China) following the manufacturers' instructions. All assays were completed within a week after preparation.

qPCR Analysis of Immune- Related and Oxidative Stress Damage Related Genes

For the purposes of investigating the effects of RSP and CoQ10 on the immunity and antioxidative capacity of *L. vannamei,* the hepatopancreas samples from 4 shrimp per each dietary were collected after low salinity challenge. The total RNA samples were extracted from hepatopancreas by RNeasy^{TM} animal RNA extraction kit (Beyotime, Shanghai, China) according to the protocol, and then transcribed to complementary DNA (cDNA) with a PrimeScript RT Reagent kit (Takara Bio, Inc.). The gene expression levels of four immune- related and oxidative stress damage related genes *HSP70*, *Caspase-3*, *SOD* and *Relish* were investigated in the hepatopancreas by the Light Cycler 480 (Roche Applied Science, Basel Switzerland). The qRT-PCR reaction program was performed as the following thermocycler condition: 95°C for 10 min, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s and 72°C for 30 s, finally at 4°C for 5 min. Specific shrimp primers of related genes used in this study were shown in **Table 2**, the expression levels of related genes were quantified using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

All results were presented as the mean ± standard error (SE). One-way ANOVA was performed to determine statistically significant differences of the different treatment using SPSS software. Multiple comparisons were made using Duncan's multiple tests. Other statistical analyses were performed with the R or VSEARCH software environment. A P-value < 0.05 was considered significant.

RESULTS

Growth Performance

The growth performance and survival rates of *L. vannamei* were shown in **Figure 1**. In the whole feeding period, shrimp fed with RSP diet had remarkably higher WG and FCR than shrimp fed with FM diet ($P < 0.05$; **Figures 1A,C**). However, survival rates were similar among all diets ($P > 0.05$; **Figure 1B**). A higher SGR was found in shrimp fed with CoQ10 diet than that in shrimp fed those diets and no significant differences were found among other diets ($P > 0.05$; **Figure 1D**). CF of shrimp fed with FM diet was remarkably lower than those of shrimp fed with RSP diet, CoQ10 diet and RSP + CoQ10 diets, while no significant differences were found among RSP diet, CoQ10 diet and RSP + CoQ10 diets ($P > 0.05$; **Figure 1E**). HSI of shrimp fed with FM diet was significantly higher than those of shrimp fed RSP diet and CoQ10 diets ($P < 0.05$), while no significant differences were found in HSI between FM diet and RSP + CoQ10 diets ($P > 0.05$; **Figure 1F**).

Muscle Proximate Composition

SA The crude lipid content in muscle was significantly lower in shrimp fed FM diet than from the other three diets ($P < 0.05$; **Table 3**). Meanwhile, no considerable differences were found in crude protein and moisture contents of muscle in all diets ($P > 0.05$).

Alpha Diversity Index Statistics and Beta Diversity Analysis of Microbial Communities

The alpha diversity parameters include ACE, Chao1, Shannon and Simpson index, which represent the extents of microbial communities' richness and diversities. Clearly, the microbial community richness from RSP diet was significantly higher than that from other diets, as supported by ACE ($P < 0.05$) and Chao1 ($P < 0.05$; **Figures 2A,B**). The Shannon index was highest in RSP diet, following in RSP + CoQ10 diet, and lowest in FM diet (**Figure 2C**). What's more, Simpson's index was lower in RSP diet than that in FM diet and CoQ10 diets (**Figure 2D**), microbial diversity in FM diet was nearly identical with CoQ10 diet (Shannon index 4.5, Simpson index 0.90). All the results manifested that 100% of fishmeal replaced by RSP can increase intestinal microbial diversity.

OTU analysis-based dilution and grade abundance curves from different treatments were given in **Figures 2E,F**. The horizontal axis of FM diet and RSP diets were broader than that of CoQ10 diet and RSP + CoQ10 diets, manifesting that the species composition from FM diet and RSP diets were more specific and richer compared with CoQ10 diet and RSP + CoQ10 diets.

Beta diversity analysis was to show the similarities and differences of microbial communities from different treatments. According to PCA analysis, a similar microbial community structure was observed among the basic diet, CoQ10 diet and RSP + CoQ10 diet (**Figure 2G**), which showed that RSP substitution of fishmeal had a bigger effect on microbial community structure. A principal coordinate analysis (PCoA) showed that the four diet treatments were divided into three parts that manifested the effects of RSP and CoQ10 on the similarity

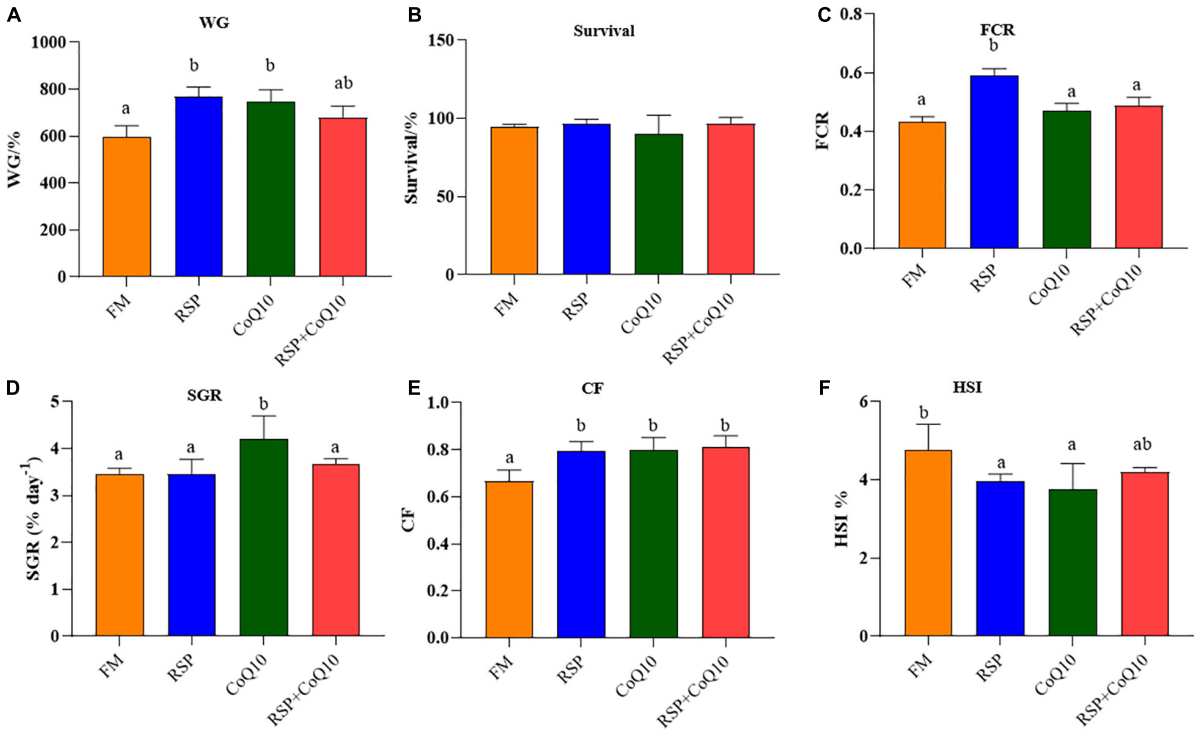

FIGURE 1 | The effect of four different diet treatments on growth performance of shrimp. **(A)** WG of shrimp in different diet treatments. **(B)** Survival rates of shrimp in different diet treatments. **(C)** FCR of shrimp in different diet treatments. **(D)** SGR of shrimp in different diet treatments. **(E)** CF of shrimp in different diet treatments. **(F)** HSI of shrimp in different diet treatments. WG, weigh gain; FCR, feed conversion ratio; SGR, specific growth rate; CF, condition factor; HSI, hepatosomatic index.

of intestinal microbial composition in *L. vannamei* (**Figure 2H**), indicating that RSP substitution had impacted *L. vannamei* microbiota significantly ($P < 0.05$) but not CoQ10 addition.

Taxonomic Compositions and Changes of Intestinal Microbiota

An OTU was defined by sequences were clustered at the 97% similarity, each OTU represented a corresponding sequence. Observed numbers of OTUs have shown that the highest OTU number of 530 was with RSP + CoQ10 diet and the lowest of 218 with CoQ10 diet with the remaining OUT numbers were in between (222–460) (**Figure 3A**). A Venn map was constructed to identify dominant OTUs presented in all diet treatments, indicating that there were 50, 141, 56, and 221 unique OTUs in FM diet, RSP diet, CoQ10 diet and RSP + CoQ10 diets, respectively, which means the dietary fishmeal replaced with RSP and the dietary CoQ10 addition can influence intestinal microbial and generate corresponding unique microbiota (**Figure 3B**).

Compared with the OTU sequences with related database, *Proteobacteria* still the only predominant phylum in all diet treatments with the abundance of 75.9, 68.4, 73.2, and 66.1% in FM diet, RSP diet, CoQ10 diet and RSP + CoQ10 diets, correspondingly, the second dominant phylum was *Bacteroidota* with an abundance of 12.6, 16.1, 21.1, and 24.6% in FM diet, RSP diet, CoQ10 diet and RSP + CoQ10 diets (**Figure 3C**). Clearly, more diverse phyla were found in RSP + CoQ10 diet, including *Thermotogae*, *Caldatribacteriota*, *Gemmatimonadota,* and *Parcubacteria*. The above results revealed that the amount of *Proteobacteria* was changed not due to RSP replacement for fishmeal, but as a result of the addition of CoQ10. On the other hand, both dietary fishmeal replaced with RSP and dietary CoQ10 addition have promoted the proliferation of *Bacteroidota*, manifesting that RSP substitution and CoQ10 supplementation could improve the intestinal microecological environment.

At bacterial class level, *Alphaproteobacteria*, *Gammaproteobacteria,* and *Bacteroidia* were confirmed to be abundant in all diet treatments. The relative abundance of *Alphaproteobacteria* revealed that a highest relative abundance of 49.9% in CoQ10 diet and a lowest of 32.6% in RSP + CoQ10 diet. Whereas the relative abundance of *Gammaproteobacteria* displayed the opposite trend that shrimp fed with RSP + CoQ10

TABLE 3 | Muscle compositions (% dry weight) of *L. vannamei* fed four different diets.

	Crude protein	Crude lipid	Moisture
FM	75.22 ± 0.03	2.80 ± 0.11a	74.09 ± 0.52
RSP	75.28 ± 0.03	4.38 ± 0.31b	75.06 ± 0.57
CoQ10	75.37 ± 0.03	5.30 ± 0.69b	75.55 ± 0.74
RSP + CoQ10	75.37 ± 0.07	5.13 ± 0.36b	75.26 ± 0.39

Values are means ± SE of three replicates. The small letters in the same column means the significant difference at $P < 0.05$.

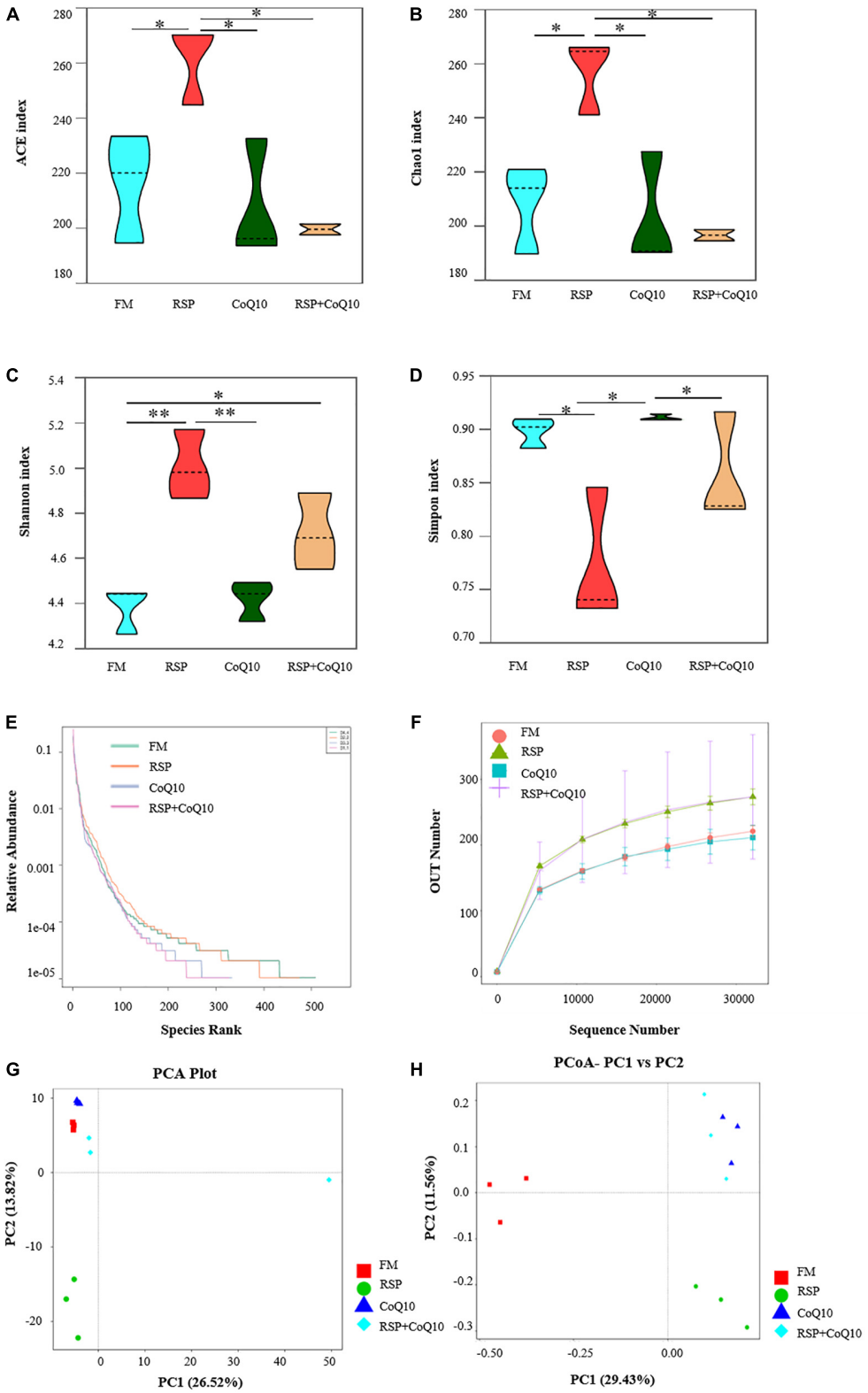

FIGURE 2 | Alpha diversity index statistics and beta diversity analysis of intestinal microbiota in *L. vannamei* fed four different diet treatments. **(A–D)** Were the Ace, Chao1, Shannon and Simpson index of OTU level separately. Statistical differences were determined using two-way ANOVA. *$P < 0.05$; **$P < 0.01$. **(E,F)** Dilution curve and grade abundance curves based on OTU analysis of *L. vannamei* intestines. **(G)** Principal co-ordinates analysis (PCoA) distribution plot. **(H)** Principal component analysis (PCoA) normalized distribution plot. PC1, the first principle component; PC2, the second principle component.

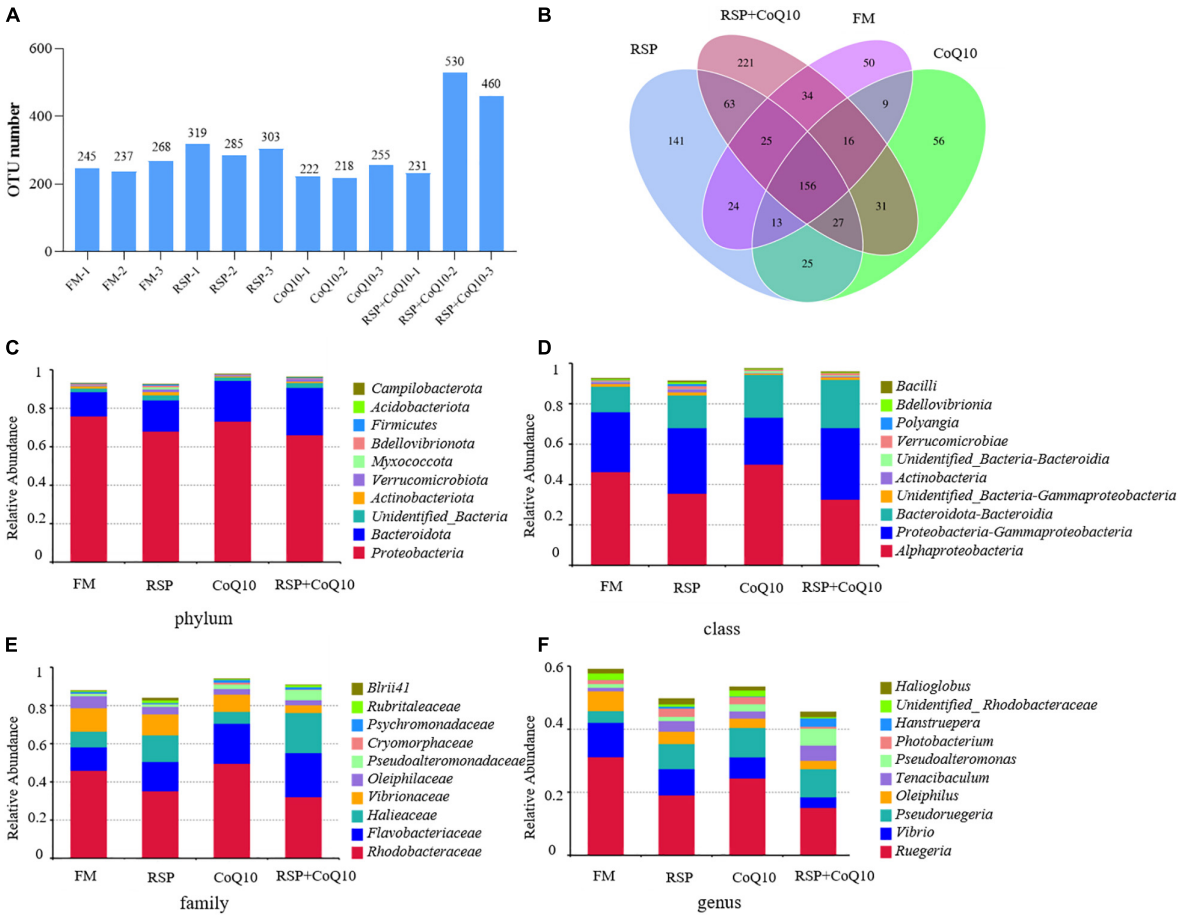

FIGURE 3 | Taxonomic analysis of intestinal microbiota in *L. vannamei* fed four different diet treatments. **(A)** Distribution map of OTU number in samples from different diet treatments. **(B)** Comparison of OTUs in the four diet treatments by Venn diagram. Relative abundance of bacterial community in all diet treatments at **(C)** phylum, **(D)** class, **(E)** family and **(F)** genus levels in different diet treatments. AL, ageratum-liquid; OTU, operational taxonomic unit (OTU).

diet has the highest abundance of 35.4% and fed with CoQ10 diet has the lowest abundance of 23.2%. The third dominant class was *Bacteroidia* with an abundance of 12.6, 16.1, 21.1, and 23.8% in FM diet, RSP diet, CoQ10 diet and RSP + CoQ10 diets (**Figure 3D**). At the family level (**Figure 3E**), *Rhodobacteraceae* was the dominant family in all diets with its relative abundance at 45.9, 35.2, 49.6, and 32.1% in FM diet, RSP diet, CoQ10 diet and RSP + CoQ10 diets, separately, indicating its predominance was weakened in RSP diet and RSP + CoQ10 diets independent of the presence of absence of CoQ10. At the genus level (**Figure 3F**), the number and abundance of predominant genus in different diets varied considerably. *Ruegeria* was the predominant genus in all diets, the relative abundance of *Ruegeria* manifested that the highest relative abundance of 31.2% was with FM diet and a lowest of 15.1% with RSP + CoQ10 diet with the rest in between (19.1–22.4%). At the end of the test, compared with FM diet, the relative abundance of *Vibrio* was decreased significantly in RSP diet, CoQ10 diet and RSP + CoQ10 diets, however, in RSP diet, CoQ10 diet and RSP + CoQ10 diets, almost an addition of *Pseudoruegeria* was observed. Altogether, the microbial compositions of predominant phyla were obviously similar among these four diet treatments. All these data indicated that RSP substitution and CoQ10 supplementation may maintain intestinal homeostasis and decrease the colonization by intestinal opportunistic pathogens, like *Vibrio*.

LefSe Analysis of Gut Microbiota

LefSe analysis was carried out to further investigate the changes of *L. vannamei*' intestinal microbial biomarkers caused by RSP substitution and CoQ10 supplementation (**Figure 4**). Based on the threshold that a Linear Discriminant Analysis (LDA) score of > 4.0, the dominant phylum, order, class, family and genus were screened. The biomarkers at different levels associated with FM diet were, in descending, *Cellvibrionales*, *Halieaceae,* and *Bacteroidia*. In RSP diet, the specific biomarkers were, *Rhodobacteraceae*, *Rhodobacterales,* and *Alphaproteobacteria*. Moreover, in CoQ10 diet, the specific biomarkers were, *Photobacterium_damselae*, *Photobacterium,* and *Actinobacteriota*, by contrast, the specific biomarkers were, *Bacteria, Protebacteria,* and *Oceanospirillales* in RSP + CoQ10 diet.

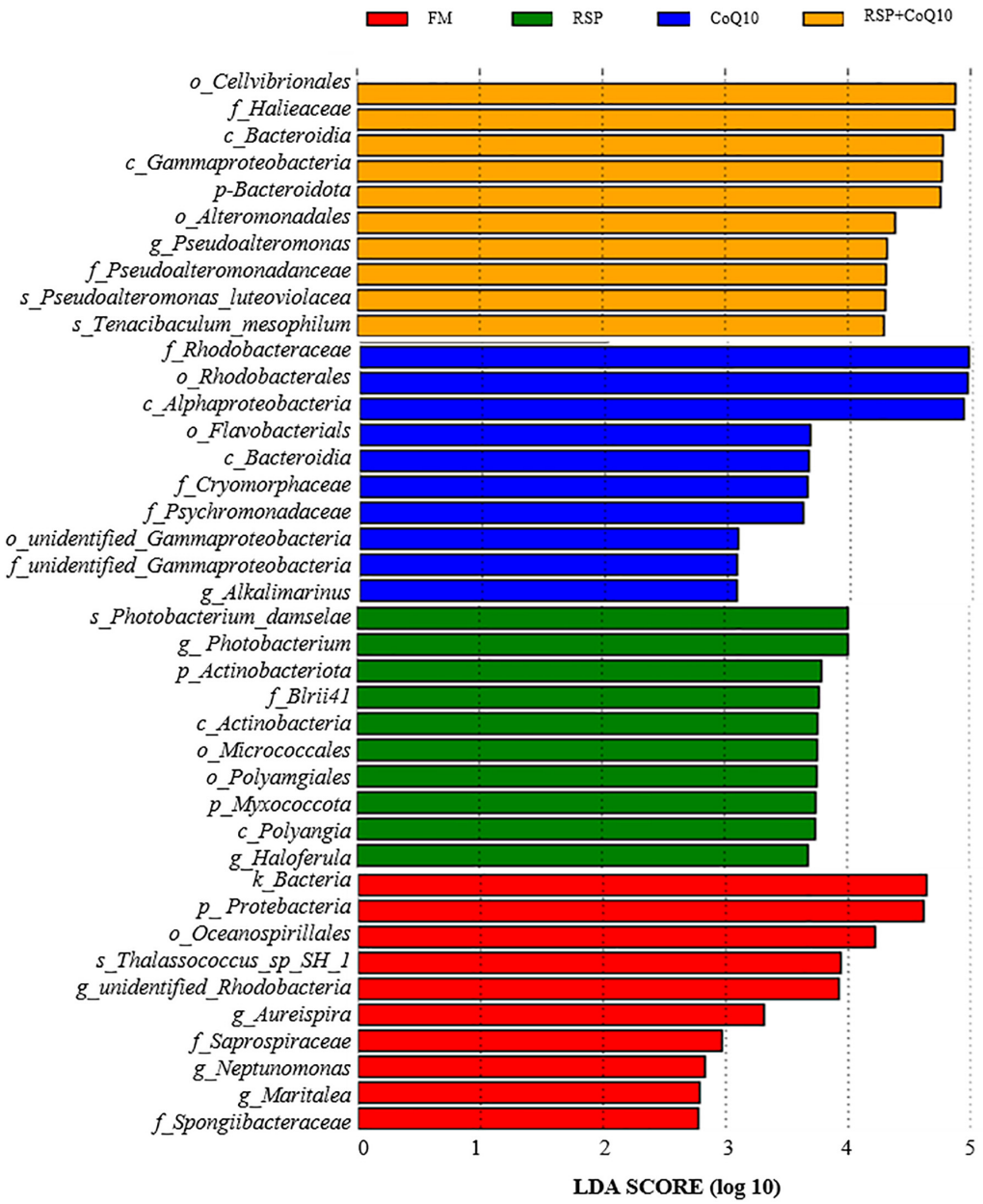

FIGURE 4 | Identified differentially abundant taxa between four diet treatments from by linear discriminant analysis coupled with LEfSe (LDA > 2.5, $P < 0.05$). Yellow box: enriched in FM diet, blue box: enriched in RSP diet, green box: enriched in CoQ10 diet, red box: enriched in RSP + CoQ10 diet. LDA, linear discriminant analysis; LefSe, LDA effect size.

Functional Prediction of the Microbiota

Based on PICRUSt, the functional capacity of the intestinal microbiota was predicted. The detailed results were given in **Figure 5**, evidently, the most abundant function was related to environmental information processing in FM diet. In RSP + CoQ10 diet, genetic information processing functions were doubled when compared to FM diet. Correspondingly, human diseases were enriched in CoQ10 diet. In combination, these COG function classification results showed that the *L. vannamei* intestinal microbial taxa presented the distinct biological functions with the presence of absence of RSP substitution and CoQ10 supplementation.

Survival Rates of After Acute Low Salinity Challenge

The survival rates of four experimental diet treatments were shown in **Table 4** after acute low salinity challenge. The survival rate in shrimp fed with RSP diet was considerably lower than those in shrimp fed with CoQ10 diet and RSP + CoQ10 diets

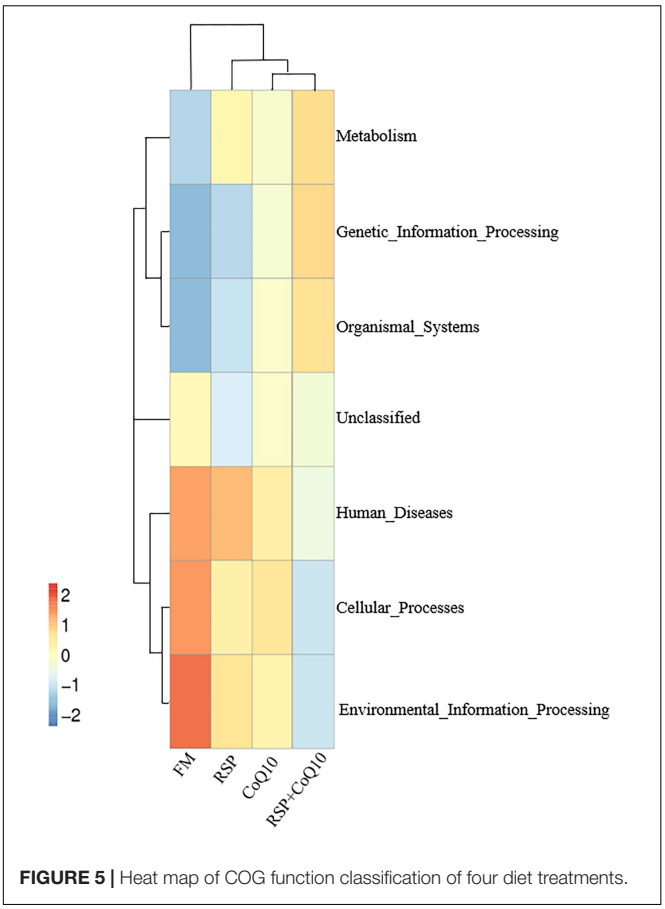

FIGURE 5 | Heat map of COG function classification of four diet treatments.

TABLE 4 | Survival rates after the acute salinity change test.

	FM	RSP	CoQ10	RSP + CoQ10
Survival (%)	66.67 ± 5.77a	65.00 ± 5.62a	77.67 ± 6.29b	80.00 ± 5.00b

Values are means ± SE of three replicates. The small letters in the same column means the significant difference at P < 0.05.

TABLE 5 | Immune parameters of *L. vannamei* challenged the acute salinity change test.

	MDA/μmol/mg	T-AOC/U/mgprot	GSH-PX/U/mgprot
FM	49.96 ± 0.13	0.76 ± 0.08a	37.10 ± 2.39
RSP	49.76 ± 0.09	0.90 ± 00.05a	35.67 ± 3.92
CoQ10	49.70 ± 0.13	1.20 ± 00.05b	36.39 ± 4.14
RSP + CoQ10	49.73 ± 0.14	1.13 ± 0.07b	36.69 ± 1.98

Values are means ± SE of three replicates. The small letters in the same column means the significant difference at P < 0.05.
MDA, malondialdehyde; T-AOC, total antioxidant capacity; GSH-PX, glutathione peroxidase.

($P < 0.05$). No statistically appreciable differences were observed in shrimp fed with FM diet and RSP diets ($P > 0.05$).

Biochemical Parameters of Hepatopancreas After Acute Low Salinity Challenge

The hepatopancreas content of MDA and activities of T-AOC and GSH-PX were illustrated in **Table 5**. The hepatopancreas activities of T-AOC in shrimp fed CoQ10 diet and RSP + CoQ10 diets were remarkably higher than that in shrimp fed FM diet and RSP diets ($P < 0.05$), while no significant differences were presented in T-AOC activity between FM diet and RSP diets ($P > 0.05$). In addition, no considerable differences were found in hepatopancreas MDA contents and GSH-PX activities among all diets ($P > 0.05$).

Relative Expression of Immune-Related and Antioxidant-Related Genes in Hepatopancreas of Shrimp After Low Salinity Challenge

As showed in **Figure 6**, after 4.5 h acute low salinity challenge, the expression level of *SOD* in hepatopancreas of shrimp fed CoQ10 diet and RSP + CoQ10 diets were remarkably higher than those of shrimp fed FM diet and RSP diets ($P < 0.05$). Relative expression of *HSP70* for shrimp fed CoQ10 diet and RSP + CoQ10 diets were higher than that for shrimp fed FM diet and RSP diets ($P < 0.05$). However, no significant differences were found in *Caspase-3* expression among all diets ($P > 0.05$). The lowest *Relish* expression was found in shrimp fed FM diet ($P < 0.05$).

DISCUSSION

The current studies of RSP as an alternative fishmeal source in *L. vannamei* feed were limited. The PNSB extracted from *Rhodobacter sphaeroides* was proved to benefit for *L. vannamei* (Chumpol et al., 2018). Our previous study has proved that RSP played a significant role in enhancing growth performance, increasing survival rates, reinforcing immune response, facilitating resistance and oxidative capacity against low salt challenge in *L. vannamei* (Liao et al., 2021). Meanwhile, CoQ10 was recognized as a natural substance with a formidable antioxidant capacity, whose natural sources varied from prokaryotes organisms to eukaryotes, comprising an abundance of bacteria (*Agrobacterium tumefaciens*, *Rhodobacter sphaeroides,* and *Paracoccus denitrificans*) (Cluis et al., 2007). With the exception of *Agrobacterium*, the great potential of a facultative photosynthetic bacteria-*Rhodobacter sphaeroides* for yielding CoQ10 had been confirmed (Choi et al., 2005). In this research, the growth performance (WG and SGR) of *L. vannamei* showed a significantly growth with CoQ10 supplementation in FM diet. Correspondingly, there were no significant differences between RSP diet and RSP + CoQ10 diets in WG and SGR. To a certain extent, RSP could serve as a complete fishmeal substitution in *L. vannamei* without compromising growth performance. Gopi et al. (2014) reported an enhancement in WG at 0.02 and 0.04 g kg^{-1} CoQ10 diet in broiler. Moreover, El et al. (2020) observed that WG and SGR of *Nile tilapia* showed no remarkable differences at the level of 0.04 g kg^{-1} CoQ10 dietary. While, 0.08 g kg^{-1} CoQ10 supplementation could significantly ameliorate growth performance of *L. vannamei* in this study.

It is well known that the intestinal microbiota has many roles in the health of aquatic animal, the majority of which were benign

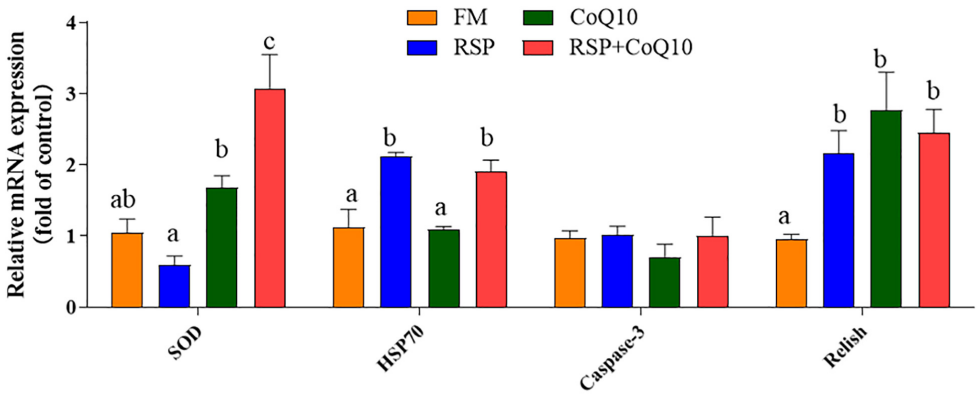

FIGURE 6 | Relative expression of immune-related and antioxidant-related genes in hepatopancreas of shrimp fed with four diet treatments after low salinity stress. Results are mean ± SEM (n = 3). The column with different superscripts manifests significant differences ($P < 0.05$). *HSP70*, heat shock protein 70; *SOD*, superoxide dismutase.

or neutral, including in immune response, nutrient absorption and intestinal morphology (Fujimura et al., 2010; Huang et al., 2015). Indeed, the intestinal microbiota would be susceptible to the food and aqueous environment (Gao et al., 2014). As an important determinant of ecosystem functioning (Johnke et al., 2020), biodiversity is generally quantified by richness, evenness or diversity (McArt et al., 2012). Balanced intestinal microbiota are important for the growth and health of *L. vannamei*. In this study, the value of Shannon index increased significantly in *L. vannamei* fed RSP diet treatment, which indicated that shrimp fed RSP diet had greater community diversity than other three diets. Meanwhile, the trend of ACE index and Chao1 index were consistent with Shannon index, while Simpson index was just opposite, all these data manifested that it was dietary RSP substitution of fishmeal that enriched the intestinal microbial community of *L. vannamei*. The PCoA score plot statistics explained that the intestinal samples from CoQ10 addition did cluster in the same district, while FM diet and RSP diets did not, indicating fluctuations in the intestinal microbial compositions of *L. vannamei* with the addition of CoQ10, which may be caused by dietary CoQ10 supplementation.

Many previous studies have proved that *Proteobacteria* was the predominant colonizer in *L. vannamei*' intestine (Xiong et al., 2015; Rungrassamee et al., 2016), the enrichment of which reflected the dysregulation of microbiota and the unstable microbial community structure (Shin et al., 2015). In this research, the abundance of *Proteobacteria* in all diets was more than 60%, and *Proteobacteria* was abundant in FM diet compared to other three dies. Accordingly, the outgrowth of *Proteobacteria* may trigger the dysbiosis, and diets of RSP substitution and CoQ10 supplementation maintain the intestinal homeostasis compared to FM diet. *Rhodobacteraceae* has been applied in shrimp aquaculture for its latent talent to promote host growth (Yamazaki et al., 2016) and ability to degrade organic compounds in aquarium (Huang et al., 2018). As beneficial bacteria, *Rhodobacteraceae* was abundant in CoQ10 diet in *L. vannamei*' intestine compared to other three diets. Correspondingly, the growth performance of CoQ10 diet was significantly higher other three diets, and the higher abundance of *Rhodobacteraceae* might be the major contributor to this difference, therefore, *L. vannamei* fed CoQ10 diet had better growth performance. In the genus levels, *Ruegeria* and *Vibrio* were the most dominant genus in *L. vannamei*' intestine fed with four diet treatments. Similarly, a significant amount of *Vibrio* was also found in some marine organisms (Guerreiro et al., 2018). *Vibrio* was affiliated to the *Vibrionaceae* (family), which was probably the most commonly bacterial pathogens (Liu et al., 2016), and excessive of *Vibrio* could impair the health status of *L. vannamei* (Xiong et al., 2017). Compared with FM diet, the relative abundance of *Vibrio* was remarkably decreased after the shrimp fed CoQ10 diet and RSP + CoQ10 diets, whereas the shrimp fed with RSP diet didn't significantly decrease, which indicated that CoQ10 addition could serve to augment the immune response of *L. vannamei* and avoid the risk of *Vibrio* infection.

Different diet treatments could shape different functional predictions of the intestinal microbiota. Function of environmental information processing, cellular processes and human diseases were more plentiful in FM diet. On the contrary, except for above functions, genetic information processing functions, metabolism and organismal systems were more enriched in RSP + CoQ10 diet. These differences in functions of intestinal microbiota would be related to *L. vannamei*' dietary supplementations. Due to the capability of microbiota (*Firmicutes* and *Bacteroidetes*) to degrade complex polysaccharide (Dudek et al., 2014), taken the abundance of intestinal microbiota into account, more abundant *Bacteroidota* in *L. vannamei* fed RSP + CoQ10 diet might be the reason of higher level of metabolism function. In addition, the higher species richness and diversity in RSP diet demonstrated that the complexity and instability of microbiota might lead to more functional categories.

As one of the most significant environmental factors in aquaculture, salinity changes have influenced the growth performance and survival rates of the shrimp (Esparza-Leal et al., 2010). The previous research has proved that no big differences were observed between FM diet and diet treatment of fishmeal

replaced with different levels of RSP in survival rates after acute low salinity challenge (Liao et al., 2021). As shown in this research, after acute low salinity challenge, the supplementation of CoQ10 to diets have significantly increased the survival rates of *L. vannamei* whether exist fishmeal substitution or not. On the other hand, antioxidant capacity of shrimp was the important defense mechanism to withstand environmental extremes. As an endogenous lipophilic antioxidant, CoQ10 plays an important role in preventing the lipid peroxidation level (Navas et al., 2007). During this research, *L. vannamei* fed with CoQ10 diet had significantly upregulated *SOD* expression in hepatopancreas after 4.5 h acute low salinity challenge. The higher expression of *SOD* could be beneficial to increase the resistance of *L. vannamei* against acute low salinity challenge (Zhang et al., 2012), which indicated that CoQ10 supplementation may increase the antioxidant capability by enhancing the release of superoxide anion. In addition, T-AOC activity in the hepatopancreas was highest in CoQ10 diet treatment after acute low salinity challenge. Low salinity had stimulated the accumulation of ROS (Rosas et al., 2001; Yeh et al., 2010), and the increase of T-AOC activity ameliorated the health status of *L. vannamei* to some extent. The improvement of health status and antioxidant capacity may directly enhance the survival rates of *L. vannamei* against acute low salinity challenge, which means that dietary CoQ10 could be used to promote resistance to environmental stress in *L. vannamei*, however, RSP replacement of FM does not influence the antioxidant ability of *L. vannamei* against acute low salinity challenge.

As an important regulator of activating the immune signaling pathway, HSP70 can induce the production of immune proteins to reinforce *L. vannamei*' resistance against a pathogen (Yik Sung and MacRae, 2013). In this study, significantly higher expression of *HSP70* were found in *L. vannamei* fed RSP diet and RSP + CoQ10 diets under low salt stress. Previous research also demonstrated that chronic exposure in the low salinity environment can decrease the immune parameters in *L. vannamei* (Lin et al., 2012). Meanwhile, CoQ10 supplementation can improve immune parameters by reducing stresses and promoting mitochondrial respiration (Feher et al., 2007). These results demonstrated that RSP substitution of fishmeal and CoQ10 supplementation enhanced resistance against low salt stress in *L. vannamei* as an immunostimulant.

CONCLUSION

In this study, the impacts of dietary RSP substitution of fishmeal and CoQ10 supplementation on the growth performance, muscle composition, intestinal microbiota, antioxidation effects and immunity capacity of *L. vannamei* after acute low salinity challenge were investigated. These beneficial effects can provide a new perspective that RSP substitution of fishmeal and CoQ10 addition may be enhance resistance at low salinity or other extreme environments.

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 in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by the Care and Use of Laboratory Animals of the National Institutes of Health (NIH) and approved by Experimental Animal Ethics Committee of Sun Yat-sen University.

AUTHOR CONTRIBUTIONS

JN and YG designed the study. ZW, YW, WZ, and XH analyzed parts of results. DW carried out the rearing trial. RY and MC analyzed the data. ZL analyzed results and wrote this manuscript with suggestions from JN and YG. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: YG was employed by Zhejiang Xinhecheng 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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Effects of Dietary Curcumin on Growth and Digestive Physiology of *Seriola dumerili*

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In order to understand the effects of dietary curcumin on the growth and digestive performance of *Seriola dumerili*, this study was conducted under laboratory conditions. The control group was added with 0% curcumin, and two treatment groups were added with 0.01% and 0.02% curcumin, respectively. Fish were fed with experimental diet for 8 weeks. The growth data, growth genes, intestinal digestive enzymes, tissue parameters, and blood lipid-related indicators were measured, and the correlation between digestive indicators, growth gene indicators, and growth characteristic indicators was analyzed. The results showed that the survival rate, visceral ratio, hepatosomatic ratio, and condition factor of *Seriola dumerili* increased first and then decreased with the increase of curcumin content in feed. The feed conversion ratio of the control group was significantly higher than that of the treatment group. With the increase of curcumin content in feed, lipase and trypsin increased first and then decreased with the increase of curcumin content. Compared with the control group, curcumin significantly increased the muscle thickness and the number of goblet cells of *Seriola dumerili*. High and low contents of curcumin can reduce the content of serum total cholesterol and triglyceride, and a low dose (0.01%) of curcumin has the strongest lipid-lowering effect. The relative gene expression of IGF1 and IGF2 reached the maximum in the 0.02% treatment group. The results showed that the addition of curcumin in feed could theoretically improve the survival rate, feed efficiency, and other growth characteristics of *Seriola dumerili*, improve intestinal digestion and structure, promote intestinal health, and reduce blood lipid levels. The best effect was achieved in the 0.01% treatment group. The results of this study provide a theoretical reference for the feasibility of adding curcumin to *Seriola dumerili* feed in production and realize the optimal proportion of feed additives for this species, which is beneficial to the sustainable development of aquaculture.

Keywords: *Seriola dumerili*, curcumin, growth performance, digestion, growth gene

INTRODUCTION

Curcumin is a polyphenolic compound extracted from rhizomes of Zingiberaceae and Araceae (Indira Priyadarsini, 2013). It has multiple functions including antioxidant, anti-inflammatory, and antibacterial effects and has the advantages of being green pollution-free and natural and having no residue (Zhang and Xiao, 2021). A previous study has shown that when using 120 mg/kg dietary level of curcumin, the lipid peroxidation of juvenile tilapia (*Oreochromis niloticus*) can be significantly reduced, and the liver can be protected from injury, while the antioxidant function of the fish body is improved (Zhang et al., 2018). The addition of curcumin in feed could reduce the serum transaminase activity and liver inflammatory factor level and improve the body color of juvenile American eel (*Anguilla rostrata*) to a certain extent (Zhu et al., 2020). A dietary supplementation of 0.06% curcumin significantly increased the serum antioxidant capacity of juvenile turbot (*Scophthalmus maximus*) (Wang et al., 2016). Giri et al. (2019) found that the addition of curcumin in diets could not only increase the expression of anti-inflammatory factors in intestine and liver of common carp (*Cyprinus carpio*), reducing the expression of pro-inflammatory factors, but also promote its growth. Mahmoud et al. (2017) proposed that dietary curcumin levels of 50–100 mg/kg could improve the growth index and feed conversion rate of *Oreochromis niloticus*, reduce the number of intestinal *Escherichia coli* and *Aeromonas*, and improve serum immunity and liver antioxidant.

Recent advances in curcumin and its other different forms being adopted as dietary supplements have broad application prospects in fish nutrition (Mahmoud et al., 2020). Curcumin could promote the growth of fish and improve the intestinal flora and tissue structure, thereby improving the antioxidant capacity of the intestinal tract and improving its own immunity. A study has shown that adding 1 or 5 g curcumin per kilogram of feed can improve digestive enzyme activities such as lipase and protease, antioxidant capacity, and digestibility of feed in crucian carp (*Carassius auratus*), thereby promoting growth (Jiang et al., 2016). Khorshidi et al. (2018) also found that the use of curcumin increased the relative abundance of lactic acid bacteria in the intestine of common carp *Cyprinus carpio*. In addition, curcumin was added to the diet in the related application experiments of rainbow trout *Oncorhynchus mykiss* (Yonar et al., 2019), large yellow croaker *Pseudosciaena crocea* (Ji et al., 2021), yellow catfish *Pelteobagrus fulvidraco* (Zhang et al., 2017), tilapia *Oreochromis mossambicus* (Sruthi et al., 2018), *Anguilla marmorata* (Xie, 2017), and Juvenile American Eel (*Anguilla rostrata*) (Zhu, 2021).

The greater amberjack *Seriola dumerili*, belonging to Carangidae *Seriola*, is a warm-water oceanodromous migration fish (Chen, 2004). Due to its high flesh quality and fast growth, the greater amberjack has become a good candidate for aquaculture (Thakur et al., 2009). With the increase in consumption demand and the continuous development of the market, the commercial culturing scale of *Seriola dumerili* has expanded considerably. However, as the consequences of large-scale intensive culturing, disease outbreaks in this species have been frequently reported and have caused considerable economic losses (Wu and Pan, 2000). In this study, a physical response of *Seriola dumerili* to different dietary curcumin levels was evaluated. Growth characteristics, intestinal digestion, and growth gene expression were used as the evaluation criteria. Results from the present study will improve our understanding on the usage of dietary curcumin in *Seriola dumerili*.

MATERIALS AND METHODS

Experimental Fish and Feeding Management

The experimental fish *Seriola dumerili* was cultivated by the Tropical Aquaculture Research and Development Center, South China Sea Fishery Research Institute, Chinese Academy of Fishery Sciences. A total of 135 healthy fish were randomly divided into 3 groups. Dietary curcumin supplemental levels of 0% (control), 0.01%, and 0.02% with three replicates were tested in this study. A total of nine tanks were used in this study, and 15 fish were tested in each tank. After transferring to the experimental tank, fish were acclimated in the experimental system for 1 week before the feeding experiment was started. The feeding experiment was carried out in a RAS system, and the tank volume was 5,000 l. The initial mean body weights of each group were 149.02 ± 12.59, 153.51 ± 4.09, and 151.78 ± 3.84g, respectively. During the experimental period, water quality was maintained at temperatures 27°C–31.5°C, salinity at 35‰, and pH at 7.5–8, and ammonia nitrogen was maintained at <0.1 mg/l, nitrite at <0.02 mg/l, and dissolved oxygen >7.0 mg/l. *Seriola dumerili* were fed twice a day at 8:00 a.m. and 16:00 p.m. The residual feeds and feces were collected 1 h after feeding. The experimental period was 8 weeks.

Preparation of Experimental Feed

In this study, 0%, 0.01%, and 0.02% curcumin were added into the feed to prepare three kinds of isonitrogenous and isolipid experimental feeds. Then, the feed feeding experiment was carried out on *Seriola dumerili*. The experimental feed was self-designed and produced by the Lingshui Tropical Aquatic Research and Development Center. Curcumin (purity >95%) was provided by Xi'an Feida Biotechnology Co., Ltd. (Xi'an, China). All ingredients were crushed and screened (0.2 mm mesh), mixed through a commercial food mixer (Guangdong Li Feng Co., Ltd., Guangzhou, China), then mixed with oil, produced through a granulator (Shandong Hengfeng Co., Ltd., Dongying, China) to produce particles with a diameter of 4 mm, and air-dried at room temperature (25°C). The prepared feed was sealed in plastic bags and stored in a -20°C refrigerator for later use (Li et al., 2020). Experimental feed formula information is shown in **Table 1**.

According to each parallel 15,000-g feed, the weight of each component of the feed is shown in the table. The control group, the 0.01% treatment group, and the 0.02% treatment group represented the experimental groups fed with 0 (control), 100 mg/kg, and 200 mg/kg curcumin, respectively; that is, the amount of curcumin in each 15,000-g feed was 0, 1.5, and 3g, respectively.

TABLE 1 | Feed formula and ingredient list.

Composition and nutritional component	The proportion of ingredients in 15,000 g feed (unit: g)		
	Control group	0.01% treatment group	0.02% treatment group
Fish meal	8,850.0	8,850.0	8850.0
Corn gluten meal	1,050.0	1,050.0	1,050.0
Soybean meal	1,200.0	1,200.0	1,200.0
Maize starch	1,200.0	1,200.0	1,200.0
Microcrystalline cellulose	750.0	750.0	750.0
Fish oil	1,050.0	1,050.0	1,050.0
Lecithin	150.0	150.0	150.0
Vitamin premix[a]	75.0	75.0	75.0
Mineral premix[b]	75.0	75.0	75.0
Choline chloride	75.0	75.0	75.0
Betaine	75.0	75.0	75.0
Carboxymethyl cellulose	450.0	450.0	450.0
DM	87.9	87.9	87.9
Crude protein	49.7	49.7	49.7
Crude fat	12.7	12.7	12.7
Crude ash	10.7	10.7	10.7
Curcumin	0.0	1.5	3.0

[a]*Vitamin premix (mg kg^{-1} diet): vitamin A 9000000 (IU kg^{-1} diet), vitamin K3 600 (IU kg^{-1} diet), vitamin D 2500000 (IU kg^{-1} diet), vitamin E 500 (IU kg^{-1} diet), vitamin B (B1 3200, B2 10900, B5 20000, B6 5000, B12 1160), vitamin C 50000, phaseomannite 1500, calcium pantothenate 200, niacin 400, folic acid 50, biotin 2.*

[b]*Mineral premix (mg kg^{-1} diet): KCl 70; KI 1.5; $MgSO_4 \cdot 7H_2O$ 300; $MnSO_4 \cdot 4H_2O$ 3; $CuCl_2$ 5; $ZnSO_4 \cdot 7H_2O$ 14; $CoCl_2 \cdot 6H_2O$ 0.5; $FeSO_4 \cdot 7H_2O$ 15; $CaCl_2$ 2.8 (g kg^{-1} diet); $KH_2PO_4 \cdot H_2O$ 4.5 (g kg^{-1} diet). The dietary energy was calculated as carbohydrate: 17.15 MJ·kg^{-1}, protein: 23.64 MJ·kg^{-1}, lipid: 39.54 MJ·kg^{-1}.*

Serum Biochemical Parameters

Feeding was stopped the day before the end of the trial, and each group was weighed separately. Using zinc oxide eugenol cement as anesthetics (Changshu Shang Dental Materials Co., Ltd., Shanghai, China), all groups were anesthetized and sampled; each parallel took 3 tails. The surface water was wiped with paper towels, and a 1-ml sterile syringe was used to extract blood from the tail veins of *Seriola dumerili*. After standing at room temperature for 4 h, blood samples were centrifuged for 10 min at 3,500 ×g, 4°C, and then the supernatants were collected. The collected supernatant was stored at −80°C for further measurement and analysis. Serum biochemical parameters were determined according to the instructions of the commercial kits (Nanjing Jiancheng Biological Co., Ltd., Nanjing, China), i.e., total cholesterol (TC) (Item No. A111-1-1); cholesterol oxidase–peroxidase aminoantipyrine method; and triglycerides (TG) (Item No. A110-1-1): phosphate oxidase–peroxidase aminoantipyrine method. All serum biochemical parameters were performed in triplicates.

Tissue Sample Collection and Growth Performance Measurement

Fish were then dissected with sterilized scalpel, tweezers, and so on, the intestines quickly separated out, and the whole operation process performed on the ice. The intestine samples were put into the 2-ml centrifuge tubes with labels. Part of them were frozen in liquid nitrogen and then transferred to a -80°C refrigerator for the determination of enzyme activity. The other part was used for making slices. Biochemical and gene expression samples were frozen in liquid nitrogen and stored at -80°C. In addition, 9 fish were randomly selected from each group, and the body length and weight were measured respectively. The liver and viscera were quickly separated and weighed to calculate the specific growth rate, weight gain rate, feed conversion ratio and condition factor, visceral ratio, and hepatosomatic ratio. The calculation formulas are as follows:

$$Weight\ gain\ rate\ (\%) = (final\ average\ weight - initial\ average\ weight)/initial\ average\ weight \times 100$$

$$Specific\ growth\ rate\ (\%/d) = (ln\ final\ average\ weight - ln\ initial\ average\ weight)/breeding\ test\ days \times 100$$

$$Visceral\ ratio\ (\%) = visceral\ weight/fish\ weight \times 100$$

$$Hepatosomatic\ ratio\ (\%) = liver\ weight/fish\ weight \times 100$$

$$Condition\ factor = body\ weight/body\ length^3 \times 100$$

$$Feed\ coefficient = feed\ weight/fish\ weight\ gain$$

$$Survival\ rate\ (\%) = number\ of\ fish\ at\ the\ end\ of\ each\ pool/number\ of\ individual\ fish\ at\ the\ beginning\ of\ each\ pool \times 100$$

In the formula, the unit of the body length is cm, the unit of the final average weight and initial average weight is g, and the unit of breeding test days is d.

Digestive Enzymes and Intestinal Structure Determination

Intestine samples from each parallel were mixed and weighed. Under the condition of ice bath, 0.9% normal saline or sample homogenate medium was added to the tissue according to the

weight volume ratio of 1:9 to make 10% homogenate. According to the requirements of the corresponding kits (Nanjing Jiancheng Biological Co., Ltd., Nanjing, China), the homogenates were centrifuged and the supernatants were extracted, and then the relevant indicators were measured. Intestinal digestion indicators included amylase (AMS) (Item No. C016-1-1), lipase (LPS) (Item No. A054-2-1), trypsin (TRYP) (Item No. A080-2-2), and total protein (TP) (Item No. A045-4-2). The starch-iodine colorimetric method, methyl halal substrate method (microplate method), colorimetric method, and BCA microplate method were used for determination, respectively.

The intestines of the three groups were fixed with 4% paraformaldehyde for 24 h, dehydrated with graded ethanol concentration, and embedded in paraffin. A Leica RM 2016 rotary microtome (Shanghai Leica Instrument Co., Ltd., Shanghai, China) was used to cut into sections of 4-μm thickness. Hematoxylin–eosin (HE) staining was used and finally permanently fixed with resinene. A Pannoramic 250/MIDI scanner and CaseViewer 2.0 (3DHISTECH Co., Ltd., Budapest, Hungary) were used to scan slices, collect images, and measure data. The height and width of intestinal folds, the height of the intestinal villus, the thickness of the muscular layer, and the number of goblet cells were quantified.

Growth Gene Expression

Genes selected for qPCR analysis were found based on the NCBI database (https://www.ncbi.nlm.nih.gov/). Primers of EF1α (internal reference gene), IGF1, and IGF2 genes were designed by the Primer Premier 5 program (**Table 2**). The method for proving the specificity and availability of synthetic primers was referred to Yang et al. (2020). RNA was extracted according to the method of Fu et al. (2019). The ND 5000 spectrophotometer (BioTeke Corporation, Beijing, China) was used to measure the absorbance at 260 and 280 nm to determine the purity and concentration of RNA extracted. Finally, agarose gel electrophoresis (1%) was used to detect the integrity of RNA. RNA was immediately used to synthesize cDNA. TransScript-Uni One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech Co., Ltd., Beijing, China) were used for reverse transcription of 1 μg total RNA. The synthesized cDNA samples were stored at -20°C for further use (Yang et al., 2020). SYBR Green (Tiangen Biotech Co., Ltd., Beijing, China) was used for real-time quantitative PCR analysis (Hangzhou LongGene Scientific Instrument Co., Ltd., Hangzhou, China) for three times for each sample (Fu et al., 2019).

Statistical Analysis

The relative mRNA expression level of the target gene was determined by the $2^{-\Delta\Delta Ct}$ method. The standard deviation (SD) of the test data was determined by using the EF1α gene as an internal reference gene. Statistical analysis was performed using SPSS Statistics 26 software. One-way analysis of variance and Duncan's test were used for comparison between groups. The significance level was set to $p < 0.05$ (Yang et al., 2020). Finally, Origin 2019 was used for mapping.

RESULTS

Effect of Curcumin on the Growth Performance of *Seriola dumerili*

Indicators related to the growth performance after 8 weeks of feeding experiment are shown in **Table 3**. It can be found that the survival rate and condition factor of *Seriola dumerili* increased first and then decreased with the increase of curcumin content in feed ($p < 0.05$). Moreover, they reached the highest level in the 0.01% treatment group ($p < 0.05$), while there was no significant difference between the control group and the 0.02% treatment group ($p > 0.05$). The feed coefficient of *Seriola dumerili* in the control group was significantly higher than that in the treatment group ($p < 0.05$), and there was no significant difference in feed coefficient between 0.01% and 0.02% treatment groups ($p > 0.05$). Different contents of curcumin diet had no significant effects on final average weight, weight gain rate, specific growth rate, visceral ratio, and hepatosomatic ratio of *Seriola dumerili* ($p > 0.05$).

Data are presented as mean ± SD. In the same row, the same lowercase letters on the right side of the data indicated no significant difference ($p > 0.05$). Different lowercase letters indicated significant difference ($p < 0.05$).

Effects of Curcumin on Digestive Enzymes and Intestinal Tissue of *Seriola dumerili*

After feeding with different levels of curcumin for 8 weeks, the intestinal digestive enzyme (lipase, trypsin, amylase) activity is as shown in **Figure 1**. The results showed that the content of curcumin in feed had a significant effect on the digestive enzyme of *Seriola dumerili* ($p < 0.05$). Among them, the intestinal amylase activity of *Seriola dumerili* decreased firstly and then increased with the increase of curcumin content ($p < 0.05$) and reached the maximum value in the control group (**Figure 1**: I). With the increase of curcumin content in diet, the activities of lipase and trypsin in the intestine of fish were firstly increased and then

TABLE 2 | Primer sequence table.

Gene abbreviation	Primer sequence (5′–3′)	Tm (°C)	Amplicon size (bp)	Accession number
IGF1	F:GTCTTCAAGAGTGCGATGTGC	58.1	189	XM_022749759.1
	R:GCCATAGCCTGTTGGTTTACTG	59.3		
IGF2	F:AGAGGAGTGTTGTTTCCGTAGC	58	131	XM_022754221.1
	R:CCTGTTTTAGTGCGGGCAT	58.5		
EF1 α	F:ATCGTTGCCGCTGGTGTT	59.3	134	XM_022744048.1
	R:TCGGTGGAGTCCATCTTGTT	57.4		

TABLE 3 | Effects of different levels of curcumin in feed on the growth performance of *Seriola dumerili*.

Growth performance	Group		
	Control group	0.01% treatment group	0.02% treatment group
survival rate (%)	62.22 ± 12.73^{b}	90.78 ± 3.24^{a}	70.00 ± 3.18^{b}
Average initial weight (g)	149.02 ± 12.59^{a}	153.51 ± 4.09^{a}	151.78 ± 3.85^{a}
Average final weight (g)	263.59 ± 83.29^{a}	327.38 ± 48.38^{a}	306.55 ± 23.95^{a}
Weight gain rate (%)	102.54 ± 9.63^{a}	112.97 ± 28.13^{a}	101.89 ± 12.90^{a}
Specific growth rate (%/d)	1.26 ± 0.09^{a}	1.34 ± 0.25a	1.25 ± 0.12^{a}
Feed coefficient	3.45 ± 0.33^{a}	2.21 ± 0.21^{b}	2.30 ± 0.31^{b}
Visceral ratio (%)	5.04 ± 0.67^{a}	5.30 ± 0.06^{a}	5.22 ± 0.26^{a}
Hepatosomatic ratio (%)	0.98 ± 0.21^{a}	0.99 ± 0.07^{a}	0.89 ± 0.02^{a}
Condition factor	2.36 ± 0.05^{b}	2.48 ± 0.06^{a}	2.27 ± 0.05^{b}

In the same row, values with same small letter superscripts or no letter superscripts mean no significant difference (P > 0.05); different small letter superscripts mean significant difference (P < 0.05).

decreased ($p < 0.05$), and the activities of these two digestive enzymes reached the minimum values in the control group and 0.02% treatment group, respectively (**Figure 1**: II and III).

As shown in **Figure 2**, compared with the control group, dietary curcumin significantly increased the thickness of the muscular layer and the number of goblet cells ($p < 0.05$) and had no significant effect on the height of the intestinal villus ($p > 0.05$). The intestinal fold width reached the maximum value in the 0.02% treatment group and the minimum value in the control group ($p < 0.05$), while there was no significant difference between the 0.01% treatment group and the control group and the 0.02% treatment group ($p > 0.05$). The intestinal fold height reached the minimum in the 0.02% group ($p < 0.05$), and there was no significant difference between the control group and the 0.01% group ($p > 0.05$).

Effects of Curcumin Content on Serum Biochemical Parameters

The results of serum triglyceride and cholesterol contents of *Seriola dumerili* are observed in **Figure 3**. It can be seen from the figure that the curcumin content in feed had a significant effect on the serum triglyceride content of *Seriola dumerili* ($p < 0.05$). With the increase of curcumin content in feed, the serum triglyceride content of *Seriola dumerili* firstly decreased and then increased ($p < 0.05$) and reached the highest value in the control group. The total cholesterol content reached the maximum value in the control group and the minimum value in the 0.01% treatment group ($p < 0.05$), and there was no significant difference between the 0.02% treatment group and the control group and the 0.01% treatment group ($p > 0.05$).

Effects of Curcumin on the Expression of Liver Growth Genes in *Seriola dumerili*

After feeding 0%, 0.01%, and 0.02% curcumin diets for 8 weeks, the relative gene expression levels of IGF1 and IGF2 in the liver of *Seriola dumerili* are shown in **Figure 4**. The relative gene expression levels of IGF1 and IGF2 reached the maximum in the 0.02% treatment group ($p < 0.05$), while there was no significant difference between the control group and 0.01% treatment group ($p > 0.05$).

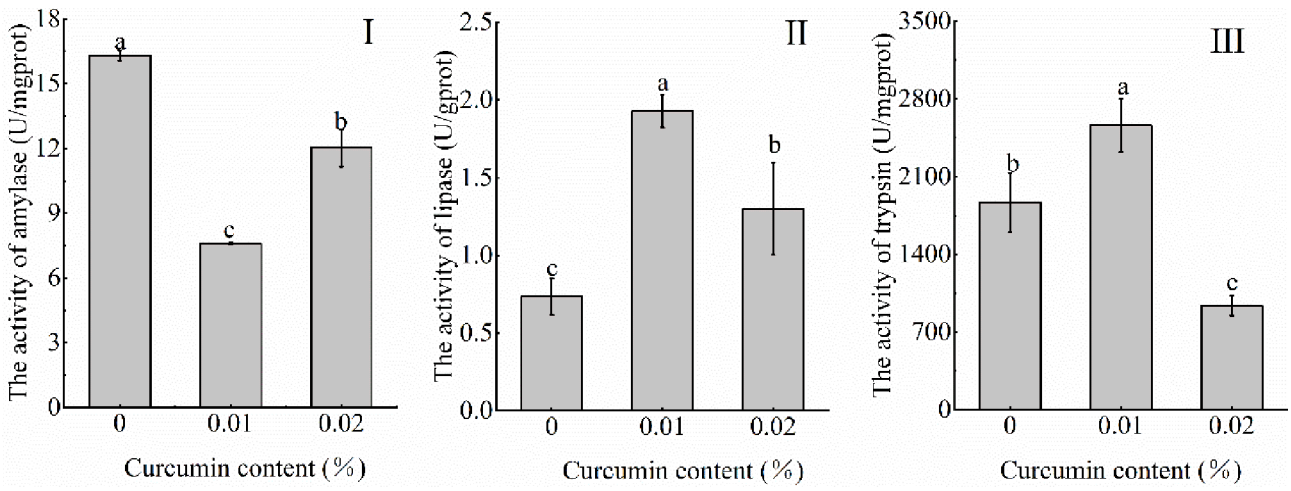

FIGURE 1 | Effects of different levels of curcumin on the activities of intestinal digestive enzymes (I, amylase activity; II, lipase activity; III, trypsin activity). Different superscript letters indicate significant differences among treatments (P < 0.05).

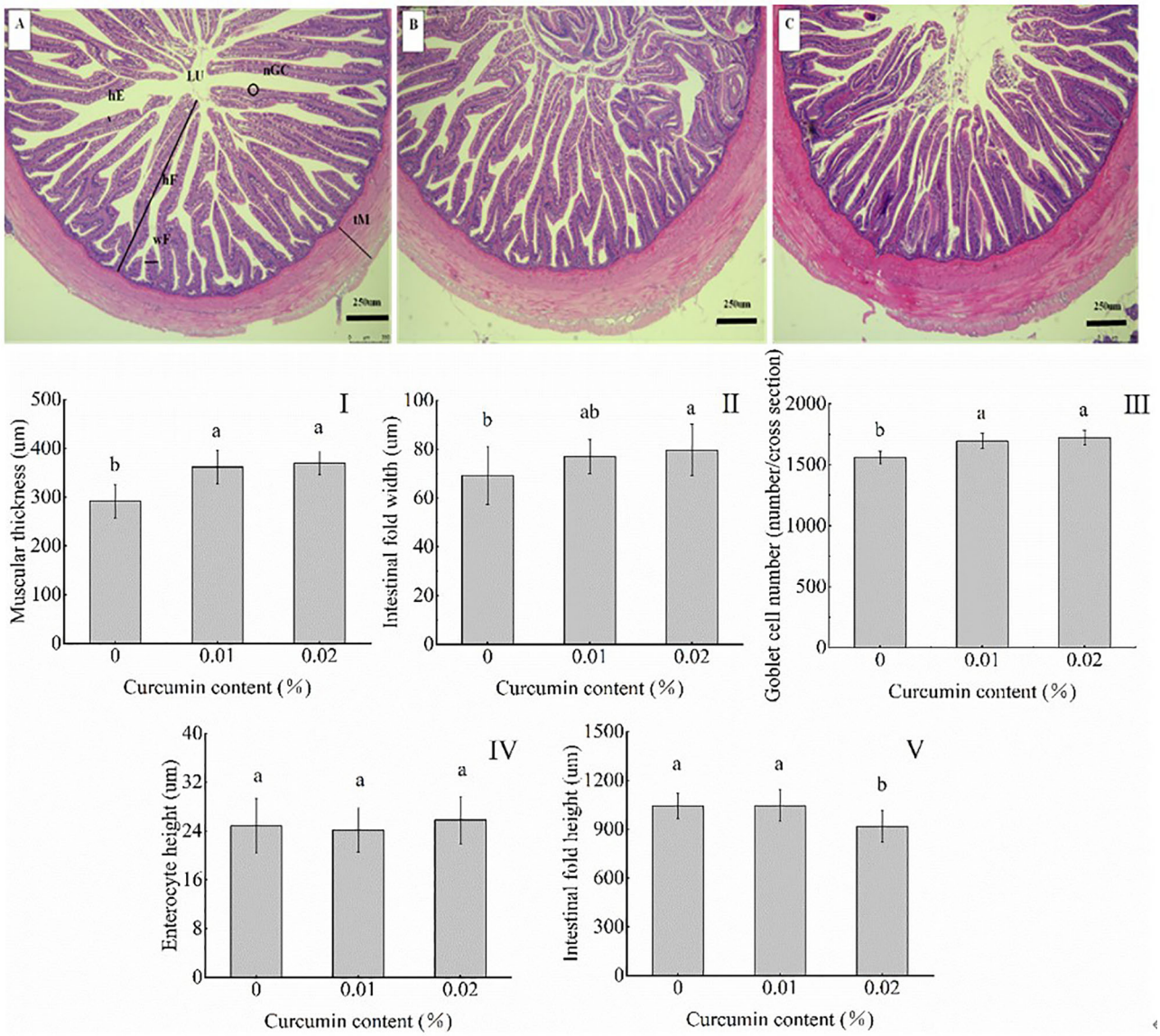

FIGURE 2 | The intestinal tract of *Seriola dumerili* was affected by diets with different curcumin contents. Representative sections of intestinal tissue (50 magnification) are shown at the top [**(A)** control group; **(B)** 0.01% treatment group; **(C)** 0.02% treatment group]. tM represents muscle layer thickness; hF represents the height of intestinal fold; wF represents the intestinal fold width; hE stands for intestinal epithelial cell height; nGC represents the number of goblet cells; LU represents lumen. (I, muscle layer thickness; II, intestinal fold width; III, number of goblet cells; IV, intestinal epithelial cell height; III, intestinal fold height). Different superscript letters indicate significant differences between different treatments ($p < 0.05$).

DISCUSSION

Curcumin has been widely used in traditional medicine for a long time because of its anti-inflammatory and antioxidant effects (Oetari et al., 1996; Nishikawa et al., 2013). In addition, recent studies have also reported that curcumin, as a popular immunostimulator, can promote the growth of aquatic animals and enhance disease resistance (Zhang et al., 2014; Guan et al., 2015; Jiang et al., 2016; Wang et al., 2016; Yonar et al., 2019; Ashry et al., 2021). Until now, a number of studies have confirmed that the addition of curcumin in diet can show great growth performance on some species of fish, including large yellow croaker (*Pseudosciaena crocea*) (Wang and Wu, 2007), grass carps (*Ctenopharyngodon idella*) (Zhongze et al., 2003), crucian carp (*Carassius auratus*) (Jiang et al., 2016), Asian see bass (*Lates calcarifer*) (Abdelwahab and El-Bahr, 2012), and Nile tilapia (*Oreochromis niloticus*) (Mahmoud et al., 2017). However, the growth of juvenile turbot (*Scophthalmus maximus*) was not significantly affected by dietary curcumin content, which may be related to various factors such as fish

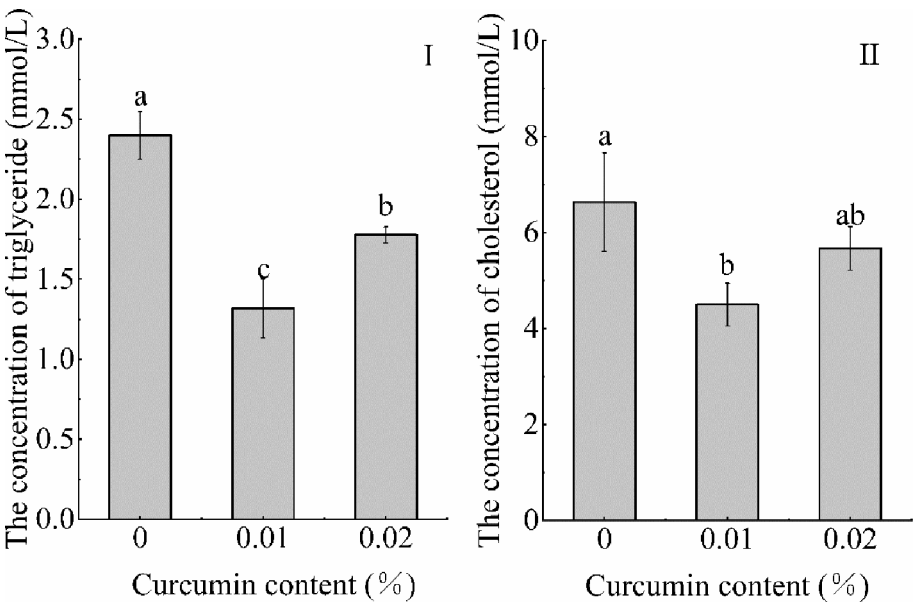

FIGURE 3 | Effects of different levels of curcumin on serum triglyceride and total cholesterol content of *Seriola dumerili* (I, triglyceride content; II, total cholesterol content). Different superscript letters indicate significant differences among treatments ($P < 0.05$).

species, size, and culture environment (Wang et al., 2016). This study found that although there was no significant difference in the final average weight, weight gain rate, specific growth rate, visceral ratio, and hepatosomatic ratio between the experimental groups, they were higher in the 0.01% treatment group than in the control group. Moreover, the 0.01% treatment also significantly increased the survival rate and condition factor and reduced the feed coefficient. However, the 0.02% treatment group had a trend to increase the survival rate, final average weight, and visceral ratio and significantly reduced the feed coefficient of *Seriola dumerili*. The results showed that the addition of curcumin in the feed had a certain promoting effect on the growth of *Seriola dumerili* and promoted the feed conversion rate, while 0.01% curcumin could better improve the growth performance of *Seriola dumerili*.

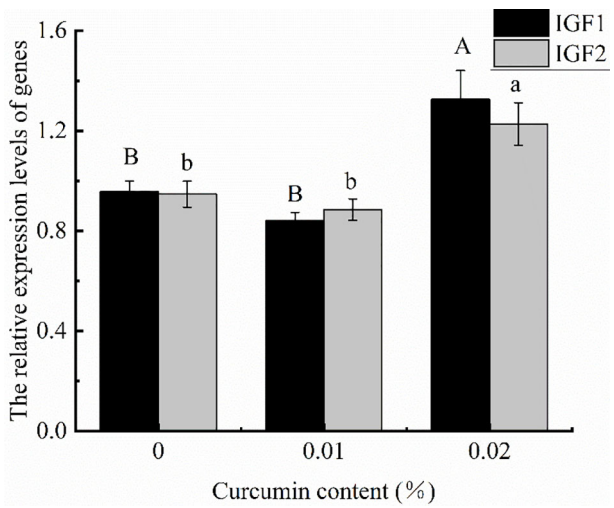

FIGURE 4 | Effects of different levels of curcumin on the quantitative expression of IGF1 and IGF2 genes in the liver of *Seriola dumerili*. Different uppercase or lowercase letters indicate significant differences between treatments ($p < 0.05$).

Improvement of growth performance has been reported to be related to the role of curcumin in enhancing intestinal digestive enzyme activity (Jiang et al., 2016; Li et al., 2020; Zhang and Xiao, 2021). Digestive enzymes play a key role in the digestion of nutrients, and their activities directly reflect the digestive ability of fish and affect the growth rate (Ling et al., 2010; Jiang et al., 2016). Moreover, because of its important role in digestive tract digestion and absorption, the intestinal tract of fish has an important impact on the growth and nutrition of fish (El-Bakary, 2010). This study showed that the 0.01% curcumin diet significantly increased the activities of lipase and trypsin in the intestine of *Seriola dumerili*. That was consistent with the findings on grass carp (Ctenopharyngodon idella) (Li et al., 2020), crucian carp (Carassius auratus) (Jiang et al., 2016), and tilapia (*Oreochromis mossambicus*) (Midhun et al., 2016). However, this study was contrary to the results of previous studies on amylase. In general, there is a strong relationship between dietary preferences and digestive ability, and high proteolytic (trypsin and pepsin), carbohydrase (cellulase and amylase), and lipase activities reflect high protein, cellulose/starch, and fat diet habits, respectively (Johnston, 2003; Johnston and Freeman, 2005; Qi-Cun et al., 2007). The results of amylase in *Seriola dumerili* may be related to

the fact that it is a carnivorous fish with less starch intake and amylase fluctuates within a low activity range. Carnivorous fish usually eat fat-rich foods that stimulate lipase activity in their digestive tract (Fu et al., 2021a). Compared with the control group, the significant increase of lipase in the treatment group may be related to the decrease of cholesterol level and the stimulation of fat digestion by dietary curcumin (Guan et al., 2015; Zhang and Xiao, 2021). In brief, this study showed that the appropriate amount of curcumin in the feed could enhance the digestive ability of the whole intestine (indicated by the activities of trypsin and lipase), and the 0.01% curcumin content in the feed was the best ratio to enhance the digestion of *Seriola dumerili*. The above analysis on growth traits confirmed this point well, indicating that curcumin may have the function as a digestive promoter, thereby improving growth performance. The attractive flavor of curcumin can also increase the palatability and intake of feed, while making it of more effective use as feed for fish, thereby improving feed utilization, which can be reflected in the feed conversion ratio. As the fermentation substrate and selective growth factor of beneficial gastrointestinal bacteria, plant chemicals and their metabolites can provide health benefits for organisms. At the same time, as a selective inhibitor of harmful intestinal bacteria, it can also play a prebiotic-like role in fish (Zheng et al., 2009; Harikrishnan et al., 2011). Curcumin contains a large number of bioactive compounds such as reducing sugars, triterpenoids, and alkaloids. These compounds may act as prebiotics which has immunomodulatory properties (Kurhekar, 2013). Therefore, curcumin may also be considered as a prebiotic that enhances the balance of the positive and negative flora in the intestine, increases intestinal absorption and digestion, and ultimately promotes fish growth and health (Midhun et al., 2016).

It has been reported that food composition strongly affects the activities of digestive enzymes and intestinal morphology of aquatic animals (Picos-Garcıía et al., 2000; Garcia-Esquivel and Felbeck, 2006; Wang et al., 2017). Intestinal tissue is the main component of the digestive system to absorb nutrients, and its morphological changes can be used as an important indicator of the health status of the organism (Wang et al., 2017; Amoah et al., 2019). According to previous studies, higher or wider intestinal folds, microvilli, and intestinal villus indicate higher nutrient intake and better health condition of aquatic animals (Li et al., 2020). In this experiment, the 0.02% treatment group significantly increased the intestinal fold width, indicating that curcumin can enhance digestive ability within a certain range. Muscle thickness, intestinal fold height, and goblet cell number are also important intestinal structural parameters, which can reflect the degree of intestinal health (Ma et al., 2018; Sun et al., 2018). Goblet cells have the function of secreting mucus and are the main source of mucin secretion; mucus can lubricate intestinal contents for easy passage, and it protects the intestinal surface from pathogens (Fu et al., 2021a). Reduced levels of mucin production and goblet cell response often occur when the intestine is impaired, such as intestinal infection, and sufficient goblet cells are required to have a possible positive impact on the intestinal host defense system (Kim and Khan, 2013). The number of goblet cells in the control group was significantly lower than in the other two treatment groups, which indicated that curcumin plays an active role in intestinal health and has a positive effect on fish intestinal morphology (Xie, 2017; Fu et al., 2021a) (Heidarieh et al., 2012). The higher feed efficiency in the treatment group may be due to the larger villi area. Curcumin can improve the intestinal digestive enzyme activities, which may be related to the increase in the number of intestinal mature cells. Studies have shown that intestinal digestive enzyme activity is positively correlated with the number of mature cells; that is, the increase in the number of mature cells leads to an increase in intestinal digestive enzyme activity (Zhu, 2021), which was consistent with the results of this study. Strikingly, the response of goblet cells was not consistent with increasing levels of turmeric supplementation and this warrants further research. At higher supplementation, minor effects observed might be due to the fact that high levels of turmeric could reduce feed intake and increased turmeric could also act as a phytoestrogen (Kaur et al., 2020). Muscle thickness is a common indicator to evaluate the body's ability to absorb and digest nutrients. The thicker the muscle is, the stronger the digestive ability is (Miao et al., 2019). Compared with the control group, curcumin significantly increased the thickness of the muscle layer and goblet cell number of *Seriola dumerili* in this study, which was similar to the results of previous studies on *Anguilla marmorata* (Xie, 2017), tilapia (Zhang et al., 2014), and *Pseudosciaena crocea* (Yu, 2016). It indicated that proper addition of curcumin could improve the morphological structure of intestinal tissue, which may also be one of the reasons for the growth-promoting effect of curcumin.

It has been reported that curcumin can also reduce serum triglyceride and total cholesterol content and promote fat metabolism (Guan et al., 2015). Total cholesterol and triglyceride are collectively referred to as blood lipid levels. Although they only account for a very small part of total fish lipid levels, they are transported between tissues and are very active in metabolism, reflecting lipid metabolism in fish (Deng et al., 2001). Previous studies have shown that the addition of curcumin reduces the contents of triglyceride (TG) and total cholesterol (TC) in serum of grass carp and tilapia, showing a favorable regulatory effect on lipid metabolism (Guan et al., 2015). That was consistent with the results of this study. In vertebrate like fish, lipids are transported as lipoproteins to deliver endogenous and dietary lipids to peripheral tissues, where lipoprotein lipase (LPL) hydrolyzes the triglycerides (TGs) of very low-density lipoprotein (VLDL) or TG-rich lipoprotein and released fatty acids (FAs) are then taken up by the tissues for oxidation or storage with reduced plasma TG level (Salmerón, 2018). However, excessive fat deposits in fish can degrade health and product quality (Salmerón, 2018). In the study of *Labeo rohita* fingerlings (Chowdhury et al., 2021), the reduced serum TG level in the fish of phyto-additive fed groups compared to non-supplemented control might be mediated through bioactive compounds of dietary phyto-additives that probably enhanced the LPL activity for lipolysis of serum TGs and utilization of released FAs for energy at the cellular level with reduced lipid storage in adipocytes as we could observe significantly reduced body lipid in the fish of phyto-additive groups. Moreover, curcumin reduced fat deposition in the

abdominal area of broiler chicks (Rajput et al., 2012) and fat content in liver of tilapia (Guan et al., 2015), respectively. The results are slightly different from those in this paper; this may be due to the different effects of curcumin on fat deposition in different tissues of different age species. Or it may be that the negative feedback effect of excessive fat deposition starts to lead to the reduction of blood lipids, so as to control the excessive accumulation of fat in fish culture. The blood lipid index measured in this study confirmed that both high and low curcumin can reduce the contents of serum total cholesterol and triglyceride, and the low-dose (0.01%) curcumin has the strongest effect on lowering blood lipid.

Insulin-like growth factors (IGFs) are mitogenic peptides, whose expression is generally regulated by exogenous factors (photoperiod and temperature) and endogenous factors (humoral factors and nutritional status) (Fu et al., 2021b). Reinecke et al. (2005) showed that blood levels of IGF-1 or tissue levels of its mRNA were positively proportional to protein content, dietary ration, and systemic growth rates in some fish. In addition, feeding methods also affect the gene expression of IGF-1 and IGF-2 (Gabillard et al., 2006). Studies have shown that polyphenol phytochemicals can increase the growth of fish by stimulating the production of IGF-1 and growth hormone and promoting the synthesis of protein, RNA, DNA, and other synthetic effects. IGFs have been proved to play an important role in fish growth, development, and nutritional metabolism and can be determined by the expression of liver mRNA (Rolland et al., 2015). For example, in the study of golden pompano (*Trachinotus ovatus*) (Tan et al., 2017), channel catfish (*Ictalurus punctatus*) (Peterson et al., 2005), clownfish (*Amphiprion ocellaris*) (Avella et al., 2010), and Barramundi (*Lates calcarifer*) (Fu et al., 2021b), it was found that IGF-1 and IGF-2 gene expression was positively correlated with fish growth performance. In this study, the expression of IGF-1 and IGF-2 in the 0.02% treatment group was significantly upregulated, and the gene expression was consistent with the growth performance (final average weight) of *Seriola dumerili*. The growth performance seems to be related to the visceral ratio and feed conversion rate. This association can also be explained by the upregulation of IGF-1, because IGF-1 can promote the proliferation and differentiation of adipose tissue (Chang et al., 2016). In the study of tilapia (*Oreochromis mossambicus*), curcumin significantly increased the real-time quantitative expression of IGF-1 and IGF-2 genes (Midhun et al., 2016), which was consistent with the results of the 0.02% treatment group in this study. Therefore, the improvement of *Seriola dumerili* growth performance may be related to the higher content of curcumin promoting IGF secretion. In addition, curcumin enhances nutrient availability, stimulates growth hormone expression, and subsequently increases the gene expression of IGF-1 and IGF-2, suggesting physiological mechanisms of growth.

CONCLUSION

In summary, the effects of different levels of curcumin in feed on growth performance, intestinal digestive enzyme activity and structural parameters, blood lipid levels, and growth genes of *Seriola dumerili* were studied. It showed that curcumin had a certain promoting effect on the growth of *Seriola dumerili*, promoted the feed conversion rate, and had no negative effect on the growth of *Seriola dumerili*. These increases may be attributed to the digestive promotion characteristics of curcumin and the potential of regulating intestinal structure and enhancing nutritional absorption and production performance. Based on these results, curcumin can promote the growth of *Seriola dumerili*. Therefore, curcumin can be safely used for fish feed, and it provides a theoretical reference for its application in *Seriola dumerili*.

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

This animal study was reviewed and approved by the Animal Welfare Committee of Chinese Academy of Fishery Sciences.

AUTHOR CONTRIBUTIONS

JY conceived the idea and wrote the manuscript. JH and ZF helped in the field experimental work. JH assisted in the data analysis. ZM supervised the manuscript. All authors contributed to the article and approved the submitted version.

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Bile Acids Reduced the Lipid Deposition in Fatty Degenerated Hepatocytes of Pearl Gentian Grouper (*Epinephelus fuscoguttatus*♂ × *Epinephelus lanceolatus*♀) *in vitro*

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Little is known about the association of bile acids (BAs) with lipid deposition and lipid metabolism of fish *in vitro*. In the present study, we established the model of fatty-degenerated hepatocytes in pearl gentian grouper (*Epinephelus fuscoguttatus*♂ × *Epinephelus lanceolatus*♀), and explored the effects and mechanism of BAs regulating on lipid metabolism in this model. The results showed that fatty-degenerated hepatocytes induced by lipid emulsion accumulated the intracellular triglyceride (TG), the enhanced expression of sterol responsive element binding protein 1 (SREBP1) protein, and the repressed expression of peroxisome proliferator-activated receptor alpha (PPARA), and phosphorylated PPARA (P-PPARA) proteins. BAs co-incubation reduced the content of TG, while increasing the expression of PPARA, farnesoid X receptor (FXR), and G protein-coupled bile acid receptor 1 (TGR5) proteins. Activation of FXR by INT-747 reduced the content of TG, while down-regulating the expression of SREBP1 and PPARA, and up-regulating the expression of P-PPARA, FXR, small heterodimer partner (SHP), and TGR5. Inhibition of FXR by guggulsterone increased the level of TG, while decreasing the expression of TGR5, increasing the expression of SHP. Activation of TGR5 by INT-777 reduced the content of TG, while down-regulating the expression of SREBP1 and SHP, up-regulated the expression of TGR5. Inhibition of TGR5 by SBI-115 elevated the level of TG, while reducing the expression of P-PPARA and TGR5. In conclusion, the FXR and/or TGR5 signaling pathways which were inhibited in fatty-degenerated hepatocytes from pearl gentian grouper, were activated after BAs co-incubation, then altering the lipid metabolism by repressing lipogenesis and enhancing lipolysis, and regulating transcriptional factors, thus reduces the lipid accumulation *in vitro*.

Keywords: FXR, tgr5, lipid deposition, fatty-degenerated hepatocytes, *in vitro*

INTRODUCTION

Groupers are the important mariculture species in China, with a total output of 192,000 tons in 2020, an increase of 4.87% over 2019 (Food Organization, 2020; China Agriculture Press, 2021). Among them, the hybrid grouper, *Epinephelus fuscoguttatus*♂ × *Epinephelus lanceolatus*♀, also known as the pearl gentian grouper, is a popular farmed marine fish in Southeast Asia and China, and is intensively cultured along the coastlines of these areas (Xu et al., 2021). High-lipid diets (>13%) are generally used in this fish species, but it will lead to abnormal lipid metabolism and fat accumulation, which results in the susceptibility of fatty liver, then impair the health of liver, and ultimately damage to the growth of fish (Shapawi et al., 2018; Zou et al., 2019). Therefore, it is of great significance to study the mechanism of lipid deposition in fish, and it is necessary to find effective ways to improve the adverse effects induced by high-fat intake.

Bile acids (BAs) are endocrine molecules with a number of basic functions, such as cholesterol catabolism, intestinal fat emulsification, lipid metabolism, and intestinal microbiota homeostasis (Xie et al., 2021b; Xu et al., 2022a). In fact, BAs have broader biological effects, as they are recognized as regulators of farnesoid X receptor (FXR) and G protein-coupled bile acid receptor 1 (TGR5), and therefore, hold important promise for altering lipid content in the treatment of metabolic diseases (Wang et al., 2016; Kumari et al., 2020). Specifically, FXR is a ligand-activated transcription factor that belongs to the nuclear receptor superfamily and plays a vital role in regulating genes network related to the maintenance of lipid homeostasis (Kumari et al., 2020). Many *in vitro* and *in vivo* studies have used mouse models to clarify the positive association of FXR with fatty liver disease and its regulatory role on lipid metabolism (Yang et al., 2010; Kumari et al., 2020). In addition, TGR5 is coupled to a stimulatory G-protein and is responsive to various BAs, such as taurolithocholic acid, lithocholic acid, deoxycholic acid, chenodeoxycholic acid, and cholic acid (Reich et al., 2016). In humans, the activation of TGR5 shows promise for the treatment of various metabolic diseases such as type 2 diabetes and obesity (Guo et al., 2016). In mice, TGR5 can prevent oxidative stress and lipid accumulation in kidney (Wang et al., 2016), and the development of steatosis in liver (Pols et al., 2011). Several studies explored the effects of dietary supplementation of BAs on fat metabolism in fish, such as largemouth bass (*Micropterus salmoides*) (Yu et al., 2019; Yin et al., 2021), tiger puffer (*Takifugu rubripes*) (Liao et al., 2020), Nile tilapia (*Oreochromis niloticus*) (Jiang et al., 2018; El-Shenawy et al., 2020), large yellow croaker (*Larimichthys crocea*) (Ding et al., 2020), grass carp (*Ctenopharyngodon idella*) (Zhou et al., 2018), and turbot (*Scophthalmus maximus*) (Huang et al., 2015); however, it is unclear whether the underlying mechanism is through the FXR and TGR5 signaling pathways.

Compared to *in vivo* experiments, *in vitro* experiments can be used as auxiliary models for whole-animal assays *in vivo* to assess the thorough effects of exogenous substances (Zhu et al., 2014). As an excellent *in vitro* platform, fish cell lines have been widely used in virology, immunology, toxicology, and nutritional analysis (Collet et al., 2018; Li et al., 2022). The liver is a critical organ in metabolic processes and plays a key role in the metabolism of nutrients (Zou et al., 2019). Therefore, fish liver cell lines from various species are commonly used for studying lipid metabolism, such as grass carp (Lu et al., 2012), pearl gentian grouper (Zou et al., 2018, 2019), yellow catfish (*Pelteobagrus fulvidraco*) (Chen et al., 2014; Song et al., 2015b), large yellow croaker (Lai et al., 2021), and zebrafish (*Danio rerio*) (Wang et al., 2019). However, so far, no research has focused on the functions of BAs in fish cells and the mechanism behind.

Therefore, the purpose of this study was to evaluate the role of BAs in the regulation of lipid deposition in primary hepatocytes from pearl gentian grouper. First, we explored the effects of BAs on lipid metabolism in a fatty-degenerated hepatocytes model. Then, we separately studied the effects of FXR and TGR5 signaling pathways on lipid metabolism *in vitro*. This study was firstly investigated the roles of BAs in the fat metabolism of hepatocytes in fish. The findings of the present study would be helpful in developing the new feed additives to improve the lipid deposition in fish.

MATERIALS AND METHODS

Reagents and Animals

The taurocholic acid sodium (TCA, CAS: 345909-26-4, T4009) was purchased from Sigma Chemical Co. (Burlington, MA, United States). INT-747 (FXR agonist, CAS: 459789-99-2, HY-12222), guggulsterone (FXR antagonist, CAS: 95975-55-6, HY-107738), SBI-115 (TGR5 antagonist, CAS: 882366-16-7, HY-111534), and INT-777 (TGR5 agonist, CAS: 1199796-29-6, HY-15677) were purchased from the MedChemExpress Co. (Monmouth Junction, United States). Lipid emulsion solution (LE, 20% soybean oil, 1.2% lecithin, and 78.8% glycerinum) was purchased from Sichuan Kelun Pharmaceutical Co., Ltd (China). MEM medium was purchased from Procell Co. (Wuhan, China). Fetal bovine serum (FBS) was obtained from Gibco Invitrogen (UK). Penicillin and streptomycin were obtained from Sigma-Aldrich (United States). The kit of Cell Counting Kit-8 (CCK-8) was purchased from Glpbio Technology Co. (California, United States). The kits of triglyceride (TG, A110-1-1) and total protein (TP, A045-4) were purchased from Nanjing Jian Cheng Bioengineering Institute (Nanjing, China). The primary antibodies against sterol responsive element binding protein 1 (SREBP1, ab28481) and phosphor-peroxisome proliferator-activated receptor alpha (P-PPARA, S12, ab3484) were purchased from Abcam Co. (Cambridge, UK); PPARA (66836-1-Ig) from Proteintech Co. (Chicago, United States); FXR (bs-12867R) and small heterodimer partner (SHP, bs-4311R) from Bioss Co. (China); TGR5 (NBP2-23669SS) from Novus Co. (China); GAPDH (2118S) from Cell Signaling Technology (MA, United States).

All animals were handled and experimental procedures were conducted in accordance with the Care and Use of Laboratory Animals in China, Animal Ethical and Welfare Committee of China Experimental Animal Society. The study was approved by the Animal Ethical and Welfare Committee of Guangdong Ocean University. The permit number for conducting animal experiments is GDOU-AEWC-20180063.

TABLE 1 | Primers designed for qPCR.

Target	Sequences forward	Sequences reverse	Fragment length (bp)	Primer efficiency (%)
6pgd	GACCACGATGAGATGGCACA	TGTACCGTCAGAGTCCCTGT	111	96.47
fxr	AGGTGCTTGTGAGTGCCATC	TTCCTCTGCGCTGTACTGTT	98	94.91
cpt1	CTTCATCCAGATCGCCCTACA	GCCCTCACGGAACAAACG	97	91.22
ppara	CATCGACAATGACGCCCTC	GCCGCTATCCCGTAAACAAC	135	99.51
pparr	GCGCCAGACACACACAATTT	CACTCGATGTTTAGCGCTGC	119	101.34
atgl	ATTGAGCACCTTCCACCCA	CCGAATCCATCCCACATCTT	213	99.41
srebp1	TGTATCCAACTGTTGAGCACCTG	CTGTGGCAGTGTGGTCCTAG	196	99.85
dgat	CATCTTCTGCTTTGGTGCTTTC	GCATTTCCCGTCCCGTTA	207	99.17
hsl	CAGCCTGGAGCCCGTTAT	TTGGCGGTGATGTAGCG	153	92.41
acc	ACTGGGGTGGTTGCTGTGG	CCTTAATAGCTTGGGCTGTTTTG	148	100.34
fas	CGGGTGTCTACATTGGGGTG	GAATAGCGTGGAAGGCGTTT	213	99.77
me	GAAGTTGTTCTACCGCTTGCTG	AGAGTCCTCGTGGTCTCCTGA	122	98.99
fabp	GTTGTCACCTCCAAGACTCCG	GACCATTTTGCCACCCTCC	129	105.68
lxr	CAGAAGCAATGCAACAAAAGG	TCAGTGAAGTGGGCGAACC	116	92.44
dgka	CATCTTCTGCTTTGGTGCTTTC	GCATTTCCCGTCCCGTTA	225	92.95
hl	GTGGTCGGTGGATGGTATGA	TGCCAATGGTGCGGGTT	155	94.56
acbp	GTTTGAGAAGATGGCAGTGGAC	CCTTTGCGAGTGTGATGTAGG	224	97.24
g6pd	GCTTCACATCCTTGTATCTGCTC	GCGTTCCTTTCATTCTCCG	246	95.96
tgr5	ATGCCATCACCATACCGCTG	CCAGGCGATGCCTAAGATGA	90	99.39

6pgd, 6-phosphogluconate dehydrogenase; acc, acetyl-CoA carboxylase; fas, fatty acid synthase; g6pd, glucose 6-phosphate dehydrogenase; me, malic enzyme; atgl, adipose triglyceride lipase; cpt1, carnitine palmitoyltransferase 1; dgat, acyl CoA diacylglycerol acyltransferase 2; dgka, diacylglycerol kinase alpha; hl, hepatic lipase; hsl, hormone-sensitive lipase; acbp; acyl-CoA binding protein; fabp, fatty acid binding protein; lxr, liver X receptor alpha; ppara, peroxisome proliferator activated receptor alpha; pparr, peroxisome proliferator activated receptor gamma; srebp1, sterol-regulator element-binding protein 1; fxr, farnesoid X receptor; tgr5, G protein-coupled bile acid receptor 1.

Cell Culture and Treatments

The pearl gentian grouper (*Epinephelus fuscoguttatus*♀ × *E. lanceolatus*♂) was obtained and domesticated according to the method of our study (Xu et al., 2022b). Primary hepatocytes were isolated from pearl gentian grouper liver as described in previous study (Zou et al., 2019). Briefly, the fish was cleared of blood, disinfected with alcohol. The liver was carefully excised, rinsed twice with phosphate buffer saline (PBS) supplemented with streptomycin (200 μg/ml) and penicillin (200 IU/ml). Next, liver was aseptically minced, digested by sterile trypsin, neutralized with MEM medium containing FBS. Then, cell suspension was filtered, collected in centrifuge tubes, centrifuged twice at low speed (100 × *g*, 5 min), and once at a lower speed (50 × *g*, 5 min). Finally, the purified hepatocytes were resuspended with MEM medium containing 15% FBS, penicillin (100 IU/ml), and streptomycin (100 μg/ml), and counted by hemocytometer. When the viability of cells was > 92%, the cells suspension was plated onto 75 cm^2 flasks at 10^6 cells/ml. The hepatocytes were cultured at 27°C with 5% (v/v) CO_2, and the medium was replaced every 2–3 days. Upon reaching 80–90% confluency, cells were harvested by trypsin, and transferred into two new flasks.

The hepatocytes was treated as follows: (1) V-CN group (MEM medium without any treatment); (2) V-HL (0.5 ml/L LE); (3) V-TCA (0.5 ml/L LE + 100 μM TCA); (4) V-T747 (0.5 ml/L LE + 100 μM TCA + 5 μM obeticholic acid); (5) V-TGU (0.5 ml/L LE + 100 μM TCA + 10 μM guggulsterone); (6) V-T777 (0.5 ml/L LE + 100 μM TCA + 10 μM INT-777); (7) V-TSBI (0.5 ml/L LE + 100 μM TCA + 1 μM SBI-115). Each treatment was performed in triplicate, and three independent experiments were carried out. In the latter four groups, the activators or inhibitors were added at 48 h after adding the LE. The 2 h later (at 50 h), in the latter five groups, the TCA was added after adding the LE. Sampling was conducted after 96 h of incubation in all groups. The concentrations and periods of LE, TCA incubation, as well as specific inhibitors and activators, were selected according to our preliminary experiment and previous studies (Zou et al., 2019).

Liver Staining, Cell Viability, and Biochemical Assays

The Bodipy 493/503 staining for liver or hepatocytes was conducted according to the established protocol (Zhao et al., 2020), and hepatocytes were observed with a laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany) to visualize the intensity of fluorescence (the green dots were defined as lipid droplet) under 400 × magnification. Cell viability was determined using the CCK-8 assay (Zou et al., 2019). The contents of TG and TP were measured using commercial kits (Nanjing Jian Cheng Bioengineering Institute, Nanjing, China) (Zou et al., 2019).

qPCR Analyses

The details of total RNA extraction, cDNA synthesis, and qPCR assays of hepatocytes sample were described in a previous study (Xu et al., 2022b). Based on transcriptome sequences obtained by our team from pearl gentian grouper (Zhang et al., 2021), the present study chose genes such as (**Table 1**): *6pgd*,

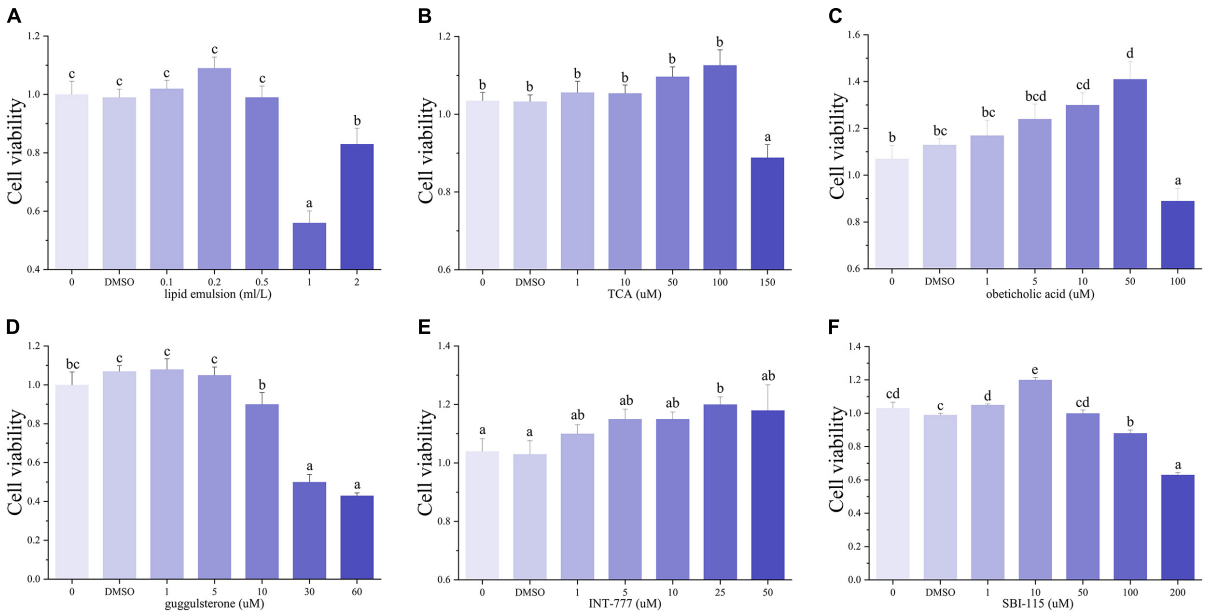

FIGURE 1 | The cell viability of hepatocytes incubated with lipid emulsion **(A)**, TCA **(B)**, obeticholic acid **(C)**, guggulsterone **(D)**, SBI-115 **(E)**, and INT-777 **(F)** at different concentrations (n = 9). TCA: taurocholic acid sodium; obeticholic acid: INT-747, FXR agonist; guggulsterone: FXR antagonist; SBI-115: TGR5 antagonist; INT-777: TGR5 agonist. Values are presented as means with standard deviation, where significant ($p < 0.05$) differences between groups are indicated by different letters (a–e).

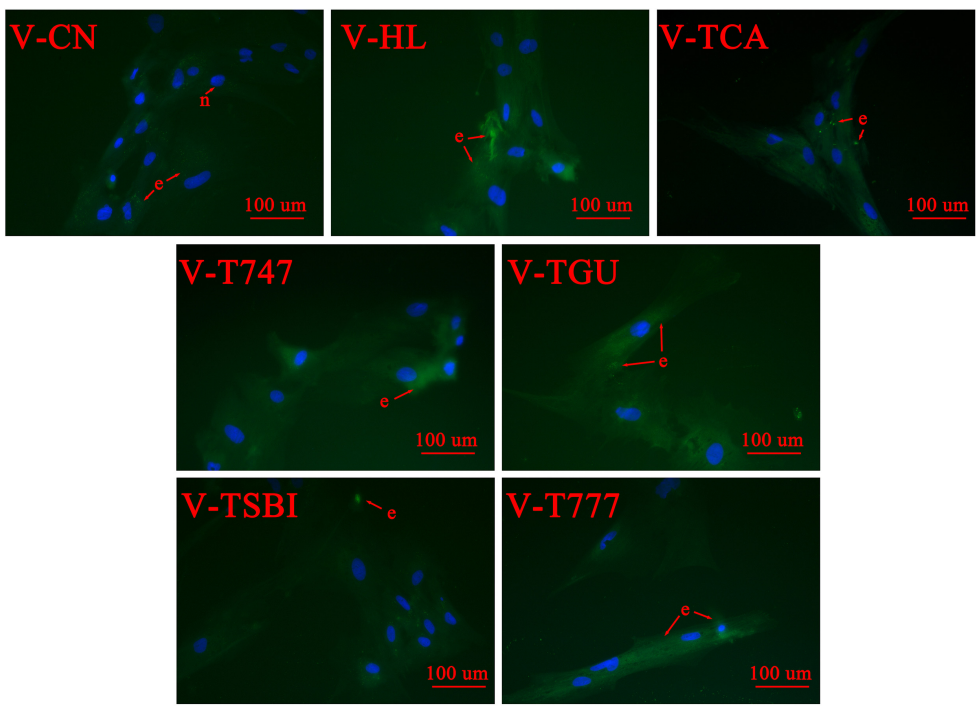

FIGURE 2 | The Bodipy 493/503 staining of different treatments in 400 × magnification (n = 9). e, lipid droplets; n, nuclei. The lipid droplets are stained green and the nuclei are stained blue.

6-phosphogluconate dehydrogenase; *acbp*, acyl-CoA binding protein; *acc*, acetyl-CoA carboxylase; *atgl*, adipose triglyceride lipase; *cpt1*, carnitine palmitoyltransferase 1; *dgat*, acyl CoA diacylglycerol acyltransferase 2; *dgka*, diacylglycerol kinase alpha; *fabp*, fatty acid-binding protein; *fas*, fatty acid synthase; *fxr*, farnesoid X receptor; *g6pd*, glucose 6-phosphate dehydrogenase;

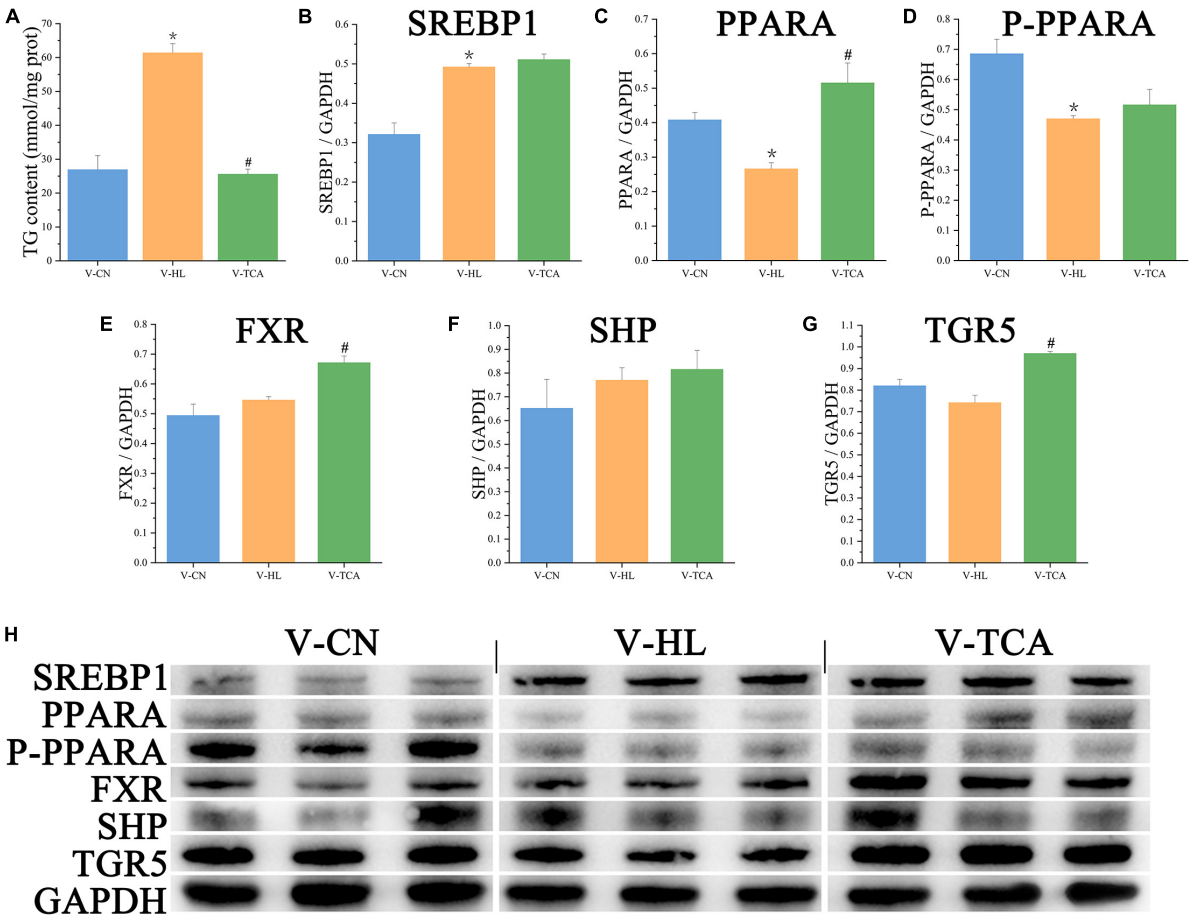

FIGURE 3 | The lipid accumulation and metabolism of hepatocytes in lipid emulsion and BAs incubation. **(A)** The content of intracellular TG (n = 9). **(B–G)** The relative quantification of protein levels of SREBP1, PPARA, P-PPARA, FXR, SHP, and TGR5 normalized to GAPDH in hepatocytes (n = 3); **(H)** the Western blot band of hepatocyte samples (n = 3). Values are presented as means with *SD*, where "*" indicates significant ($p < 0.05$) differences in the V-HL group (compared to the V-CN group), and "#" indicates significant ($p < 0.05$) differences in the V-TCA group (compared to the V-HL group).

hl, hepatic lipase; *hsl*, hormone-sensitive lipase; *lxr*, liver X receptor alpha; *me*, malic enzyme; *ppara*; *pparr*, peroxisome proliferator-activated receptor-gamma; *srebp1*; *tgr5*. The references genes *18s* (F: AGCAACTTTAGTATACGCTATTG; R: CCTGAGAAACGGCTACCACATC) and β-*actin* (F: TACGAGC TGCCTGACGGACA; R: GGCTGTGATCTCCTTCTGC) were selected as the reference gene on the basis of its expression stability (Xu et al., 2022b). We calculated the relative expression of genes using the $2^{-\Delta\Delta CT}$ method (Xu et al., 2022a).

Western Blot Analyses

According to our previous methods (Xu et al., 2022b), phosphorylase inhibitors, protease inhibitors, cell lysate, PBS, and phenylmethanesulfonyl fluoride (PMSF) were added to tissues for fragmentation. After the concentration of protein was determined, loading buffer and PBS were added to make the final concentration at 4 mg/ml, then degeneration. Each gel hole was added 32 mg total protein in SDS-PAGE separation: electrophoresis running with 90 V for 30 min; transferring to PVDF membrane (Billerica, MA, United States); electrophoresis running with 110 V for 80 min. The membrane incubated with primary antibody overnight at 4°C, washed three times with TBST, and incubated with secondary antibody for 1 h. After visualizing with ECL reagents (Billerica, MA, United States), the Western bands were quantified using Image J (version 1.42, National Institutes of Health). The following antibodies were performed: SREBP1 (1:800), PPARA (1:1000), P-PPARA (1:800), FXR (1:500), SHP (1:800), TGR5 (1:500), and GAPDH (1:1000).

Statistical Analysis

The data were tested for normality and homogeneity of variances using the Shapiro–Wilk and Levene's tests, and evaluated by one-way ANOVA and further analyzed by the Duncan's multiple range tests. For the comparison between two groups, Student's t-tests were used (unpaired, two-tailed). The analyses were performed with SPSS 23.0 (IBM, Armonk, NY, United States). Results were presented as means ± *SD* and statistically significant was set as $p < 0.05$.

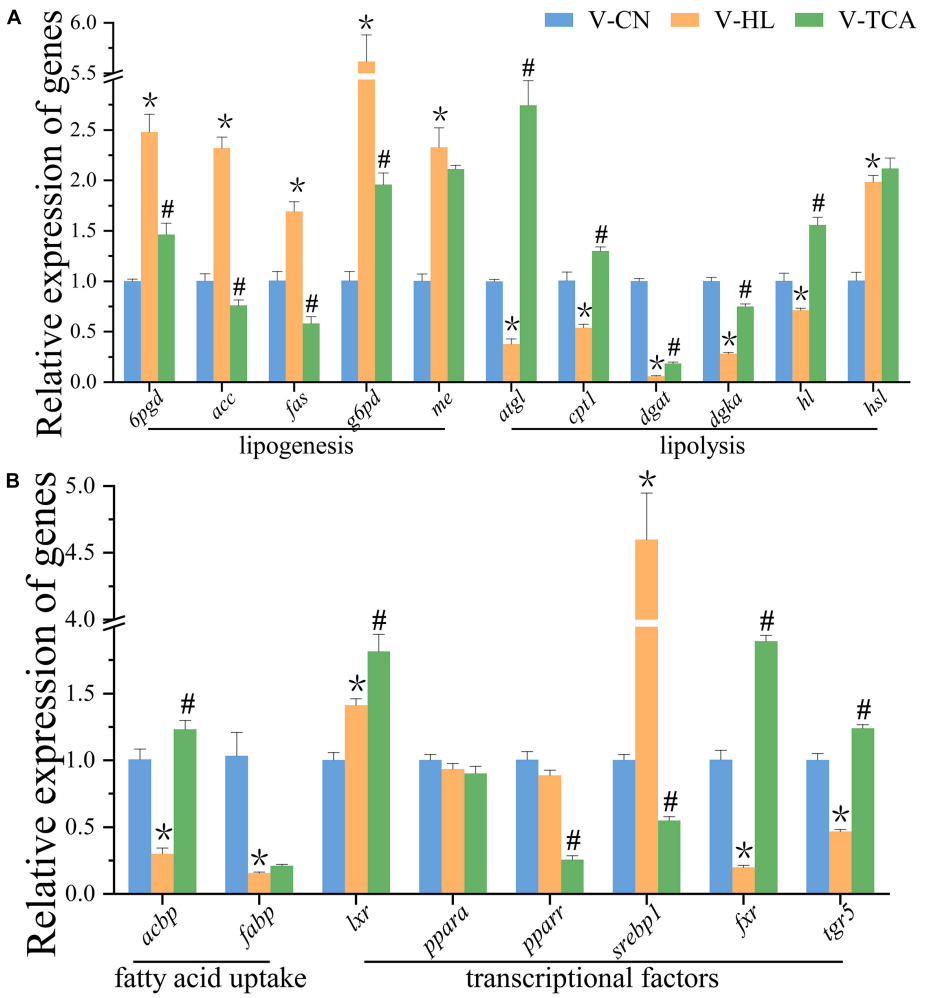

FIGURE 4 | The relative expression of genes associated with lipid metabolism of hepatocytes normalized to *18s* and β-*actin* in lipid emulsion and BAs incubation **(A,B)** (n = 9). Values are presented as means with SD, where "*" indicates significant ($p < 0.05$) differences in the V-HL group (compared to the V-CN group), and "#" indicates significant ($p < 0.05$) differences in the V-TCA group (compared to the V-HL group).

RESULTS

Fatty-Degenerated Hepatocytes Increased, While Bile Acids Co-incubation Reduced the Lipid Accumulation of Pearl Gentian Grouper *in vitro*

The CCK-8 assay showed that LE concentrations of 0.1–0.5 ml/L had no significant influence on the viability of hepatocytes (**Figure 1**). Thus, we chose the dose of 0.5 ml/L LE for the 48 h incubation to build the fatty degenerated hepatocytes model (FDH). The FDH was then incubated with LE for another 48 h (V-HL group). Same as the features of fatty degeneration in grass carp hepatocytes (Lu et al., 2012), the V-HL group increased the lipid accumulation (the area of green dots in Bodipy 493/503 staining and the level of TG) of hepatocytes compared to the V-CN group (hepatocytes without any treatment) (**Figures 2**, **3A**). The V-HL group significantly up-regulated the expression of SREBP1 protein, significantly down-regulated the expression of PPARA and P-PPARA proteins (**Figure 3**). In addition, compared to V-CN treatment, the V-HL treatment significantly increased the expression of lipogenesis genes (*6pgd*, *acc*, *fas*, *g6pd*, and *me*) and their transcriptional factors genes (*lxr* and *srebp1*), whereas significantly decreased the expression of lipolysis genes (*atgl*, *cpt1*, *dgat*, *dgka*, and *hl*), fatty acid uptake genes (*acbp* and *fabp*), *fxr*, and *tgr5* (**Figure 4**).

According to the results of CCK-8 assay, the FDH were co-incubated with 100 μM TCA for another 48 h (V-TCA group) (**Figure 1**). The TG concentration of the V-TCA group was significantly decreased, compared to the V-HL group (**Figure 3A**). The TCA incubation significantly elevated the expression of PPARA, FXR, and TGR5 proteins. Meanwhile, in V-TCA group, the expression of lipogenesis genes (*6pgd*, *acc*, *fas*, and *g6pd*) and their transcriptional factors genes (*pparr* and *srebp1*) were significantly decreased, whereas the

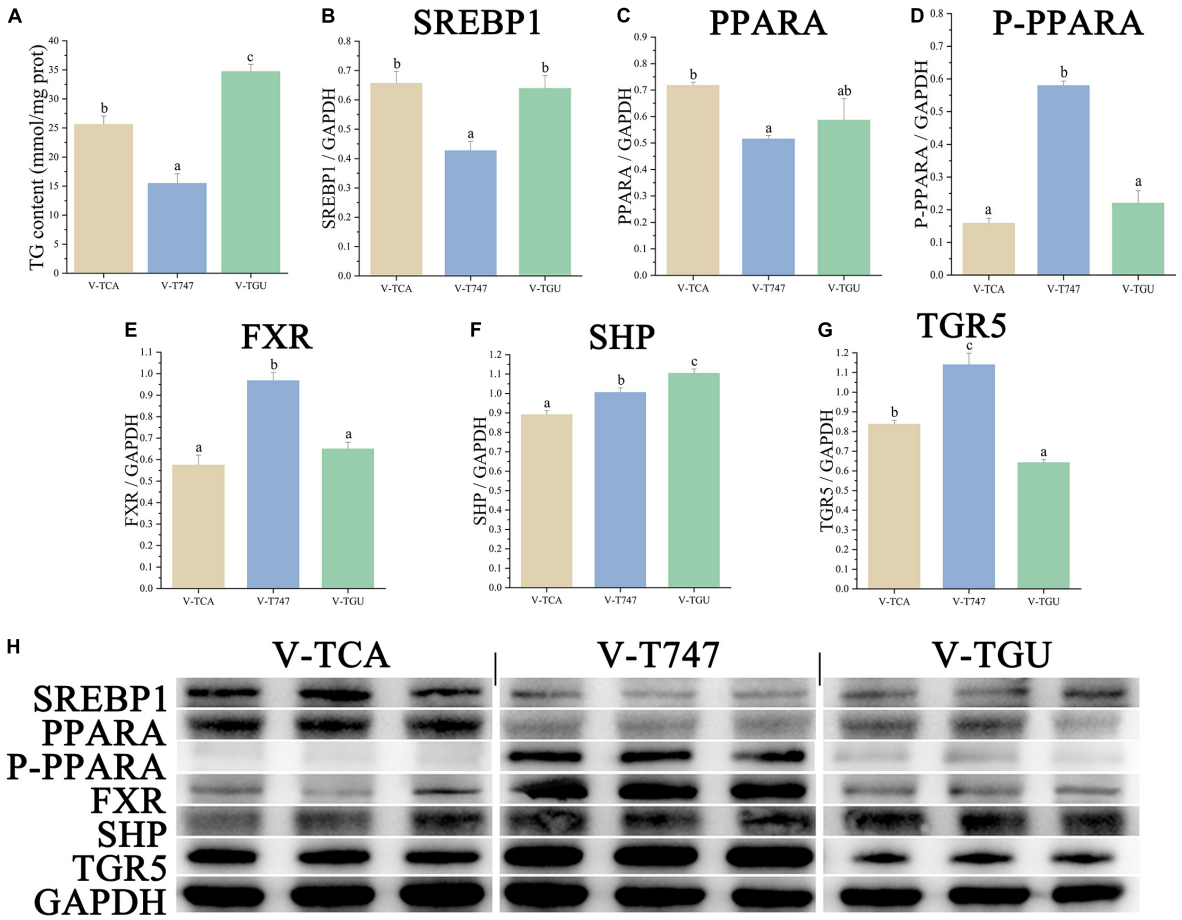

FIGURE 5 | The lipid accumulation and metabolism of hepatocytes in FXR agonist or antagonist incubation. **(A)** The content of intracellular TG (n = 9). **(B–G)** The relative quantification of protein levels normalized to GAPDH in hepatocytes (n = 3). **(H)** the Western blot band of hepatocyte samples (n = 3). Values are presented as means with *SD*, where significant ($p < 0.05$) differences between groups are indicated by different letters.

expression of lipolysis genes (*atgl*, *cpt1*, *dgat*, *dgka*, and *hl*), as well as *fxr* and *tgr5*, significantly increased (**Figure 4**). These results showed that fatty-degenerated hepatocytes induced by LE increased the lipid accumulation *via* up-regulating the lipogenesis and down-regulating the lipolysis, also repressed the expression of *fxr* and *tgr5*; BAs co-incubation improved the lipid metabolism and reduced the fat accumulation of fatty-degenerated hepatocytes, while activated the FXR and TGR5 signaling pathways.

Regulation of Bile Acids on Lipid Deposition of Hepatocyte Was Dependent on the Activation of Farnesoid X Receptor Signaling *in vitro*

According to the CCK-8 assay, the 5 μM obeticholic acid and 10 μM guggulsterone had no significant influence on the viability of hepatocytes (**Figure 1**). Prior to the TCA incubation, the FDH pre-treated with obeticholic acid for 2 h (V-T747 group) or guggulsterone (V-TGU group) enhanced or alleviated the TCA-induced down-regulation of lipid accumulation, respectively (**Figures 2**, **5A**). Compared to the V-TCA group, the V-T747 group exhibited a significantly decreased expression of SREBP1 and PPARA proteins, whereas a significantly increased expression of P-PPARA, FXR, SHP, and TGR5 proteins (**Figure 5**). Meanwhile, the V-T747 treatment significantly down-regulated the expression of lipogenesis genes (*6pgd*, *acc*, *fas*, and *me*) and their transcriptional factors genes (*lxr*, *pparr*, and *srebp1*), whereas significantly up-regulated the expression of *fxr*, fatty acid uptake gene (*fabp*), lipolysis genes (*cpt1*, *dgat*, *dgka*, *hl*, and *hsl*), and their transcriptional factors gene (*ppara*) (**Figure 6**).

Compared to the V-TCA group, the V-TGU group significantly increased and decreased the expressions of SHP and TGR5 proteins, respectively. The V-TGU treatment significantly enhanced the expression of lipogenesis genes (*6pgd*, *acc*, *fas*, and *g6pd*) and their transcriptional factors gene (*srebp1*), significantly repressed the expression of *fxr*, fatty acid uptake genes (*acbp* and *fabp*), lipolysis genes (*cpt1*, *dgat*, *dgka*, *hl*, and *hsl*), and their transcriptional factors gene (*ppara*). These results showed that the lipid deposition of hepatocytes was decreased by the agonist of FXR, and increased by the antagonist of FXR,

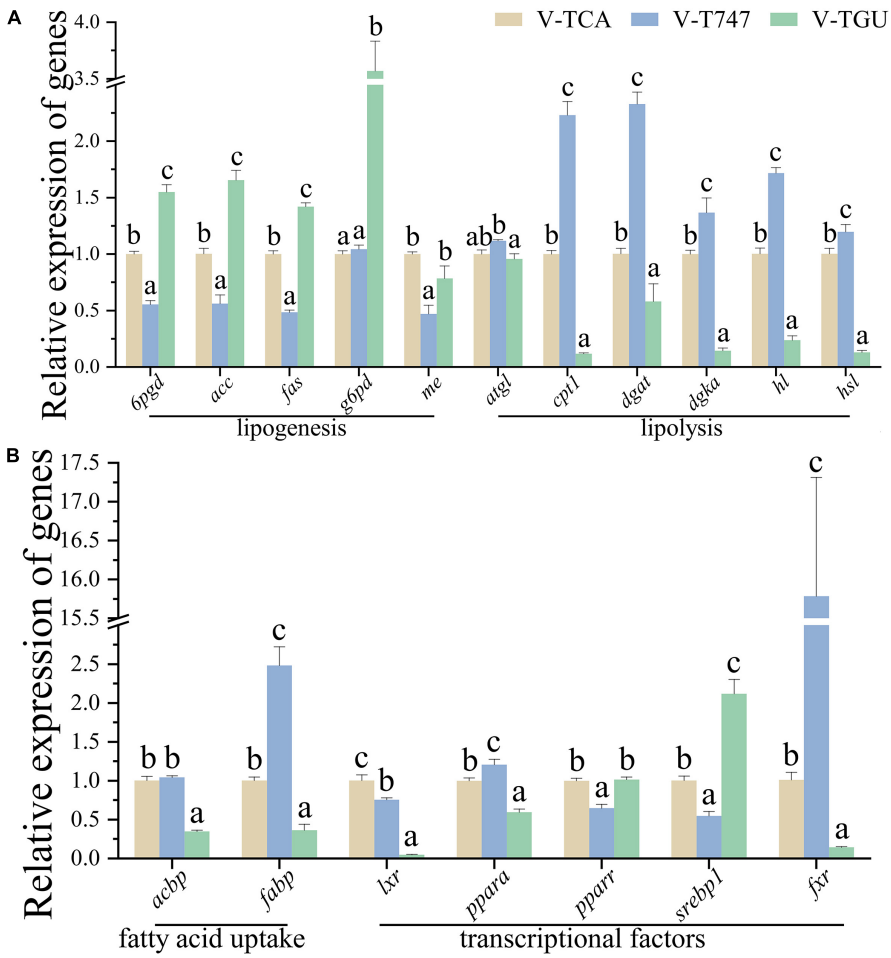

FIGURE 6 | The relative expression of genes associated with lipid metabolism of hepatocytes normalized to 18s and β-*actin* in FXR agonist or antagonist incubation **(A,B)** (n = 9). Values are presented as means with *SD*, where significant ($p < 0.05$) differences between groups are indicated by different letters.

indicating that the effects of the BAs on lipid accumulation may be mediated through the FXR signaling pathway.

Regulation of Bile Acids on Lipid Deposition of Hepatocyte Was Dependent on the Activation of G Protein-Coupled Bile Acid Receptor 1 Signaling *in vitro*

According to the results of CCK-8 assay, the 1 μM SBI-115 and 10 μM INT-777 had no significant influence on the viability of hepatocytes (**Figure 1**). Before the TCA incubation, the FDH pre-treated with SBI-115 for 2 h (V-TSBI group) or INT-777 (V-T777 group) alleviated and enhanced the TCA-induced down-regulation of lipid accumulation (**Figures 2**, **7A**), respectively. Compared to V-TCA treatment, the V-T777 treatment significantly increased the expression of TGR5, whereas significantly reduced the expression of SREBP1 and SHP proteins (**Figure 7**). In addition, the V-T777 group showed a significantly increased expression of *tgr5* and lipolysis genes (*atgl*, *cpt1*, *dgat*, *dgka*, *hl*, and *hsl*), whereas a significantly decreased expression of lipogenesis genes (*6pgd*, *acc*, *fas*, *g6pd*, and *me*) (**Figure 8**).

Compared to V-TCA group, the V-TSBI treatment significantly repressed the expression of P-PPARA and TGR5 proteins (**Figure 7**). Besides, the V-TSBI treatment significantly repressed the expression of *tgr5*, fatty acid uptake gene (*fabp*), lipolysis genes (*atgl*, *cpt1*, and *hsl*), and their transcriptional factors gene (*ppara*), whereas significantly enhanced the expression of lipogenesis genes (*6pgd*, *acc*, *fas*, *g6pd*, and *me*) and their transcriptional factors gene (*srebp1*) (**Figure 8**). These results showed the lipid deposition of hepatocytes was reduced by the agonist of TGR5, and elevated by the antagonist of TGR5, indicating that the effects of the BAs on lipid accumulation may be mediated through the TGR5 signaling pathway.

DISCUSSION

Firstly, we established the model of fatty-degenerated hepatocytes in pearl gentian grouper. In grass carp, the hepatocytes of fatty

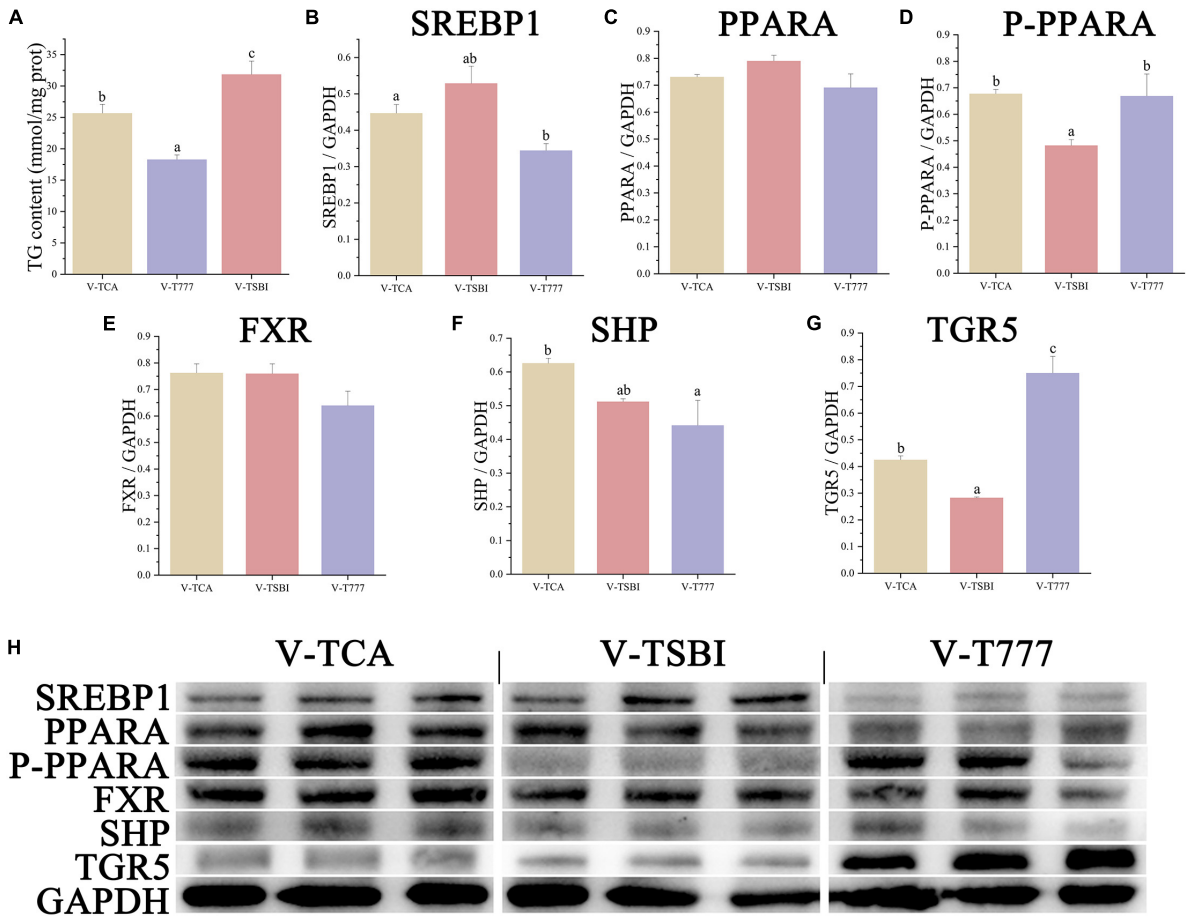

FIGURE 7 | The lipid accumulation and metabolism of hepatocytes in TGR5 agonist or antagonist incubation. **(A)** The content of intracellular TG (n = 9). **(B–G)** The relative quantification of protein levels normalized to GAPDH in hepatocytes (n = 3). **(H)** The Western blot band of hepatocyte samples (n = 3). Values are presented as means with SD, where significant ($p < 0.05$) differences between groups are indicated by different letters.

degeneration were induced with media containing LE (Lu et al., 2012), which showed a significant accumulation of intracellular TG. Similarly, after exposing to LE in hybrid grouper liver cells, lipid droplets in Oil Red O staining and TG content were increased significantly (Zou et al., 2019). These observations were consistent with our results, that is, BODIPY 493/503 staining showed a large number of lipid droplets accumulated in hepatocytes in V-HL group, which can be proved by the increase of intracellular TG content. Generally, fat accumulation is caused by the imbalance of fatty acids synthesis and fat catabolism, and many key enzymes are involved in these processes (Song et al., 2015a). As providing NADPH essential compounds for fatty acids synthesis in the pentose phosphate pathway, G6PD and 6PGD act important roles in lipogenesis (Song et al., 2015b). As catalyzing the carboxylation of acetyl-CoA to malonyl-CoA, ACC act a rate-limiting enzyme for the synthesis of long-chain fatty acids (Zhu et al., 2014). In this study, the V-HL treatment up-regulated the mRNA level of these genes, as well as *fas* and *me*, indicating that the lipogenesis was enhanced in a fatty-degenerated hepatocytes model. Our results are similar to previous study in which LE induced the expression of adipogenesis genes (*g6pd*, *me*, and *fas*) in pearl gentian grouper hepatocytes (Zou et al., 2019). As catalyzing the conversion of fatty acids-CoAs into fatty acids-carnitine, CPT1 is regarded as a main regulatory enzyme in fatty acid oxidation (Xie et al., 2021a; Xu et al., 2022b). The reduced the mRNA level of *cpt1* in V-HL group, as well as *atgl*, *dgat*, *dgka*, and *hl*, suggesting that lipolysis was suppressed in fatty-degenerated hepatocytes model. Similarly, LE co-incubation decreased the expression of lipolytic genes (*atgl* and *lpl*) in another reports in pearl gentian grouper hepatocytes (Zou et al., 2019). Furthermore, *ppara* is a ligand-dependent transcriptional activation of genes related to fatty acids oxidation pathway (Ribet et al., 2010; Zhu et al., 2014). While *pparr* and *srebp1* are the major regulators of fatty acids/lipids biosynthesis genes, both of which promote lipid storage (Minghetti et al., 2011; Zhu et al., 2014). Same LE treatment decreased the expression of *ppara* (Zou et al., 2019), in the present study, the V-HL treatment significantly enhanced the expression of SREBP1 gene and protein, and repressed the expression of PPARA and P-PPARA proteins. Overall, compared to normal cell, fatty-degenerated hepatocytes increased lipid accumulation, and impaired lipid metabolism by enhancing

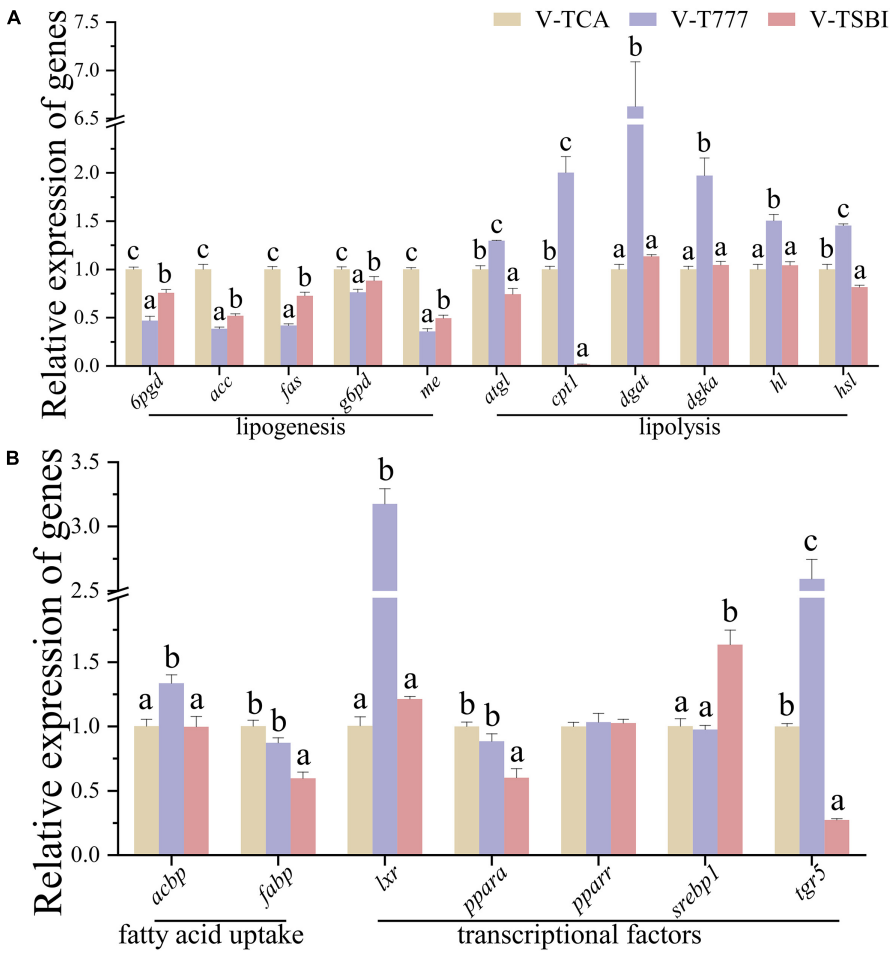

FIGURE 8 | The relative expression of genes associated with lipid metabolism of hepatocytes normalized to 18s and β-*actin* in TGR5 agonist or antagonist incubation **(A,B)** (n = 9). Values are presented as means with *SD*, where significant ($p < 0.05$) differences between groups are indicated by different letters.

lipogenesis, repressing lipolysis, and regulating transcriptional factors in pearl gentian grouper.

Then, we explored the effects of BAs on lipid deposition in a model of fatty-degenerated hepatocytes. Unlike 200 μM TCA did not affect the viability of hepatocytes in mice (Allen et al., 2011), the CCK-8 assay showed the TCA concentrations below 100 μM had no significant influence on the viability of hepatocytes in this study. This discrepancy might be due to the different tolerance of animals to BAs (Jia et al., 2018; Kumari et al., 2020). *In vivo*, exogenous BAs could decrease fat deposition caused by a high-lipid diet and improve the hepatic lipid metabolism in large yellow croaker and largemouth bass (Ding et al., 2020; Yin et al., 2021). Similarly, the V-TCA treatment reduced the lipid deposition in fatty degeneration liver cells. Furthermore, in present study, the increased expression of lipolysis genes, and decreased lipogenesis genes, and associated transcriptional factors, suggesting that TCA co-incubation could improve lipid metabolism by repressing lipogenesis and enhancing lipolysis. Notably, the low expression of *fxr* and *tgr5* genes in the V-HL group, but high expression of both genes and proteins in the V-TCA group, might indicate that the fatty-degenerated hepatocytes inhibited, but TCA co-incubation activated, the FXR and TGR5 signaling pathways, thus altering the lipid metabolism and lipid accumulation *in vitro*.

Next, we explored whether the effects of BAs on lipid deposition were dependent on the activation of FXR signaling. The INT-747 (10 μM) is a classical FXR agonist that can significantly increase the luciferase activity of FXR reporter gene in HepG2 cells (Gao et al., 2018). Similarly, we also found the INT-747 (5 μM) co-incubation up-regulated the expression of FXR gene and protein in hybrid grouper hepatocytes, accompanied by a reduction in lipid accumulation. Our results are consistent with other studies in which INT-747 (1 μM) treatment significantly reduced TG level in mouse liver (AML12 cells) and human liver (Fa2N-4 cells) (Miyazaki et al., 2018). In human intestinal cells, the activation of FXR inhibited the expression of *lxr* and *srebp1* genes, thereby decreasing the expression of *fas* gene and lipid synthesis *via* the SHP pathway (Watanabe et al., 2004; Yang et al., 2010). In addition, the activation of FXR stimulated the fatty acid β-oxidation by

inducing the expression of PPARA (Alfaro-Viquez et al., 2018; Kumari et al., 2020). In accordance with this, we also found that the activation of FXR promoted the lipolysis and inhibited the lipogenesis. Same as the present study, the guggulsterone (20 μM) treatment significantly increased the content of TG in primary hepatocytes from yellow catfish (Wu et al., 2020). Thus, antagonizing FXR by guggulsterone, eliminated the lipid-lowering effects of TCA, suggesting that the activation of FXR might be the key step toward TCA-induced lipid-lowering outcome.

Besides, we explored whether the effects of BAs on lipid deposition was dependent on the activation of TGR5 signaling. The INT-777 had been discovered as a specific agonist for TGR5 (Guo et al., 2016). As with the human podocytes (Wang et al., 2016), the results of CCK-8 assay also showed 10 μM INT-777 had no significant effects on the viability of hepatocytes in this study. Since INT-777 treatment enhanced the expression of TGR5 and decreased the level of plasma TG in mice (Bianco et al., 1988; Wang et al., 2016), we also found the activation of TGR5 by INT-777 reduced the lipid deposition in hybrid grouper hepatocytes. In human podocytes cultured with high glucose, INT-777 co-incubation suppressed mRNA level of *srebp1*, induced the mRNA level of *ppara* and *cpt1*, leading to an increase in fatty acid β-oxidation (Wang et al., 2016). Partially agreeing with these, we observed that INT-777 treatment increased the expression P-PPARA protein and *cpt1* gene in this study. At the same time, the inhibition of TGR5 by SBI-115 promoted lipid accumulation and impaired lipid metabolism of hybrid grouper hepatocytes. Our results were consistent with the results of another *in vivo* experiment, the *ppara* and *cpt1* mRNA level were decreased in TGR5$^{-/-}$ mice (Wakil and Abu-Elheiga, 2009). Since TCA could active TGR5 in various cells (HEK293, Kupffer, and Sinusoidal endothelial cells) (Keitel et al., 2007, 2008), and TGR5 negatively regulated lipid deposition, we inferred that the activation of TGR5 might be a key step toward TCA-induced lipid-lowering outcome.

CONCLUSION

In pearl gentian grouper, we revealed: (1) a model of fatty degenerated hepatocytes induced by LE is characterized by increasing lipid accumulation and impairing lipid metabolism; (2) BAs co-incubation could improve lipid deposition and metabolism in fatty degenerated hepatocytes model; (3) the inhibition of FXR or TGR5 promoted lipogenesis and inhibited lipolysis *in vitro*; (4) the activation of FXR and/or TGR5 might be the key step toward the lipid-lowering effects of BAs. Understanding the functions of BAs may help to develop of management strategies to improve lipid metabolism and deposition in the liver of hybrid grouper.

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 Animal Ethical and Welfare Committee of Guangdong Ocean University. The permit number for conducting animal experiments is GDOU-AEWC-20180063.

AUTHOR CONTRIBUTIONS

JX: conceptualization, methodology, formal analysis, investigation, data curation, writing – original draft, and writing – review and editing. XY: data curation, investigation, and writing – original draft. XL: investigation, data curation, and visualization. SX: conceptualization, resources, and visualization. SC: methodology and conceptualization. SZ: conceptualization and funding acquisition. JC: methodology, conceptualization, and funding acquisition. BT: methodology, conceptualization, writing – review and editing, and funding acquisition. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.861117/full#supplementary-material

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Effects of L-carnitine Supplementation in High-Fat Diet on Growth, Antioxidant Capacity and Lipid Metabolism of Golden Pompano (*Trachinotus ovatus*)

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A 6-week feeding trial was carried out to investigate whether dietary L-carnitine supplementation could alleviate the adverse effects of high-fat diet on the growth, antioxidation and lipid metabolism of *Trachinotus ovatus*. Four isonitrogenous experimental diets were formulated supplementing with or without L-carnitine (low fat diet (D1): 13% lipid; high fat diet (D2): 20% lipid; D3: 20% lipid with 0.02% L-carnitine; D4: 20% lipid with 0.06% L-carnitine). The final body weight, weight gain rate, specific growth ratio and feed intake in fish fed with D2 diet were significantly lower than that of fish fed with D1, D3 and D4 diets ($P < 0.05$). The weight gain rate and specific growth ratio of D2 diet were as low as 167.21% and 2.41% initial body weight/d, respectively. However, experimental diets were unable to change the feed conversion ratio and survival rate of fish ($P > 0.05$). The lowest value of crude protein and the highest value of crude lipid of whole-body were found in fish fed with D2 diet ($P < 0.05$), which were 31.35% and 17.41% wet weight respectively. Meantime, the level of crude lipid of whole-body in fish fed with D3 and D4 diets was significantly higher than that of fish fed with D2 diet ($P < 0.05$). Besides, there were significantly higher levels of triglyceride and total cholesterol in the D2 diet when compared to the other diets ($P < 0.05$). Additionally, the superoxide dismutase activity, malondialdehyde content and the mRNA levels of manganese superoxide dismutase and glutathione peroxidase of the liver in fish fed with D2 diet were significantly higher than those fed with D1, D3 and D4 diets ($P < 0.05$). The mRNA levels of carnitine palmitoyl

transferase 1 and peroxisome proliferator-activated receptors-alpha of the liver in fish fed with D2 diet were significantly higher than those fed with D1, D3 and D4 diets ($P < 0.05$). In conclusion, L-carnitine supplementation in high-fat diet improved the growth performance and health of *T. ovatus* by promoting lipid hydrolysis, improving cholesterol transport and antioxidant capacity. Therefore, we recommended the 0.02% addition level of L-carnitine for *T. ovatus* diet.

Keywords: L-Carnitine, growth performance, antioxidation, lipid metabolism, *Trachinotus ovatus*

INTRODUCTION

In animals, L-carnitine is synthesized from essential amino acids lysine and methionine supported by vitamin C and other secondary chemical compounds produced in the body (Sabzi et al., 2017). Its main function is as a cofactor to transport long-chain unsaturated fatty acids to mitochondria matrix for β-oxidation to generate energy (Chen et al., 2020). Thus, L-carnitine plays an important regulatory role in energy metabolism and lipid metabolism (Selcuk et al., 2010; Chen et al., 2020). Although organisms can synthesize L-carnitine, dietary supplementation is usually required when the lipid concentration in the body exceeds the synthetic ability of organisms (Li et al., 2019). Besides, the L-carnitine synthesis capacity of juvenile fish is limited due to the mechanism of L-carnitine synthesis is under developed (Li et al., 2019). Therefore, L-carnitine is considered to be an essential nutrient for animals under specific conditions.

In view of the key regulatory role of L-carnitine in metabolism, the addition of L-carnitine to fish feed has drawn extensive attention to its potential to improve growth, feed efficiency, and reduce body-fat deposition. L-carnitine supplementation has been proved to increase the yield of cultured fish, which promoted weight gain and feed efficiency and decreased lipid deposition (Desai et al., 2010; Haji-abadi et al., 2010; Ozorio et al., 2010 Zheng et al., 2014). However, some studies showed contradictory results. Dietary supplementation with L-carnitine has no effect on growth performance and feed efficiency of several species of cultured fish, but altered the lipid metabolism in liver (Gaylord and Gatlin, 2000; Yang et al., 2009; Selcuk et al., 2010; Ozorio et al., 2012). The above results indicated that the effects of dietary L-carnitine supplementation on fish are species-specific. In addition, the contradictory results may also be different from the growth stages, feed composition (lipid, protein, lysine and L-carnitine content) and environmental conditions (water quality and water temperature) (Ozorio et al., 2012; Li et al., 2019).

The protein content of aquatic feed is the most important factor affecting fish growth and feed costs. Dietary lipid is the second important nutrient after protein for aquatic animals and play a key regulatory role in maintaining growth and normal physiological function of fish. It is well known that marine carnivorous fish preferentially use protein as energy source, and lipids are the main source of dietary energy and essential fatty acids (López et al., 2009). Therefore, partial replacement of protein with lipid is one of the main strategies to reduce feed costs. Within a certain range, increasing the dietary lipid level can not only improve feed efficiency, but also spare protein (Schuchardt et al., 2008; López et al., 2009). However, excessive lipid levels in the diet might cause adverse effects on fish, such as poor growth performance, abnormal lipid metabolism, oxidative stress and immunosuppression (Guo et al., 2019; Xie et al., 2020; Xu et al., 2021).

Trachinotus ovatus is widely distributed in southern China, Southeast Asia, Japan and Australia (Zhao et al., 2021). This species is popular because of its high nutritional value, rapid growth and delicious meat. Due to the increasing market demand, *T. ovatus* has become a vital economically marine fish widely cultured in the southern coast of China. However, in order to reduce the culturing cost, high-fat diet has been widely used to feed *T. ovatus* in practical aquaculture conditions, which causes high mortality and economic losses. Therefore, in this study, L-carnitine was supplemented in the high-fat diet to study its effect on growth, antioxidant capacity, body composition and lipid metabolism of *T. ovatus*. The results obtained in this study can provide reference for the research and development of high-fat diet formulas for *T. ovatus*.

MATERIALS AND METHODS

Experimental Diets

The formulation, proximate composition and nutrient levels of experimental diets were shown in **Table 1**. Four isonitrogenous experimental diets were performed as following: low fat diet (D1) contained 13% lipid level; high fat diet (D2) contained 20% lipid level; D3 diet contained 20% lipid with 200 mg/kg L-carnitine; D4 diet contained 20% lipid with 600 mg/kg L-carnitine. The experimental diets were prepared and manufactured according to the procedure described by Zhao et al. (2020). Briefly, all dry ingredients were finely ground and thoroughly mixed. Then soybean lecithin and fish oil were gradually added and mixed constantly. Subsequently, 50 mL of water for each 100 g of the mixture was slowly blended. The 2.5-mm-diameter puffed pellets were produced by using a puffing apparatus (Institute of Chemical Engineering, South China University of Technology, Guangdong, China). The puffed pellets were oven-dried at 60°C to approximately 10% moisture and then stored at -20°C until feeding.

TABLE 1 | Composition and nutrient levels of the experimental diets (%DM basis).

Items	D1	D2	D3	D4
Ingredients				
Fish meal	25	25	25	25
Soybean protein concentrate	15	15	15	15
Soybean meal	20	20	20	20
Wheat flour	16	16	16	16
Carboxymethyl cellulose	9	2	1.98	1.94
Fish oil	8	15	15	15
Soybean lecithin	2	2	2	2
$Ca(H_2PO_4)_2$	2	2	2	2
Vitamin premix[a]	1	1	1	1
Mineral premix[b]	1	1	1	1
Choline	0.5	0.5	0.5	0.5
Methionine	0.4	0.4	0.4	0.4
Lysine	0.1	0.1	0.1	0.1
L-carnitine[c]	0	0	0.02	0.06
Total	100	100	100	100
Nutrient levels[d]				
Moisture	9.13	9.79	9.47	9.82
Crude protein	40.31	40.75	39.96	40.51
Crude lipid	12.98	20.17	20.32	20.54

[a]*Vitamin premix provides the following per kg of diet: vitamin B_1 25 mg, vitamin B_2 45 mg, vitamin B_6 20 mg, vitamin B_{12} 0.1 mg, vitamin K_3 10 mg, pantothenic acid 60 mg, niacin 200 mg, folic acid 20 mg, biotin 1.2 mg, retinal acetate 32 mg, vitamin D_3 5 mg, vitamin E 120 mg, choline chloride 2.5 g, ethoxyquin 150 mg, coarse flour 14.012 g.*

[b]*Mineral premix provides the following per kg of diet: NaF 2 mg, KI 0.8mg, $CoCl_2$•$6H_2O$ (1%) 50 mg, $CuSO_4$•$5H_2O$ 10 mg, $FeSO_4$•H_2O 80 mg, $ZnSO_4$•H_2O 50 mg, $MnSO_4$•H_2O 60 mg, $MgSO_4$•$7H_2O$ 1200 mg, $ZnSO_4$•H_2O 50 mg, $Ca(H_2PO_4)_2$•H_2O 3000 mg, NaCl 100 mg, zeolite powder 15.447g.*

[c]*L-carnitine was purchased from Sigma-Aldrich (C0158).*

[d]*Measured values.*

Experimental Fish and Feeding Management

Juvenile *T. ovatus* were obtained from a commercial farm (Lingshui, Hainan, China). The feeding experiment was performed in the Bay (Lingshui, Hainan, China) using sixteen seawater cages (1.0 m × 1.0 m × 1.5 m). Prior to the feeding experiment, fish were fed the D1 group diet for 2 weeks to acclimate to the experimental conditions and facilities. At the beginning of the feeding experiment, a total of 320 healthy fish (average body weight 7.60 ± 0.06 g) were distributed randomly into sixteen seawater cages at 20 fish per cage. The fish were slowly hand-fed to apparent satiation two times daily at 08:00 and 16:00, and the feeding experiment lasted for six-weeks. Feed consumption, fish death amounts and weight were recorded for each sea cage every day. After six-weeks feeding experiment, all survival fish were starved for 24 h, and then weighed in batches after anesthesia (MS-222, Sigma, St Louis, MO, USA).

Sample Collection

After six-weeks feeding experiment, all survived fish were starved for 24 h and anesthetized (MS-222, Sigma, St Louis, MO, USA). Then, four fish from each cage were randomly collected and frozen in liquid nitrogen, and then stored at - 80 °C for whole body composition analysis. Four individuals from each cage were randomly collected for obtaining the blood sample. Serum was obtained based on procedures of Li et al. (2014). Briefly, the blood was collected from the tail sinus with 1 ml sterile syringes and then centrifuged (5000 rpm, 10 min) at 4°C. Then, the serum was separated and stored in the liquid nitrogen for analysis of the haematological biochemical index. The livers were removed from six fish per cage, and immediately frozen in liquid nitrogen and stored at - 80 °C for enzyme activity and RNA expression analysis.

Chemical Analysis of the Experimental Diets and Whole body

The crude lipid, crude protein and moisture of experimental diets and whole body were determined and analyzed according to the standard procedures of AOAC (1995). Briefly, crude protein content was detected using the Kjeldahl method (1030-Auto-analyzer, Tecator), crude lipid was determined by the Soxhlet extractor method (Soxtec System HT6, Tecator), moisture was analysed by drying in an oven at 105°C to acquire dry weight and ash was examined after combustion in a muffle furnace at 550°C for 24 h.

Survival Rate and Growth Performance

During the feeding experiment, the number of dead fish were recorded to calculate the survival rate. The weight gain rate (WGR), feed conversion ratio (FCR), specific growth ratio (SGR) and feed intake (FI) were calculated as follows: WGR (%) = 100 × (final body weight - initial body weight)/initial body weight; SGR (% initial body weight/day) = 100 × (*Ln* final mean weight - *Ln* initial mean weight)/number of days; FCR = dry feed intake/(final body weight - initial body weight); FI (g/fish) = dry feed intake/number of fish in each cage].

Serum Parameters Assays

Serum levels of triglyceride (TG) and total cholesterol (T-CHO) were determined on Chemray 240 automatic chemistry analyser (Rayto Life Science Co., Ltd.) with the commercial kits (Huili Biotech Co., Ltd.).

Antioxidant Enzyme Activity Analysis

Liver samples were homogenized in ice-cold phosphate buffer (pH 6.4, 1:10 dilution). Afterward, the above homogenate was centrifuged (10 min, 4°C, 1200 g) to collect supernatant for antioxidant enzyme activity analysis. Superoxide dismutase (SOD)(A001-1) activities and malondialdehyde (MDA) (A003-1) contents in liver were determined following the instructions of the reagent kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). SOD was determined by the xanthine oxidase method. One unit of enzyme activity was defined as the amount of enzyme exhibiting 50% inhibition of the SOD per milligram of protein in l mL solution. MDA was determined using the thiobarbituric acid method.

Total RNA Extraction and Gene Expression Analysis

Liver RNA extraction and real-time quantitative PCR analysis were performed according to our previously published methods (Zhao et al., 2020). Briefly, the livers from each cage were pooled for the isolation of total RNA using Trizol® reagent (Invitrogen, USA). 1% agarose gel electrophoresis and spectrophotometer

TABLE 2 | Sequences of primers used for real-time quantitative PCR.

Gene name	Primer sequence (5′-3′)	Reference
CPT1	F-CTTTAGCCAAGCCCTTCATC R-CACGGTTACCTGTTCCCTCT	Zhao et al., 2020
PPARα	F-AATCTCAGCGTGTCGTCTT R-GGAAATGCTTCGGATACTTG	KP893147
Mn-SOD	F-AGCCAGCCTCAGCCAAACT R-GGCGGTGACATCTCCCTTT	Tan et al., 2017
CAT	F-AGTTTTACACCGAGGAGGGC R-TGTGGGTTTGGGGATTGC	Tan et al., 2017
GSH-PX	F-AAGTATGTCCGTCCTGGAAATG R-AAGTATGTCCGTCCTGGAAATG	Tan et al., 2017
β-actin	F-TACGAGCTGCCTGACGGACA R-GGCTGTGATCTCCTTCTGC	Tan et al., 2017

(NanoDrop 2000, Thermo Fisher, United States) were used to ascertain the quality and quantity of RNA. Then, cDNA was synthesized using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China) following the manufacturer's instructions. Primers for real-time quantitative PCR were shown in **Table 2**. Real-time quantitative PCR assays were quantified on the Light Cycler 480 (Roche Applied Science, Basel Switzerland) using an SYBR® Premix Ex TaqTM II (Takara, Dalian, China). The real-time PCR conditions were as follows: 95°C for 1 min, followed by 40 amplification cycles at 95°C for 5 s, 60°C for 15 s and 72°C for 20s. The relative expression levels of target genes were quantified using $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

All data in this study were shown as means ± standard error (SE). All data were analyzed in SPSS 22.0 (SPSS, Chicago, IL, USA) and followed by one-way analysis of variance (ANOVA) as well as Duncan's multiple range test. A *P*-value < 0.05 was deemed to be statistically significant.

RESULTS

Growth Performance and Feed Utilization

The growth performance (WGR, SGR), SR and FCR of T. ovatus were shown in **Table 3**. The final body weight (FBW), WGR and SGR of fish fed with D2 diet were significantly lower than that of other diet groups ($P < 0.05$). Dietary L-carnitine supplementation was unable to change the FCR of fish ($P > 0.05$), however, FI in fish fed with D2 diet was significantly lower than those fed with D1, D3 and D4 diets ($P < 0.05$). The SR was 90% approximately, and there was no significant difference among all diet treatments ($P > 0.05$).

TABLE 4 | Effects of L-carnitine on whole body composition (% wet weight) of *Trachinotus ovatus*.

Items	D1	D2	D3	D4
Moisture	72.31 ± 1.25	71.80 ± 0.79	71.35 ± 1.64	71.71 ± 1.58
Crude protein	16.68 ± 0.37^{a}	15.16 ± 0.46^{b}	16.53 ± 0.42^{a}	16.57 ± 0.44^{a}
Crude lipid	6.13 ± 0.20^{a}	8.39 ± 0.32^{b}	7.41 ± 0.23^{c}	7.32 ± 0.26^{c}
Ash	4.40 ± 0.23	4.13 ± 0.18	4.20 ± 0.20	4.09 ± 0.24

Values are means ± SE of four replicates. The superscript small letters (a,b,c) in the same column means the significant difference at P<0.05.

Whole-Body Composition

As showed in **Table 4**, experimental diets significantly altered the whole-body composition of T. ovatus. The lowest value of crude protein was found in fish fed with D2 diet, and significantly lower than those fed the D1, D3 and D4 diets ($P < 0.05$). Meantime, fish fed with D2 diet showed higher crude lipid and significantly higher than those fed with D1, D3 and D4 diets ($P < 0.05$). However, there was no significant difference in the moisture among all diet treatments ($P > 0.05$).

Antioxidant Capacity

Antioxidant enzyme activities and expression levels of antioxidant related-genes were presented in **Figures 1**, **2**, respectively.

The SOD activity and MDA content in fish fed with D2 diet were significantly higher than those fed with D1, D3 and D4 diets ($P < 0.05$) (**Figure 1**).

The mRNA levels of manganese superoxide dismutase (*Mn-SOD*) and glutathione peroxidase (*GSH-PX*) in fish fed with D2 diets were significantly higher than those fed with D1, D3 and D4 diets ($P < 0.05$). However, there was no significant difference in the mRNA level of catalase (*CAT*) among all diet treatments ($P > 0.05$) (**Figure 2**).

Serum Parameters

Higher levels of TG and T-CHO were found in D2 diet feeding fish than that of the other group ($P < 0.05$) (**Figure 3**).

Expression Levels of Genes Related to Lipid Metabolism

The mRNA levels of carnitine palmitoyl transferase 1 (*CPT1*) and peroxisome proliferator-activated receptors-alpha (*PPARα*) in fish fed with D2 diet were significantly higher than those fed with D1, D3 and D4 diets ($P < 0.05$) (**Figure 4**).

TABLE 3 | Effects of L-carnitine on growth performance of *Trachinotus ovatus*.

Items	IBW/g	WGR/%	SGR/(% initial body weight/day)	SR/%	FCR	FI/(g/fish)
D1	7.61 ± 0.11	207.32 ± 11.71^{a}	2.73 ± 0.10^{a}	89.11 ± 3.63	1.41 ± 0.04	22.77 ± 1.09^{a}
D2	7.59 ± 0.12	167.21 ± 10.05^{b}	2.41 ± 0.09^{b}	84.33 ± 4.19	1.43 ± 0.09	18.45 ± 0.82^{b}
D3	7.54 ± 0.12	220.31 ± 17.12^{a}	2.90 ± 0.12^{a}	90.00 ± 5.32	1.39 ± 0.07	21.89 ± 0.71^{a}
D4	7.67 ± 0.11	213.51 ± 14.27^{a}	2.85 ± 0.13^{a}	88.13 ± 4.16	1.40 ± 0.07	21.52 ± 0.63^{a}

Values are means ± SE of four replicates. The superscript small letters (a,b) in the same column means the significant difference at P<0.05.
IBW, initial body weight; WGR, weight gain rate; SGR, specific growth ratio; SR, survival rate; FCR, feed conversion ratio; FI, feed intake.

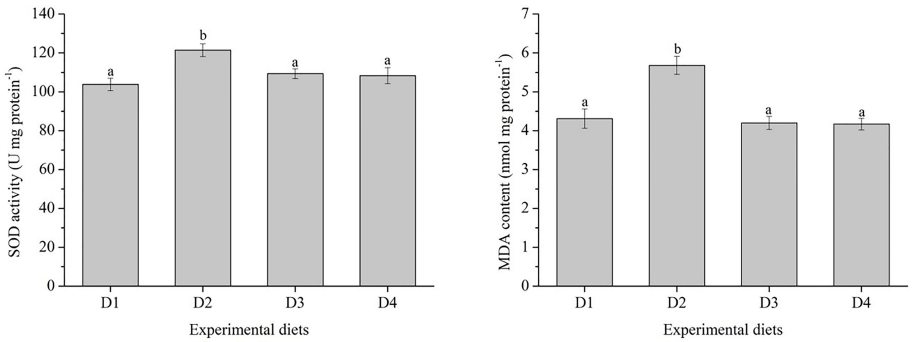

FIGURE 1 | Specific activities of liver antioxidant enzymes of *Trachinotus ovatus* fed experimental diets for 56 days. Different superscript letters in the same row means the significant difference at $P < 0.05$. SOD, superoxide dismutase; MDA, malondialdehyde.

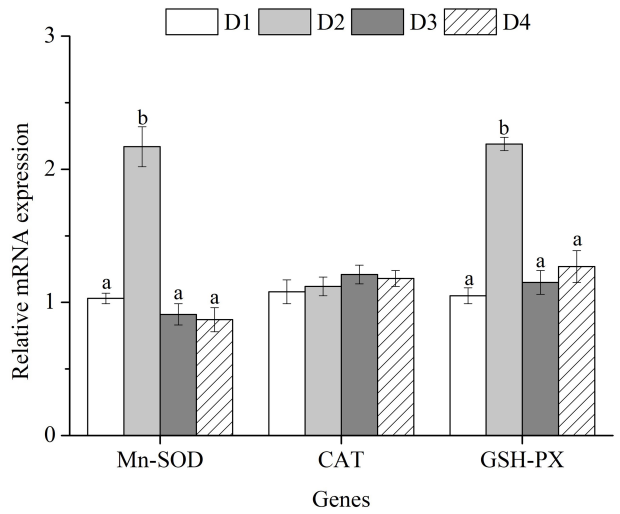

FIGURE 2 | The expression profiles of antioxidant-related genes in the liver of *Trachinotus ovatus* fed experimental diets for 56 days. Different superscript letters in the same row means the significant difference at $P < 0.05$. Mn-SOD, manganese superoxide dismutase; CAT, catalase; GSH-PX, glutathione peroxidase.

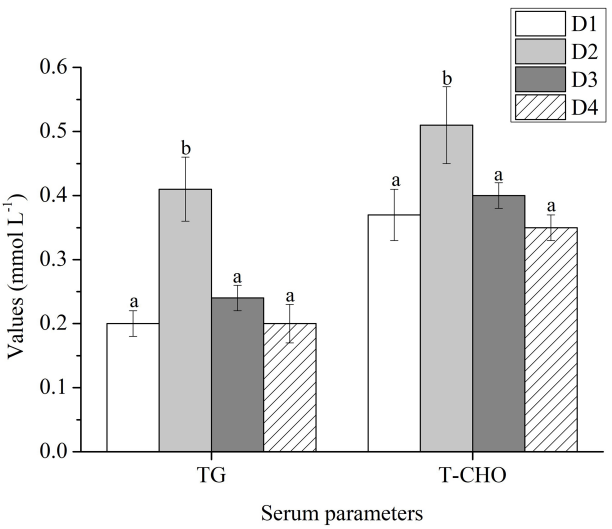

FIGURE 3 | Serum parameters of *Trachinotus ovatus* fed experimental diets for 56 days. Different superscript letters in the same row means the significant difference at $P < 0.05$. TG, triglyceride (mmol L^{-1}); T-CHO, total cholesterol (mmol L^{-1}).

DISCUSSION

In this study, the results showed that the growth performance (WGR and SGR) of T. ovatus exposed to a high-fat diet without L-carnitine (D2 diet) was significantly lower than that of the low-lipid diet (D1 diet), indicating that excessive lipid intake reduced the growth performance of T. ovatus. Similarly, the poor growth performance caused by high-fat diet has been reported in studies on *Atractoscion nobilis* (López et al., 2006), *Ctenopharyngodon idellus* (Li et al., 2016), *Micropterus salmoides* (Zhou et al., 2020) and *T. ovatus* (Fang et al., 2021). The poor growth of fish caused by high-fat diet is mainly attributed to lower feed intake and metabolic disorders caused by lipid deposition in the liver (Wang et al., 2005; Song et al., 2009; Ding et al., 2020). Similar results were obtained in this study. *T. ovatus* fed the high-fat diet without L-carnitine (D2 diet) showed lower FI, which can be used to explain that the high-fat diet reduced the growth of *T. ovatus*. Meantime, the present results showed that the growth performance (WGR and SGR) and FI of *T. ovatus* in the low-lipid group (D1) was similar to that in the high-lipid supplemented with L-carnitine groups (D3 and D4), which demonstrated that dietary L-carnitine supplementation was helpful to alleviate the adverse effects caused by high-fat diet on the growth of *T. ovatus*. Normal physiological functions are essential for the growth of fish, and L-carnitine can alleviate the adverse effects of high-fat diet on the growth of fish may be

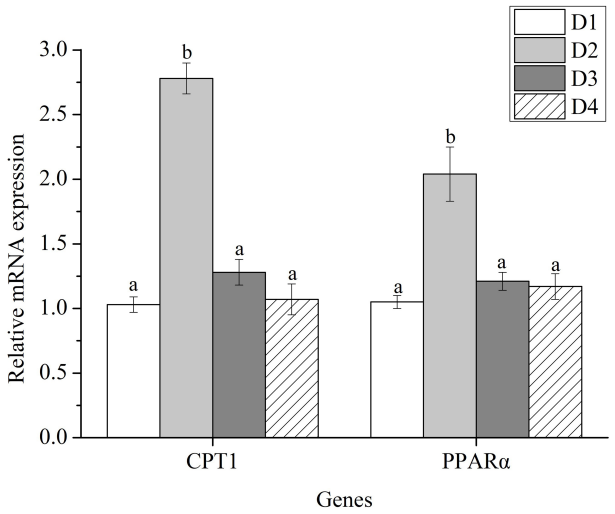

FIGURE 4 | The expression profiles of lipolysis-related genes in the liver of *Trachinotus ovatus* fed experimental diets for 56 days. Different superscript letters in the same row means the significant difference at $P < 0.05$. CPT1, carnitine palmitoyl transferase 1; PPARα, peroxisome proliferator-activated receptors-alpha.

attributed to its ability to promote lipid hydrolysis, improve lipid utilization and depress lipid peroxidation (Dikel et al., 2010; Sabzi et al., 2017; Li et al., 2019).

Regarding whole-body composition, the present results indicated that *T. ovatus* fed high-fat diet (D2) obtained higher lipid and lower protein concentration in whole-body than *T. ovatus* fed low-lipid diet (D1). Similar results have been reported in studies on *T. ovatus* (Fang et al., 2021), *Scortum barcoo* (Song et al., 2009) and *Salmo salar* (Bjerkeng et al., 1997). Excessive lipid intake by fish resulted in excessive fat deposition in visceral cavity and tissues, which not only has an adversely impact on the growth and metabolism function of fish, but also reduce the commercial value of the fish products (Bjerkeng et al., 1997). In the present study, it was found that the whole-body protein was significantly increased and the whole-body lipid was significantly reduced in the high-fat diet supplemented with L-carnitine groups (D3 and D4) when compared to the high-lipid group (D2). The present results indicated that the addition of L-carnitine in high-fat diet was helpful to reduce whole-body lipid concentration and promote protein deposition. Dietary L-carnitine supplementation can promote the oxidative metabolism of lipids, thereby reducing the accumulation of lipids in the body (Yang et al., 2012). Similarly, dietary supplementation with L-carnitine can promote protein deposition and decrease whole-body lipid concentration, which has been reported in studies on *C. carpio* (Sabzi et al., 2017), *Bidyanus bidyanus* (Yang et al., 2012), *Oncorhynchus mykiss* (Haji-abadi et al., 2010) and hybrid tilapia (Yang et al., 2009). However, some studies conversely found that dietary L-carnitine supplementation did not alter the lipid content of fish, such as *Dicentrarchus labrax* (Dias et al., 2001) and *Morone saxatilis* male × *M. chrysops* female (Gaylord and Gatlin, 2000). Species differences may be an important reason for inconsistent results. In addition, different experimental results may also be related to fish growth stage, feed formula and environmental factors. At present, it is not easy to explain the causal mechanisms of the effects of dietary L-carnitine supplementation on body composition of fish.

TG and T-CHO concentrations in the serum of *T. ovatus* were significantly higher in the high-fat diet group (D2) than in the low-lipid diet group (D1). These results were in accordance with data in *C. carpio* (Sabzi et al., 2017), *B. bidyanus* (Yang et al., 2012) and *Ctenopharyngodon Idella* (Du et al., 2006). The results showed that high-fat diet could promote the deposition of TG and T-CHO in blood. However, this study results found that L-carnitine supplementation in high-fat diet (D3 and D4) significantly reduced the contents of TG and T-CHO in serum compared to the high-fat diet without L-carnitine supplementation (D2). Dietary L-carnitine supplementation decreased the concentrations of TG and T-CHO in plasma have also been found in *D. labrax* (Santulli et al., 1988), hybrid tilapia (Yang et al., 2009) and *C. carpio* (Sabzi et al., 2017). The results indicated that dietary L-carnitine supplementation could reduce intravascular cholesterol and triglyceride deposition and improve fish health by increasing the transport capacity of cholesterol.

Additionally, Gou et al. (2016) reported that the addition of L-carnitine in the diet promoted the lipid hydrolysis capacity of the liver by increasing the activity of lipoprotein lipase and lipase. Similar results were obtained in this study, the present results found that the mRNA levels of *CPT1* and *PPARα* in fish fed with high-fat diet (D2) were significantly higher than those fed with low-lipid (D1) and high-lipid with L-carnitine supplementation (D3 and D4) diets. The main function of CPT1 is to transport long-chain fatty acids to the mitochondrial region for β-oxidation (Chen et al., 2020). PPARα can catalyse the hydrolysis of lipoproteins and participate in lipolysis (Pawlak et al., 2015). The results indicated that T. ovatus responded to the adverse effects of high-fat diet by increasing the expression of lipid hydrolysis related genes (*CPT1* and *PPARα*), which was helpful to promote lipid hydrolysis and reduce lipid deposition in the liver. However, the addition of L-carnitine in the high-fat diet decreased the expression levels of *CPT1* and *PPARα* compared to the high-fat diet group. The results demonstrated that L-carnitine promoted liver lipolysis and improved liver health, so it did not induce the expression of *CPT1* and *PPARα* to deal with the adverse effects of liver lipid deposition. Similar conclusions have also been reported by Chen et al. (2020), their study found that dietary L-carnitine supplementation could enhance lipid metabolism and decrease lipid deposition in the whole-body of fish.

For fish, high-fat diet intake is easy to cause lipid accumulation and peroxidation in liver (Zhou et al., 2020; Fang et al., 2021). In response to lipid deposition and peroxidation, cells can produce reactive oxygen species (ROS) to reduce oxidative stress and damage, but excessive production of ROS will destroy the integrity of organelles and cause damage to cells and tissues (Zhao et al., 2020; Zhou et al., 2020). SOD is an important enzymatic antioxidant and GSH-PX is a non-enzymatic antioxidant in cellular antioxidant system. SOD and GSH-PX can alleviate cells from oxidative stress by scavenging ROS (Fang et al., 2021). MDA is the final product of lipid

peroxidation, and its accumulation may cause cell toxicity and accelerate cell and tissue damage (Liu et al., 2021). The present results found that T. ovatus fed with high-fat diet (D2) showed higher SOD activity, MDA content and mRNA levels of Mn-SOD and GSH-PX, and significantly higher than those fed with low-lipid (D1) and high-lipid with L-carnitine supplementation (D3 and D4) diets. The results indicated that high-fat diet without L-carnitine supplementation led to lipid deposition and peroxidation, which activated the antioxidant system of T. ovatus to alleviate the oxidative pressure. However, the increase of SOD activity and mRNA levels of Mn-SOD and GSH-PX did not eliminate excessive ROS, thus maintaining the MDA content at a high level in the high-lipid group. Previous studies have reported that dietary L-carnitine supplementation inhibited lipid peroxidation and improved fish resistance to oxidative stress by increasing the activities of antioxidant enzymes (Mohseni and Ozorio, 2014; Sabzi et al., 2017; Zhou et al., 2020). Similarly, the present results found that T. ovatus fed with high-fat diet with L-carnitine supplementation showed lower SOD activity, MDA content and mRNA levels of Mn-SOD and GSH-PX when compared to the high-fat diet group. Above results demonstrated that *T. ovatus* were not exposed to oxidative stress caused by high-fat diet, which may be attributed to L-carnitine supplementation promoting lipid hydrolysis, improving cholesterol transport and scavenging excessive ROS. Therefore, *T. ovatus* did not need to produce SOD and induce the expression of *Mn-SOD* and *GSH-Px* to deal with the oxidative stress caused by high-fat diet intake.

CONCLUSION

In conclusion, L-carnitine supplementation in high-fat diet can alleviate the adverse effects of high-fat diet on the growth performance of *T. ovatus*. Besides, dietary L-carnitine supplementation can also improve the health of *T. ovatus* by promoting lipid hydrolysis, improving cholesterol transport and antioxidant capacity.

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 care and use of laboratory animals in Sun Yat-sen University and the National Institutes of Health Guide for Care and Use of Laboratory Animals. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

The authors thank the participants who gave their time to the trial. WZ, JW and JNdesigned the study. XC carried out the rearing work and measured experimental parameters. WZ and XC analyzed the results and wrote the paper with contributions from the other authors. All authors contributed to the article and approved the submitted version.

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Effects of Dietary Zymosan-A on the Growth Performance and Intestinal Morphology, Digestive Capacity, and Microbial Community in *Litopenaeus vannamei*

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The effects of dietary zymosan A on the growth performance and histological structure, digestive capacity, and microbiota were investigated in *Litopenaeus vannamei*, an important shrimp species used in aquaculture. *L. vannamei* (initial body weight = 0.41 ± 0.05 g) were fed diets supplemented with zymosan-A at doses of 0 mg/kg (Z0, control), 1 mg/kg (Z1), 5 mg/kg (Z5), 25 mg/kg (Z25), 125 mg/kg (Z125), or 625 mg/kg (Z625) for 8 weeks. The results showed that doses of 25 and 125 mg/kg significantly increased the final body weight, weight gain rate (WGR), and specific growth rate (SGR) and significantly decreased the feed conversion rate compared with the controls ($p < 0.05$). Analyses of the WGR and SGR revealed that the optimum dietary zymosan-A levels were 308.56 and 300.00 mg/kg, respectively. Compared with the controls, the intestinal villus height was significantly increased by the addition of zymosan-A at doses ≥25 mg/kg ($p < 0.05$). An obvious upregulation of the activities of trypsin and amylase was observed in all groups that received zymosan-A supplementation, while the activity of lipase was significantly increased in groups Z125 and Z625 ($p < 0.05$). Consistently, the gene expressions of trypsin and lipase were significantly higher in group Z125, while the gene expression of amylase was significantly increased in all zymosan-A-supplemented groups ($p < 0.05$). Analysis of the intestinal microbiota showed significant differences in the alpha diversity between group Z125 and controls. The supplemented groups showed altered intestinal bacterial community structures and compositions at the phylum, family, and genus levels, with statistical differences observed in the relative abundances of some dominant species. Tax4Fun predictions of the microbiota indicated that multiple intestinal functions were significantly altered in all zymosan-A-supplemented groups, except Z1. Among these groups, the functions related to transport and catabolism, substance dependence, cardiovascular disease, and signaling molecules and interactions were obviously increased by the addition of zymosan-A at different levels. In conclusion, dietary

supplementation of the optimum amount of zymosan-A can improve growth and intestinal function in *L. vannamei*, which will be valuable in aquaculture.

Keywords: *Litopenaeus vannamei*, zymosan-A, growth performance, digestive enzyme, intestinal microbiota

INTRODUCTION

Litopenaeus vannamei is a decapod crustacean species of the Penaeidae family that is economically important in global aquaculture. Shrimp production has increased with the rapid development of shrimp farming; however, diseases such as white spot disease (WSD) and acute hepatopancreatic necrosis disease (AHPND) have caused huge economic losses and resource wastage in this industry (Thitamadee et al., 2016). Antibiotics are a common form of disease control in aquaculture, yet may enhance the resistance of numerous pathogenic microorganisms, reduce the quality and safety of shrimp products, and threaten human health (Holmstrom et al., 2003). Alternatively, immunostimulants such as probiotics, prebiotics, hormones, cytokines, and polysaccharides, which can improve growth and immune responses while controlling disease, may be a better choice for use in aquaculture (Carbone and Faggio, 2016; Dawood et al., 2018; Mohan et al., 2019).

β-glucan is a type of polysaccharide present in the cell walls of bacteria, fungi, algae, and plants. It has various structural types consisting of β-(1,3/1,4) or β-(1,3/1,6)-D-linked glucose units (Barsanti et al., 2011). β-glucan is a well-known immunomodulator that has been administered to invertebrates and vertebrates *via* bath treatment, injection, and diet (Kim et al., 2000; De Oliveira et al., 2019). In humans, β-glucan has been reported to have anticancer, antidiabetic, anti-inflammatory, and immunomodulating effects (Jayachandran et al., 2018). Recent findings have suggested that β-glucan has significant impact on changes in the gut microbiota and, in turn, on the health of humans, pigs, and mice (Xu et al., 2020; Golisch et al., 2021; Wu et al., 2021). Currently, β-glucan is used as an immunomodulatory food supplement that is beneficial to immunity in commercial aquaculture species (Rodrigues et al., 2020). The beneficial effects of dietary β-glucan on growth, health status, and immunity have been reported in *Oreochromis niloticus* (Fabiana et al., 2017), *Paramisgurnus dabryanus* (Zhu and Wu, 2018), *L. vannamei* (Li et al., 2019), and *Cyprinus carpio* (Harris et al., 2020). Intake of β-glucan can also optimize the intestinal microbiota of aquatic animals, including *O. niloticus*, *C. carpio*, *Scophthalmus maximus*, *Apostichopus japonicus*, and *L. vannamei* (Kühlwein et al., 2014; Yang et al., 2015; Miest et al., 2016; Li et al., 2019; Xu et al., 2020). However, the structures of β-glucan have not been classified in most of the previous studies and their sources are also diverse.

Zymosan-A is a purified β-1,3-glucan obtained from a naturally insoluble polysaccharide macromolecule extracted from the cell walls of *Saccharomyces cerevisiae*. As a type of β-glucan, zymosan-A has been reported to play an important role in regulating the immune response of several species (Stuyven et al., 2009; Lei et al., 2013; Yu et al., 2014). In mice, zymosan-A enhances the mucosal adjuvant activity of poly(I:C) in a nasal influenza vaccine and also affects the composition and biodiversity of the gut microbiota (Ainai et al., 2010; Jeong et al., 2021). However, studies using zymosan-A as a dietary supplement for aquatic animals are rare. In addition, knowledge of its effects on the intestinal microbiota of aquatic animals remains lacking.

In this study, the effects of dietary supplementation of zymosan-A on the growth performance, histological structure, digestive enzyme activities and gene expressions, and microbiotic structure of *L. vannamei* were analyzed to evaluate the growth and intestinal functional responses to zymosan-A. These results have implications for improvement of the nutritional regulation of shrimp farming.

MATERIALS AND METHODS

Diets and Experimental Design

Six experimental diets were formulated; a proximate analysis is given in **Table 1**. Group Z0 was used as the control, while groups Z1, Z5, Z25, Z125, and Z625 were supplemented with graded levels of zymosan-A (1, 5, 25, 125, or 625 mg/kg, respectively; Z4250; supplied by Sigma-Aldrich, St. Louis, MO, USA). Dietary ingredients were ground through an 80-mesh screen, weighed, and thoroughly mixed to homogeneity (M-256, South China University of Technology, Guangzhou, China). Each diet was weighed according to the test formula and mixed step by step using a twin-screw extruder (F-26, South China University of Technology, Guangzhou, China) to make pellets with particle sizes of 1.0 and 1.5 mm. The pellets were heated in an electric oven at 60°C for 30 min, then dried at room temperature to a moisture content of about 10%, sealed in Ziploc bags, and stored at −20°C until required.

Shrimp Feeding Trial and Sample Collection

The entire experiment was carried out in an indoor breeding system at the Marine Biology Research Base of Guangdong Ocean University (Zhanjiang, China). *L. vannamei*, with average body weight 0.41 ± 0.05 g, were obtained from Guangdong Haixing Agriculture Group Co., Ltd. (Zhanjiang, China). Before starting the feeding experiment, the shrimp were allowed to adapt to the experimental conditions for 1 week before feeding with commercial feed. The acclimated shrimp were randomly distributed into 300-L fiberglass tanks at 40 shrimp per tank. Shrimp were fed the experimental diets to apparent satiation four times daily (0700, 1100, 1700, and 2100 hours) for 8 weeks. During the experimental period, the temperature range was 29.0–30.0°C, salinity was 27–30 g/L, the dissolved oxygen level was at least 6.0 mg/L, the pH value was

TABLE 1 | Ingredient and proximate composition of the basal diet.

Ingredient	Percentage (%)
Brown fish meal	20
Soybean meal	20
Shrimp head meal	4
Peanut meal	9
Corn gluten meal	10
Wheat meal	25
Fish oil	2
Corn oil	2
Soybean lecithin	0.5
Vitamin premix[a]	0.2
Mineral premix[b]	0.5
Choline chloride	0.5
Antioxidants	0.03
Lunar agent	0.1
Calcium phosphate	1.5
Vitamin C	0.05
Microcrystalline cellulose	4.62
Total	100
Crude protein[c]	41.13
Ether extract[c]	7.72
Ash	12.53

[a]*Vitamin premix(per kg diet): Vitamin B1, 25 mg; Vitamin B2, 45 mg; Vitamin B3, 60 mg; Vitamin B5, 200 mg; Vitamin B6, 20 mg; Vitamin B7,1.20 mg; Vitamin B12, 0.1 mg; Inositol, 800 mg; Folic acid, 20 mg; Vitamin A, 32 mg; Vitamin E, 120 mg; Vitamin D3, 5 mg; Vitamin K3, 10 mg.*

[b]*Mineral premix(per kg diet): Sodium fluoride, 2 mg; Potassium iodide, 0.8 mg; Cobalt chloride(%), 50 mg; Cupric sulphate, 10 mg; Ferrous sulphate,80 mg; Zinc sulphate, 50 mg; Manganese sulphate, 60 mg; Magnesium sulfate, 1200 mg; Sodium chloride, 100 mg; Zeolite powder, 1447.2 mg.*

[c]*Crude protein and crude lipid contents were measured value.*

7.7–8.0, and the ammonia nitrogen level was <0.05 mg/L. During the trial, each tank was individually aerated and 60% of the water was exchanged daily.

At the end of the 8-week period, shrimp were fasted for 24 h before collection of samples. After being counted and weighed to determine survival and weight gain, six shrimp from each tank were randomly sampled for each of the following analyses: histological sectioning, digestive enzyme activity analysis, gene expression analysis, and intestinal microbiome analysis. The samples were rapidly removed and frozen in liquid nitrogen until analysis.

Histological Structure of the Intestine

At the end of the feeding trial, the midguts were sampled from four shrimp from each tank and fixed with 4% paraformaldehyde in 5-ml Eppendorf tubes to remove the intestinal content. Following fixation, the intestinal tissue was embedded in paraffin, sliced, and stained with hematoxylin–eosin (H&E) using standard histological techniques, then examined for intestinal morphology parameters under an optical microscope (Olympus BX51, serial no. 9K18395, Tokyo, Japan). The electronic images were further analyzed using ImageJ software to assess the dimensions of intestinal villus height (VH), villus width (VW), and muscle thickness (MT).

Digestive Enzyme Analysis

Intestine samples were homogenized in ice-cold phosphate buffer (1:10 dilution). The homogenate was then centrifuged for 20 min (4°C, 3,000 rpm), and aliquots of the supernatant were used to quantify the digestive enzymes. All indices, including amylase (AMS), lipase, and trypsin, were measured with commercial assay kits (Nanjing Jian Cheng Bioengineering Institute, Nanjing, China) in accordance with the manufacturer's instructions.

Gene Expression Analysis

Total RNA was extracted from the intestines without feces of three shrimp from each tank using TransZol Up Plus RNA kits (TransGen, Beijing, China) following the manufacturer's protocol. Spectrophotometric analysis (Nanodrop 2000) was used to assess the RNA quality and concentration. Complementary DNA (cDNA) was synthesized using a PrimeScriptTM RT reagent kit with gDNA Eraser (Takara, Shiga, Japan) according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed to the first-strand cDNA using reverse transcriptase (Accurate Biology, Changsha, China) following the manufacturer's instructions.

Real-time PCR for the target genes was performed using a SYBR® Green Premix Pro Taq HS qPCR Kit II (Accurate Biology, Changsha, China) and quantified on a LightCycler 480 (Roche Applied Science, Mannheim, Germany) using the following program: 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 nuclease-free water to make a final volume of 10 μl; denaturation at 95°C for 30 s, 40× 5 s amplification cycles, denaturation at 95°C, and 30 s annealing at 60°C, followed by melting curve analysis and cooling to 4°C. The elongation factor 1α (EF1α; GenBank accession no. GU136229) was used as the internal control. All samples were tested in triplicate. The primer sequences are listed in **Table 2**.

Intestinal Microbial Analysis

The total genomic DNA of microbes from the intestinal samples was extracted using HiPure Soil DNA Kits (or HiPure Stool DNA Kits; Magen, Guangzhou, China) according to the manufacturer's protocols. Then, the V3+V4 region of the bacterial 16S ribosomal RNA (rDNA) gene was amplified using a pair of barcoded specific primers: 341F (5′-CCTACGGGNGGC WGCAG-3′) and 806R (5′-GGACTACHVGGGTATCTAAT-3′). The PCR amplification began with 2 min at 94°C, followed by 30 cycles at 98°C for 10 s, 62°C for 30 s, 68°C for 30 s, and a final extension at 68°C for 5 min. PCR reactions were performed in triplicate 50 μl mixtures containing 5 μl of 10× KOD buffer, 5 μl of 2 mM dNTPs, 3 μl of 25 mM $MgSO_4$, 1.5 μl of each primer (10 mM), 1 μl of KOD polymerase, and 100 ng of template DNA. Related PCR reagents were obtained from Toyobo (Osaka, Japan). The amplified products were purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions. The purified and amplified products were mixed equivalently and ligated with the sequencing adapter, then sequenced with a Hiseq2500 PE250 machine (Illumina, San Diego, CA, USA). The raw data have been deposited in the NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

The data were filtered for noisy sequences, checked for the presence of chimaeras, and clustered with a threshold of 97% sequence similarity. To determine the level of sequencing depth,

TABLE 2 | Primers used for quantitative real-time PCR.

Gene names	GenBank no.	Primer names	Sequences (5'-3')
Elongation factor 1α	GU136229	EF1α-F	GAAGTAGCCGCCCTGGTTG
		EF1α-R	CGGTTAGCCTTGGGGTTGAG
Trypsin	X86369	TRYP-F	CGGAGAGCTGCCTTACCAG
		TRYP-R	TCGGGGTTGTTCATGTCCTC
Lipase	DQ858927	LIP-F	ACTGTCTCCTCTGCTCGTC
		LIP-R	ATGGTTTCTGGAATAGGTGTTT
Amylase	AH013375	AMY-F	CTCTGGTAGTGCTGTTGGCT
		AMY-R	TGTCTTACGTGGGACTGGAAG

rarefaction curves were produced by plotting the number of observed operational taxonomic units (OTUs) against the number of sequences. The tag sequence with the highest abundance was selected as a representative sequence within each cluster. Between-group Venn analysis was performed in R software (version 3.4.1) to identify unique and common OTUs. The representative sequences were classified into organisms by a naive Bayesian model using the RDP classifier, version 2.2 (Wang, 2007), based on the SILVA database (https://www.arb-silva.de/) (Quast et al., 2012), with confidence threshold values of 0.8–1. Alpha diversity indices [observed species (S_{obs}), Shannon, Simpson, Chao1, and the abundance-based coverage estimator (ACE)] were calculated using QIIME software. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the OTUs was inferred using Tax4Fun, version 1.0 (Aßhauer et al., 2015).

Statistical Analysis and Calculations

The experimental results are presented as the mean ± standard deviation (SD). Statistically significant differences were established using one-way analysis of variance (ANOVA) at a 5% level of probability, and differences between means were compared using Tukey's tests. Statistical analysis was carried out in SPSS for Windows, version 22 (SPSS Inc., Chicago, IL, USA). The formulae employed were as follows:

$$Survival\ rate\ (SR\ in\ \%) = final\ fish\ number/initial\ fish\ number \times 100$$

$$Weight\ gain\ rate\ (WGR\ in\ \%) = (final\ body\ weight - initial\ body\ weight)/initial\ weight \times 100$$

$$Specfic\ growth\ rate\ (SGR\ in\ \%/day) = [ln(final\ weight) - ln(initial\ weight)]/days\ of\ feeding\ trial \times 100;\ and$$

$$Feed\ conversation\ ratio\ (FCR) = weight\ of\ dry\ diet\ fed/wet\ weight\ gain$$

RESULTS

Growth Performance

After the feeding experiment, all *L. vannamei* had a high SR, which was unaffected by supplementation with zymosan-A (**Table 3**). The final body weight (FBW), WGR, and SGR were significantly higher and the FCR significantly lower in groups Z25 and Z125 than those in group Z0 ($p < 0.05$). According to the line chart analysis using WGR or SGR as the evaluation index, the optimal amounts of zymosan-A supplementation were 308.56 and 300.00 mg/kg, respectively (**Figure 1**).

Histological Observation of the Intestine

The effects of dietary zymosan-A supplementation on the intestinal morphological parameters are shown in **Figure 2**. Photomicrographs of cross-sections of the intestinal tract are shown in **Table 4**. When the amount of zymosan-A reached 25 mg/kg, the intestinal VH in *L. vannamei* was significantly higher compared to that of group Z0 ($p < 0.05$). Zymosan-A supplementation did not obviously change the intestinal VW and MT. In addition, the intestinal structure was destroyed in group Z625.

Digestive Enzyme Activities in the Intestine

As shown in **Figures 3A, C**, the activities of both the intestinal trypsin and amylase were significantly increased in all zymosan-A-supplemented groups ($p < 0.5$), with the highest levels in group Z125, followed by group Z25. For lipase activity, compared with group Z0, obvious increases were only observed in groups Z125 and Z625, being highest in group Z125 (**Figure 3B**).

Gene Expressions of Digestive Enzymes in the Intestine

Compared with the controls, the relative expression levels of the digestive-related genes, including trypsin, lipase, and amylase, were increased by supplementation with zymosan-A (**Figure 4**). The gene expressions of both trypsin and lipase were significantly induced in group Z125, while amylase expression was obviously higher in all zymosan-A-supplemented groups than those in controls ($p < 0.5$).

Microbiota Community Characterization in the Intestine

The raw data of this study have been deposited in the NCBI's Sequence Read Archive (SRA) database with the accession

TABLE 3 | Growth performance and feed utilization of *L. vannamei* fed the experimental diets for 8 weeks.

Items	Groups					
	Z0	**Z1**	**Z5**	**Z25**	**Z125**	**Z625**
SR (%)	93.33±2.20	92.5±1.44	90.00±1.44	91.67±0.83	91.67±2.20	93.33±3.63
FBW (g)	6.75±0.01^{ab}	6.65±0.05^{a}	7.20±0.09^{b}	7.68±0.21^{c}	8.12±0.20^{d}	7.09±0.11^{b}
WGR (%)	1545.89±18.80^{ab}	1507.91±15.81^{a}	1658.41±1.67^{bc}	1757.80±68.10^{c}	1879.22±43.16^{d}	1633.53±8.44^{b}
SGR (% /d)	5.00±0.02^{ab}	4.96±0.02^{a}	5.12±0.01^{bc}	5.22±0.07^{c}	5.33±0.04^{d}	5.09±0.01^{b}
FCR	1.48±0.03^{bc}	1.52±0.04^{c}	1.44±0.03^{bc}	1.32±0.03^{a}	1.24±0.02^{a}	1.41±0.05^{b}

SR, Survival rate; FBW, Final body weight; WGR, Weight gain rate; SGR, Specific growth rate; FCR, Feed conversion ratio. Values in the same column with different superscript letters are significantly different (P < 0.05, Tukey's test) among treatments. Absence of letters indicates no significant differences among treatments.

number PRJNA808041. After quality control and read assembly, the effective tags were >90%. All OTUs of the comparison group with an average abundance >1 were selected for Venn diagram analysis. A Venn diagram was constructed to identify the core and different OTUs existing in different groups of shrimp samples. In this regard, 432 OTUs were shared among all the intestine samples (**Figure 5**). In contrast, 341, 158, 261, 272, 321, and 281 OTUs were unique to groups Z0, Z1, Z5, Z25, Z125, and Z625, respectively. Group Z1 had the minimum number of unique OTUs, while group Z125 had the maximum.

As shown in **Table 5**, significant differences in the diversity and richness of the intestinal microbiota were observed among the dietary treatments, as evaluated using the alpha indices, including the observed species (S_{obs}), Chao1, Shannon, and Simpson indices, and ACE. Compared to those in group Z0, the S_{obs} and Chao1 were significantly higher in groups with zymosan-A addition of >5 mg/kg ($p < 0.05$). Similarly, only the Shannon and Simpson indices of group Z125 were significantly higher than those of group Z0 ($p < 0.05$). The ACE values were significantly higher in each zymosan-A-supplemented group than those in group Z0 ($p < 0.05$).

At the phylum level, the average intestinal microbiotic community of *L. vannamei* was dominated by Proteobacteria (48.29%), Bacteroidetes (25.32%), Planctomycetes (7.97%), and Verrucomicrobia (7.20%). Actinobacteria (3.99%) and Chlamydiae (2.83%) were subdominant (**Figure 6A**). Moreover, Acidobacteria, Cyanobacteria, Firmicutes, and Chloroflexi were also among the top 10 phyla in the intestine. Of the top 10 phyla, Verrucomicrobia, Acidobacteria, Cyanobacteria, and Chloroflexi were significantly different in the zymosan-A-supplemented groups compared with the controls (**Figure 6B**). The relative abundance of Verrucomicrobia was significantly greater in groups Z1 and Z625 than in controls. The relative abundance of Acidobacteria was obviously higher in all zymosan-A-supplemented groups, except group Z1, being highest in group Z125. The relative abundances of Cyanobacteria and Chloroflexi were significantly reduced and increased in all zymosan-A-supplemented groups, respectively.

At the family level, the nine known families of the top 10 families, from high to low relative abundance, were Flavobacteriaceae (20.50%), Rhodobacteraceae (17.77%), Vibrionaceae (9.34%), Rubritaleaceae (6.54%), Pirellulaceae (4.51%), Halieaceae (4.38%), Rubinisphaeraceae (2.71%), Demequinaceae (2.44%), and Psychromonadaceae (2.33%) (**Figure 7A**). As **Figure 7B** shows, there were significant between-group differences in the relative abundance rates of Rhodobacteraceae, Vibrionaceae, Rubinisphaeraceae, and Psychromonadaceae ($P < 0.05$). Unlike the various degrees of significant increases in Rhodobacteraceae, Rubinisphaeraceae, and Psychromonadaceae in the zymosan-A-supplemented groups, the relative abundance of Vibrionaceae was significantly reduced in all supplemented groups compared with the controls.

At the genus level, nearly half of the genera (49.27%) were unclassified. The top 10 genera were *Vibrio*, *Haloferula*, *Ruegeria*, *Hoppeia*, *Pir4_lineage*, *Tenacibaculum*, *Demequina*, *Motilimonas*, *Actibacter*, and *Pseudoalteromonas*, with average community richness values of 8.60%, 5.89%, 4.37%, 2.67%, 2.64%, 2.46%, 2.18%, 2.12%, 1.63%, and 1.63%, respectively (**Figure 8A**). *Vibrio*, *Haloferula*, *Ruegeria*, *Motilimonas*, and *Pseudoalteromonas* were the five genera with significant differences between the zymosan-A-supplemented and control

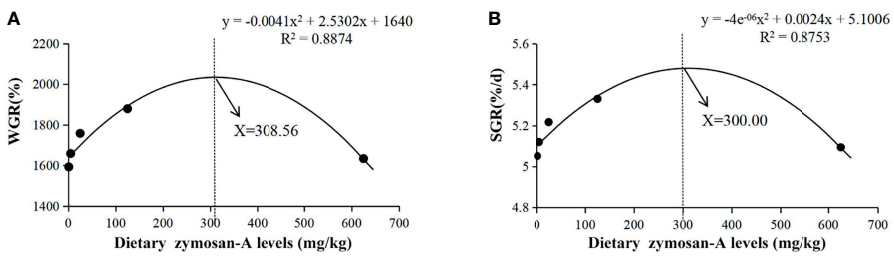

FIGURE 1 | Relationships between the level of dietary zymosan-A and weight gain rate (WGR) **(A)** and specific growth rate (SGR) **(B)** in *Litopenaeus vannamei* fed experimental diets for 8 weeks (with fitted second-order polynomial models).

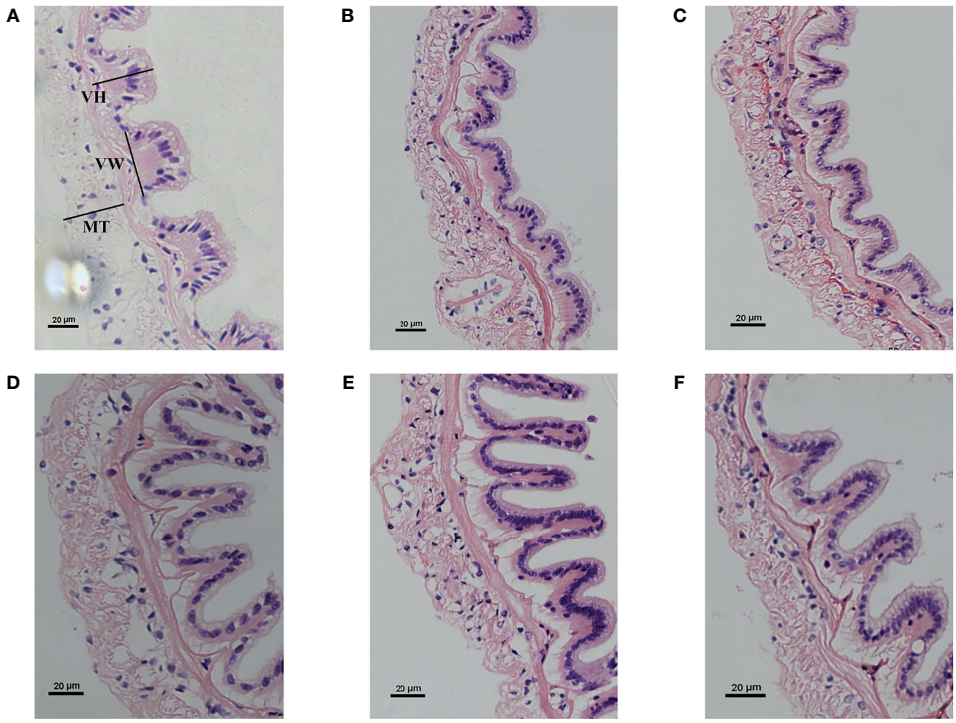

FIGURE 2 | Intestinal histology of *Litopenaeus vannamei* fed different levels of zymosan-A. **(A–F)** Groups Z0 (control), Z1, Z5, Z25, Z125, and Z625, respectively. *VH*, villus height; *VW*, villus width; *MT*, muscle thickness.

groups (**Figure 8B**). *Vibrio* was the only genus that was significantly reduced in all zymosan-A-supplemented groups.

Functional Prediction of the Intestinal Microbiota

Changes in the presumptive functions of the intestinal microflora were examined using Tax4Fun software to predict the metagenomes. As shown in **Figure 9A**, the top 10 predicted functions with their relative abundance rates were as follows: membrane transport (13.2%–13.8%), carbohydrate metabolism (12.8%–13.1%), amino acid metabolism (12.2%–12.5%), signal transduction (7.3%–7.5%), metabolism of cofactors and vitamins (6.9%–7.1%), energy metabolism (6.8%–6.9%), nucleotide metabolism (5.2%–5.3%), translation (4.1%–4.2%), xenobiotic biodegradation and metabolism (3.8%–4.1%), and replication and repair (3.8%–3.9%). Welch's *t*-tests showed that there were no significantly changed functions between groups Z0 and Z1. In the other four zymosan-A-supplemented groups, several predicted pathways were significantly enriched in the microbiota in KEGG level 2 (95% confidence intervals, $p < 0.05$). Compared with group Z0, transport and catabolism were significantly enriched in group Z5, while endocrine and metabolic diseases were the opposite (**Figure 9B**). In group Z25, transport and catabolism and substance dependence were the only two significantly enriched functions (**Figure 9C**). Comparison of groups Z0 and Z125 indicated that the functions of the nervous system, signaling molecules and interaction, cardiovascular diseases, and cell communication were significantly enriched in group Z125 (**Figure 9D**). As **Figure 9E** shows, folding, sorting and degradation, transport and catabolism, environmental adaptation, substance dependence, excretory system, cardiovascular diseases, and

TABLE 4 | Intestinal structure of *L. vannamei* fed the experimental diets.

Items	Groups					
	Z0	Z1	Z5	Z25	Z125	Z625
VH (μm)	41.73±0.87^{a}	43.54±0.58^{a}	50.93±4.72^{a}	57.68±4.34^{b}	72.69±1.40^{d}	66.57±1.52^{c}
VW (μm)	44.06±1.58^{ab}	44.58±6.22^{b}	42.36±0.70^{ab}	35.34±3.94^{a}	37.36±1.42^{ab}	36.76±2.23^{ab}
MT (μm)	40.17±4.38	34.96±2.84	37.28±1.05	41.99±4.35	39.81±3.50	35.46±4.13

VH, villus height; VW, villus width; MT, muscle thickness. Values in the same column with different superscript letters are significantly different (P < 0.05). Absence of letters indicates no significant difference among treatments.

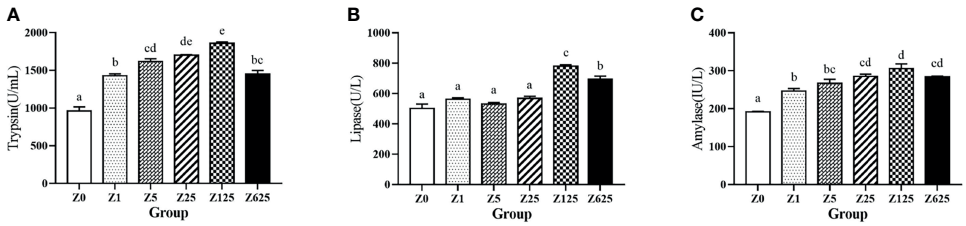

FIGURE 3 | Effect of different levels of zymosan-A on the activities of trypsin **(A)**, lipase **(B)**, and amylase **(C)** in the intestine of *Litopenaeus vannamei*. *Different letters* indicate significant differences between treatments ($p < 0.05$).

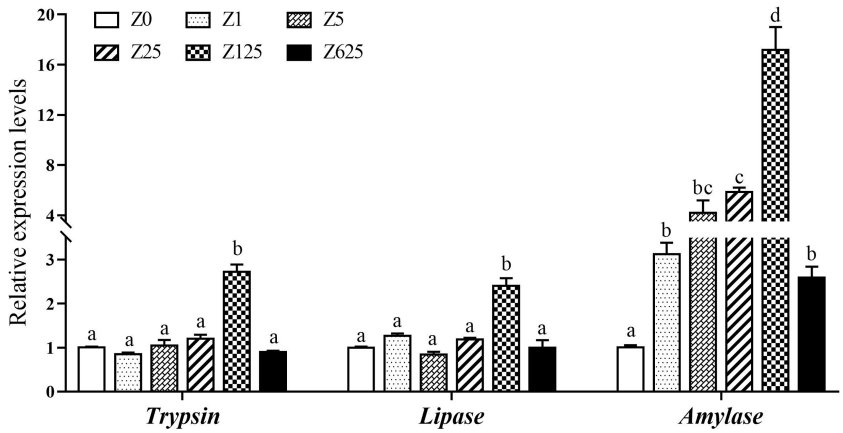

FIGURE 4 | Effect of different levels of zymosan-A on the expressions of *trypsin*, *lipase*, and *amylase* genes in the intestine of *Litopenaeus vannamei*. *Different letters* indicate significant differences between treatments ($p < 0.05$).

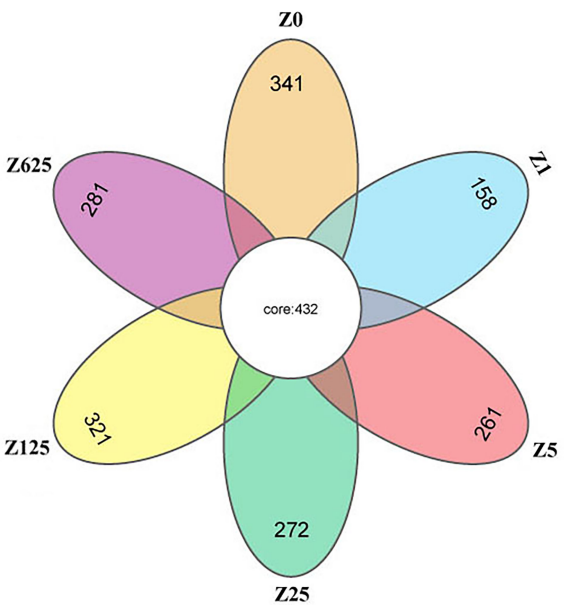

FIGURE 5 | Venn diagram for the comparison of operational taxonomic units (OTUs).

TABLE 5 | The summary of intestinal microbiota Alpha diversity indices.

Items	Groups					
	Z0	Z1	Z5	Z25	Z125	Z625
Sobs	1158.67±224.88^{a}	1387.67±91.42^{ab}	1556.67±91.68^{bc}	1700.00±69.38^{bc}	1797.33±47.52^{c}	1610.00±96.39^{bc}
Chao1	1804.69±429.38^{a}	2414.46±196.78^{ab}	2520.44±247.20^{b}	2793.70±190.72^{b}	3039.32±118.65^{b}	2577.39±120.95^{b}
Shannon	5.46±0.51^{a}	6.03±0.15^{ab}	6.64±0.86^{ab}	6.30±0.34^{ab}	7.14±0.61^{b}	6.53±0.35^{ab}
Simpson	0.93±0.02^{a}	0.96±0.00^{ab}	0.97±0.02^{ab}	0.95±0.01^{ab}	0.98±0.01^{b}	0.96±0.01^{ab}
ACE	1918.65±418.74^{a}	2450.47±140.41^{b}	2626.56±207.32^{b}	2833.41±243.70^{b}	3029.14±103.86^{b}	2663.03±136.51^{b}

Values (Means ± SD) in the same line having different superscript letters are significantly different (P<0.05) among treatments. The lack of a superscript letter indicates no significant differences among treatments (P>0.05).

signaling molecule functions were significantly enriched in group Z625 compared with the controls.

DISCUSSION

Several studies have established the beneficial effects of β-glucan supplementation in aquatic animals (Fabiana et al., 2017; Ji et al., 2017; Nieves-Rodríguez et al., 2018; Zhu and Wu, 2018; Harris et al., 2020). However, the complexity of the structure of β-glucan and the diversity of its sources are often ignored. Furthermore, studies using high-throughput sequencing to assess the impact of β-glucan on the intestinal flora are also uncommon. In this study, the effects of zymosan-A, which is a purified β-1,3-glucan, on *L. vannamei* were investigated. The results indicated that an optimum amount of dietary zymosan-A improves growth performance and intestinal function from the aspects of histological structure, digestive capacity, and microbiota.

Numerous studies have indicated a positive role of β-glucan in aquatic animal growth. (Ji et al., 2017) confirmed that *Oncorhynchus mykiss* fed 100–200 mg/kg β-glucan showed significantly improved growth parameters and feeding efficiency. Additionally, dietary supplementation with 200 mg/kg β-glucan promoted growth in *O. niloticus*, while supplementation with 50 and 100 mg/kg β-glucan significantly enhanced the growth and survival rates in *Trachinotus ovatus* (Linnaeus) (Whittington et al., 2005; Do Huu et al., 2016). Furthermore, *L. vannamei* fed 200–400 mg/kg β-glucan exhibited higher growth performance (Bai et al., 2010; Boonanuntanasarn et al., 2016; Li et al., 2019). Consistent with previous research, the present study found that diets supplemented with zymosan-A not only increased the growth performance but also decreased the FCR of *L. vannamei*. The optimal supplementation amount was approximately 300 mg/kg using either WGR or SGR as the evaluation index. The results indicated that zymosan-A, which is a purified β-1,3-glucan, possesses the same functionality as other types of β-glucan in improving growth. The optimal amount identified in the present study for *L. vannamei* is consistent with those of previous studies.

Several parameters are closely related to growth performance, including intestinal structure and digestive enzymes.

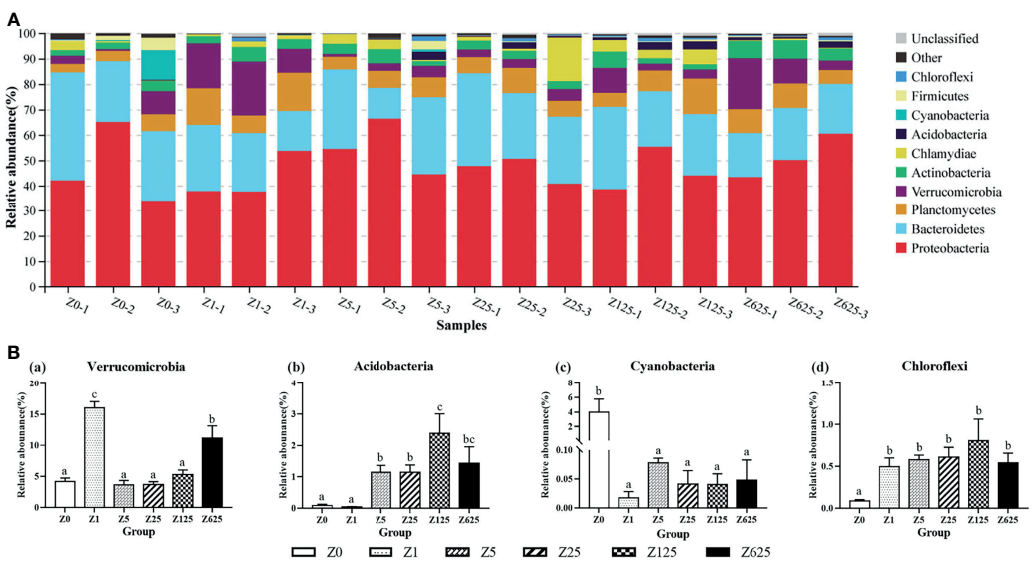

FIGURE 6 | Structure and composition of the intestinal bacterial communities in *Litopenaeus vannamei* fed different levels of zymosan-A (phylum level). **(A)** Species distribution stacking of the treatment groups. **(B)** Relative abundances of the top 10 phyla. *Different letters* indicate significant differences ($p < 0.05$).

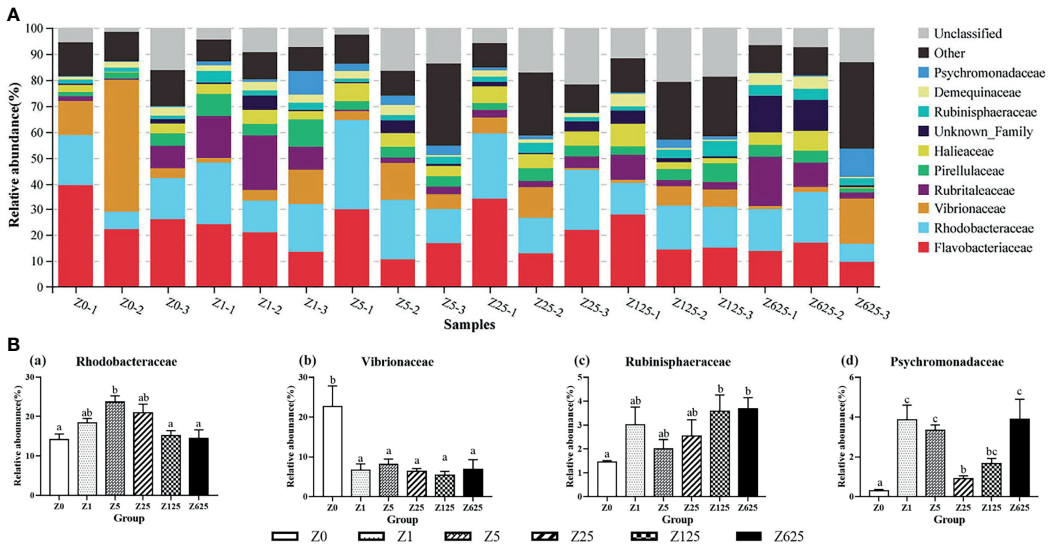

FIGURE 7 | Structure and composition of the intestinal bacterial communities in *Litopenaeus vannamei* fed different levels of zymosan-A (family level). **(A)** Species distribution stacking of the treatment groups. **(B)** Relative abundances of the top 4 families. *Different letters* indicate significant differences ($p < 0.05$).

An increased intestinal villus length increases the area of contact between the intestine and ingested nutrients, which, in turn, improves growth performance (Kühlwein et al., 2014; Sewaka et al., 2019). In the present study, dietary zymosan-A supplementation increased the intestinal villus height and body weight. This is consistent with observations in *L. vannamei*, *O. niloticus*, and *Tarphops oligolepis* (Bleeker, 1858) fed dietary β-glucan (Boonanuntanasarn et al., 2016; Dawood et al., 2019; Gu et al., 2021). Another factor closely related to growth parameters is digestive enzyme activity, which reflects the degree of feed utilization and digestion. There are three key enzymes known to be related to nutrient metabolism in shrimp (Wang, 2007; Anand et al., 2013): trypsin (which catalyzes the hydrolysis of proteins into smaller peptides), lipase (known to catalyze the hydrolysis of fats/lipids), and amylase (known to catalyze the hydrolysis of starch into sugars). In the present study, the activities of trypsin, amylase, and lipase in the zymosan-A-supplemented groups were positively correlated

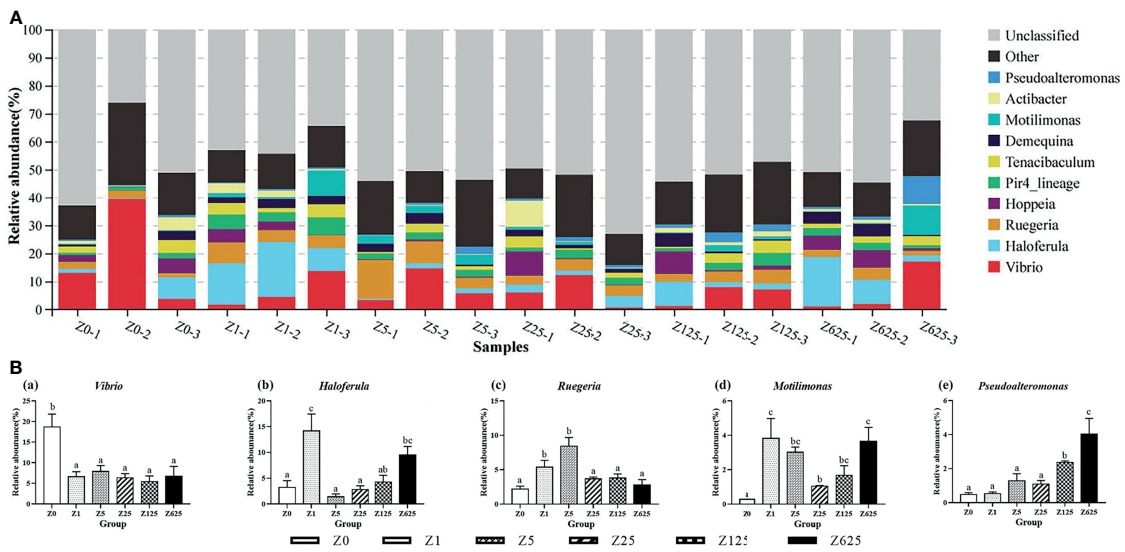

FIGURE 8 | Structure and composition of the intestinal bacterial communities in *Litopenaeus vannamei* fed different levels of zymosan-A (genus level). **(A)** Species distribution stacking of the treatment groups. **(B)** Relative abundances of the top 5 genera. *Different letters* indicate significant differences ($p < 0.05$).

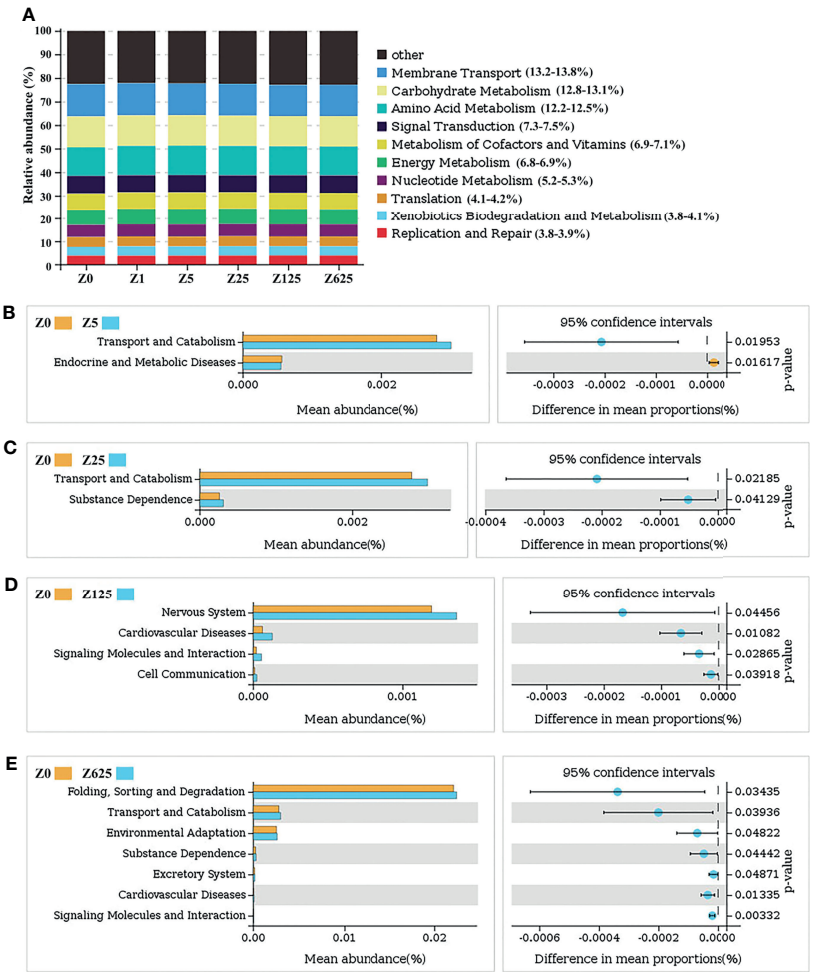

FIGURE 9 | **(A)** Relative abundances of the top 10 predicted functions. **(B–E)** Welch's *t*-tests of the significantly different functions at level 2.

with the concentration of zymosan-A in the diet. These results are similar to those of (Li et al., 2019), in which dietary addition of 400 mg/kg β-glucan improved the activities of protease and amylase in *L. vannamei*. However, our study found that 125 and 625 mg/kg zymosan-A can also improve lipase activity. Our results also agree with studies on fish. *Lutjanus peru* fed 0.2% β-glucan had greater aminopeptidase, trypsin, and chymotrypsin activities than those fed 0.1% β-glucan diet (Guzmán-Villanueva et al., 2014). In *Atractosteus tropicus*, diets supplemented with 1.0% and 1.5% β-glucan increased the activity of chymotrypsin (Nieves-Rodríguez et al., 2018). The increased activities of digestive enzymes were further confirmed by the gene expressions of the intestinal digestive enzymes; hence, the gene expressions of trypsin, lipase, and amylase could be significantly induced by optimum addition of zymosan-A.

It is well known that the intestinal microbiota plays a vital role in the digestion and absorption processes of shrimp, as well as a supportive role in intestinal digestive activities, which, in turn, determine their growth traits (Dai et al., 2017; Xiong et al., 2017). In the present study, Illumina sequencing showed that the intestinal microbial diversity and richness were higher in zymosan-A-supplemented groups than those in controls. At the phylum level, Proteobacteria, Bacteroidetes, and Planctomycetes were dominant in the intestines, which is consistent with previous studies on fish and shrimp (Rungrassamee et al., 2014; Xiong et al., 2015; Dehler et al., 2017; Amoah et al., 2019). The distinguishing feature of the changes in the intestinal microbiota, both at the family and genus levels, is that pathogenic bacteria decreased significantly while beneficial bacteria increased significantly. The study of Yang et al. showed that the intestinal microbial community in β-glucan-fed *A. japonicus* was widely dominated by Flavobacteriaceae, Rhodobacteraceae, and Vibrionaceae, while dietary β-glucan supplementation reduced the relative abundance of Flavobacteriaceae and promoted Rhodobacteraceae (Yang et al., 2015). This is consistent with the present study, where Flavobacteriaceae, Rhodobacteraceae, and Vibrionaceae were the top 3 dominant families in *L. vannamei* and where the relative abundances of Flavobacteriaceae and Rhodobacteraceae were reduced and increased by the addition of zymosan-A, respectively. Furthermore, the dominant family Vibrionaceae

was significantly decreased, while Rhodobacteraceae, Rubinisphaeraceae, and Psychromonadaceae were significantly increased. It is worth noting that Vibrionaceae and Rhodobacteraceae are considered pathogenic bacteria and are probiotics in aquatic animals (Zhou et al., 2007; Suphoronski et al., 2019). At the genus level, the microbial community was consistent with those at the family and phylum levels. The most obvious result was that the dominant genus *Vibrio* (family Vibrionaceae) was significantly reduced in all zymosan-A-supplemented groups. A common bacteria in aquatic animals, *Vibrio* has been characterized as the dominant member of the shrimp gastrointestinal tract, while *Vibrio* infection can cause serious diseases in shrimp (Rungrassamee et al., 2014; Tzuc et al., 2014; Huang et al., 2016; Zoqratt et al., 2018; Suphoronski et al., 2019). The significantly decreased abundance of *Vibrio* suggests that dietary zymosan-A could effectively reduce the risk of disease in *L. vannamei*. In addition, *Haloferula* (phylum Verrucomicrobia) was significantly increased by zymosan-A supplementation. In general, a low abundance of *Haloferula* in the intestine indicates an interrupted intestinal function that may lead to an energy imbalance (Zeng et al., 2021). In the present study, the increased abundance of *Haloferula* in supplemented *L. vannamei* indicates that zymosan-A might be helpful in maintaining normal intestinal function in this species. In addition, the predicted functions of the intestinal microflora showed that metabolic-related functions comprised most of the top 10 functions and are closely related to improved growth in *L. vannamei*. Overall, the changes in the intestinal microbiota showed that dietary zymosan-A can improve the intestinal microbial community and, in turn, promote growth by regulating metabolism in *L. vannamei*.

Considering all the observed effects of dietary zymosan-A on *L. vannamei*, this study provides further evidence that β-glucan plays a positive role in improving growth performance. This is probably due to the regulation of intestinal function in terms of morphology, digestive capacity, and the microbial community. Considering that zymosan-A is a purified form of β-1,3-glucan, it can be speculated that β-1,3-glucan is an important part of the function of β-glucan.

CONCLUSION

The present study demonstrated that dietary supplementation of zymosan-A improved not only the growth performance but also the intestinal morphology and the activities and gene expressions of digestion enzymes and the microbiome. Using the growth index as the evaluation factor, the optimal amount of zymosan-A addition to the diet of *L. vannamei* was approximately 300 mg/kg. These results provided detailed information for using β-glucan in shrimp and for achieving precise nutritional regulation in aquaculture.

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: NCBI [accession: PRJNA808041].

AUTHOR CONTRIBUTIONS

SZ and BT conceived and designed the experiments. YZ analyzed the data and wrote the paper. YZ, CH, ZY, JC, and HW collected samples and performed the experiments. All authors contributed to the article and approved the submitted version.

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Dietary Supplementation of Bile Acids in Tongue Sole (*Cynoglossus semilaevis*): A Promising Strategy to Improve Hepatic Health Status

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A 10-week feeding trial was conducted to investigate the beneficial effects of bile acids (BAs) on hepatic health status in tongue sole (*Cynoglossus semilaevis*). Three experimental diets were prepared with different levels of BA inclusion in the commercial basal diet: control group (CT: 0 mg/kg basal diet), low-level group (BA1: 300 mg/kg basal diet), and high-level group (900 mg/kg basal diet). At the end of the feeding trial, growth performance and survival rate were measured, as well as numerous physiological and biochemical parameters of the liver, including four hepatic function indices, five antioxidant indices, five lipid metabolism parameters, and three digestive enzyme activities. Moreover, the mRNA expression levels of three growth-related genes and three immune-related genes in the liver were assayed. Results showed that growth performance and survival were substantially improved in both low- and high-level BA inclusive groups. Further, the tested liver physiological and biochemical parameters combined with the quantitative real-time PCR results revealed enhanced antioxidant capacity, energy metabolism, digestive ability, and immune response. Finally, these findings provide a wider spectrum of the beneficial effects of dietary BAs on liver health in tongue sole.

Keywords: hepatic health, antioxidant capacity, immune response, digestive enzyme activity, hepatic function

INTRODUCTION

Bile acids (BAs), synthesized from cholesterol in the liver, can act as emulsifiers to promote lipid digestion and absorption in animals (Hofmann et al., 2010). Other roles of BAs include enhancing glucose absorption and transport, regulating cholesterol levels, and modulating the immune response in the liver and intestine (Houten et al., 2006; Nguyen and Bouscarel, 2008; Fiorucci et al., 2010; Fiorucci and Distrutti, 2015; Xie et al., 2021; Fu et al., 2022; Xu et al., 2022). International research on the BAs as feed additives in livestock and poultry started in the 1970s, and BAs were soon used in aquaculture species in the 1980s. In China, BA-related products have been proved as animal feed additives since 2014 by the Ministry of Agriculture.

Indeed, BAs as feed additives have shown much effectiveness in solving a series of adverse effects caused by unbalanced diets or antinutritional factors in feeds (e.g., high lipid diets, high starch diets, and high plant protein-based diets) (Romano et al., 2020). Recently, the beneficial effects of dietary BAs on liver health have been investigated in many aquaculture species. New studies have shown remarkable

promise to enhance liver health and then further improve productivity by dietary BA supplementation in fishes, including large yellow croaker (*Larimichthys crocea*) (Ding et al., 2020), tiger pufferfish (*Takifugu rubripes*) (Liao et al., 2020), largemouth bass (*Micropterus salmoides*) (Yu et al., 2019; Guo et al., 2020; Yin et al., 2021), black seabream (*Acanthopagrus schlegelii*) (Jin et al., 2019), tilapia (*Oreochromis niloticus*) (Jiang et al., 2018), and grass carp (*Ctenopharyngodon idella*) (Zhou et al., 2018).

The liver has long been acknowledged as one of the essential metabolic organs and is extensively involved in maintaining normal energy metabolism in the body through sophisticated processes, such as lipid digestion, glucose homeostasis, and antioxidant defense (McBride and Kelly, 1990; Li et al., 2012; Wu et al., 2013; Xie et al., 2016). Further, the liver is the major lymphoid organ containing many innate immune effectors that undertake immune defense when exposed to infectious diseases in teleost (Zapata et al., 2006). Thus, a good physiological status of the liver contributes to building the hepatic antioxidant and immune defense system, which is significantly important for health maintenance.

In view of these perspectives, it is a salient interest point to reveal the beneficial effects of BAs on the fish hepatic status by dietary supplementation. However, studies focused on this field are still scarce in aquaculture species, especially in flatfishes. Tongue sole (*Cynoglossus semilaevis*) is an important flatfish that is of high economic values and widely cultured in coastal areas in China (Li et al., 2020; Li et al., 2021b; Li et al., 2021c; Li et al., 2021d). The objectives of this study were mainly focused on the effect of BA administration on liver health. Numerous parameters, including growth performance, physiological and biochemical indices of the liver, and immune gene expression levels, were investigated by dietary BA inclusion through a 10-week feeding trial using tongue sole juveniles. This research sheds light on a potential strategy for improving hepatic health status in tongue sole.

METHODS AND MATERIALS

Experimental Diet Preparation

In this study, a diet formulated containing ~53% crude protein, ~8% crude fat, ~3% crude fiber, and ~16% crude ash was used as the basal diet in the control group (CT), while the other two experimental diets were formulated by adding 300 (BA1) and 900 mg/kg (BA2) of BAs (Longchang Group, China; purity 99%; containing 69.9% hyodeoxycholic acid, 18.9% chenodeoxycholic acid, and 7.8% hyocholic acid) to the basal diet. The additive contents of BAs were determined according to previous studies (Li et al., 2021a). The compound of BAs was extracted from viscera of animals like livestock and poultry by our group. The experimental diets were commercially manufactured by our partner (Sangtong Bio-engineering (Weifang) Co. Ltd, Weifang, China), and the main ingredients and proximate compositions of the basal diet are as described in previous studies (Li et al., 2021e).

Feeding Trial

Juvenile tongue sole fish were obtained from a flatfish aquaculture company in Tangshan, China. After a 15-day acclimation (fed with a control diet), fish were randomly distributed to nine rearing tanks (1 m^3), each tank has 180 (N_0) individuals, and each group has three triplicate tanks. Then fish were hand-fed to apparent satiation with an amount of 1%–2% of wet body weight twice daily at 06:00 and 18:00 for 10 weeks. For each meal, in most cases, there were little feed residuals within 0.5 h after feeding, but residuals would be eaten up in the extended 0.5 h. The daily amount was fine-tuned weekly according to the total wet weight of the fish in each tank. Throughout the whole experimental period, the physicochemical parameters of water were as follows: temperature 22.5°C–23.5°C, salinity 26‰–30‰, pH 7.6–8.3, ammonia nitrogen <0.06 mg/L, dissolved oxygen 6–8 mg/L, and daily water exchange rate 500%.

Growth Performance Measurements

At the beginning and end of the feeding trial, 30 individuals from each tank were randomly selected and measured after 24-h starvation to obtain the initial body weight (BWi) and final body weight (BWf). The survival fish (N_1) of each tank were counted at the same time. The weight gain rate (WGR), specific growth rate (SGR), hepatosomatic index (HSI), and survival rate (SR) are calculated as follows: $\mathrm{WGR} = \frac{BWf - BWi}{BWi} \times 100\,\%$; $\mathrm{HSI} = \frac{liver\ \ weight}{body\ \ weight} \times 100\,\%$; $\mathrm{SGR} = (e^{\frac{\log_e(BWf) - \log_e(BWi)}{days}} - 1) \times 100$; $\mathrm{SR} = \frac{N1}{N0} \times 100\,\%$. Herein, the BWi is the average of the initially measured body weight of 30 individuals.

Sample Collection

At the end of the feeding trial, fish were fasted for 24 h, and then 10 fish from each tank were randomly selected and anesthetized with MS-222 (30 mg/L) for sampling. Briefly, the body weight of 10 fish from each tank was individually measured, and then the whole liver of each fish was dissected and measured for weight promptly. After that, three of these liver samples from each tank were divided in half, separately pooled in 2 ml of sterilized RNase-free tubes, immediately frozen in liquid nitrogen, and then stored at −80°C for later use. All the scientific activities were performed in accordance with the protocols and guidelines approved by the Care and Use of Laboratory Animals of Shandong Longchang Animal Health Product Co., Ltd.

Physiological and Biochemical Indices in Liver

Four hepatic function indices, five lipid metabolism parameters in the liver, five antioxidant parameters in the liver, and three digestive enzyme activity indices in the liver were assayed by commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The detailed parameters are listed in **Table 1**.

RNA Isolation and Quantitative Real-Time PCR Analysis

For detecting the immune responses in the liver, the expression levels of three immune-related genes (i.e., IL-8, TGF-β1, and TNF-α) and three growth-related genes (insulin-like growth

TABLE 1 | The assayed hepatic parameters in this study.

Item	Index (activity or content)	Version
Hepatic function	Urea nitrogen (UN)	C013-2-1
	Albumin (ALB)	A028-2-1
	Alanine transaminase (ALT)	C009-2-1
	Aspartate aminotransferase (AST)	C010-2-1
Antioxidant index	Superoxide dismutase (SOD)	A001-3-2
	Catalase (CAT)	A007-2-1
	Total antioxidant capacity (TAOC)	A015-2-1
	Glutathione peroxidase (GSH-PX)	H545-1-2
	Malondialdehyde (MDA)	A003-3-1
Lipid metabolism	Total bile acid (TBA)	E003-2-1
	Triacylglycerol (TG)	A110-1-1
	Total cholesterol (TC)	A111-1-1
	High-density lipoprotein cholesterol (HDL-C)	A112-1-1
	Low-density lipoprotein cholesterol (LDL-C)	A113-1-1
Digestive enzyme	Protease	A080-2-2
	Lipase	A054-1-1
	Amylase	C016-2-1

factor (IGF-I), FAS, and G6PD) were analyzed by qRT-PCR. Total RNA of liver samples was extracted using TRIzol Reagent (Takara, Maebashi, Japan) according to the manufacturer's instructions. The RNA quality and quantity were determined by agarose gel electrophoresis (1.5%) and spectrophotometric analysis (Nanodrop 2000, Thermo Fisher Scientific, Waltham, MA, USA). Then RNA samples were reversely transcribed into complementary DNA for qRT-PCR analysis using Servicebio® RT reagent kit with gDNA Eraser (Servicebio, Wuhan, China). Primers were designed by Premier 5.0 and listed in **Table 2**. β-Actin gene has been validated as a stable internal reference gene and is widely used in various tissue samples of tongue sole (Li et al., 2010). The qRT-PCR amplification was performed based on QuantStudio™ 5 system (Thermo Scientific, USA). The detailed reaction system and programs were described by Wei et al. (2019). The gene expression levels were normalized by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), and the CT was used as the reference group.

Statistical Analysis

Data were shown as mean ± SE. Data expressed in percentage were arcsine square-root transformed and followed by one-way ANOVA to determine significant differences in various parameter indices in R software (v4.1.0). Tukey's test was used for significance comparison between different dietary groups. The significance level was set as $p < 0.05$.

RESULTS

Growth, Hepatosomatic Index, and Survival

The growth performance parameters, HSI, and survival rate are presented in **Table 3**. The inclusion of BAs in diets substantially enhanced the growth performance of tongue sole; parameters including BWf, WGR, and SGR were significantly improved in both BA supplementary groups, especially in the high addition group ($p < 0.05$). In the BA1 group, the HSI increased slightly ($p > 0.05$), while in the BA2 group, it was significantly improved as compared to both the CT and BA1 groups. For SR, a significant improvement was observed only in the BA2 group ($p < 0.05$).

Hepatic Function Index

Effects of dietary BAs on hepatic function indices are shown in **Table 4**. The urea nitrogen (UN) content in the liver was only significantly increased in the BA2 group ($p < 0.05$). Compared with the CT group, the albumin (ALB) content in the liver was significantly improved in both dietary BA supplementation groups ($p < 0.05$). However, when BAs were added to the basal diet, the hepatic alanine transaminase (ALT) activity decreased dramatically ($p < 0.05$), where the value was the lowest in the BA1 group, while for aspartate aminotransferase (AST) activity, it significantly declined ($p < 0.05$) when 300 mg/kg of BAs was added to the basal diet (BA1) and further slightly declined ($p > 0.05$) when 900 mg/kg of BAs was added (BA2).

Lipid Metabolism in Liver

Five lipid metabolism-related parameters in the liver are summarized in **Table 5**. The content of total bile acid (TBA)

TABLE 3 | Growth performance, hepatosomatic index, and survival of tongue sole fed diets supplemented with bile acids for 10 weeks.

Group	CT	BA1	BA2
BWi (g)	13.02 ± 2.38	13.12 ± 2.40	13.15 ± 2.40
BWf (g)	52.05 ± 2.09^{c}	60.40 ± 2.65^{b}	71.34 ± 2.25^{a}
WGR (%)	299.80 ± 10.57^{c}	360.39 ± 27.75^{b}	442.51 ± 12.15^{a}
SGR	2.00 ± 0.04^{c}	2.20 ± 0.09^{b}	2.44 ± 0.03^{a}
HSI (%)	1.01 ± 0.04^{b}	1.03 ± 0.03^{b}	1.15 ± 0.05^{a}
SR (%)	92.18 ± 1.07^{b}	93.34 ± 1.89^{ab}	96.64 ± 0.44^{a}

Data are expressed as mean ± SE (n = 3 for SR, n = 10 for HSI, and n = 90 for others). BWi initial body weight; BWf, final body weight; WGR, weight gain rate; SGR, specific growth rate; HSI, hepatosomatic index; SR, survival rate. CT, basal diet; BA1 and BA2, bile acids were supplemented at the level of 300 and 900 mg/kg of basal diet.

^{a,b,c}Values with different superscript letters in the same row show significant differences ($p < 0.05$).

TABLE 2 | Primers used in quantitative real-time PCR (qRT-PCR).

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product length	Accession no.
β-Actin	CCAACAGGGAAAAGATGACC	TTCTCCTTGATGTCACGCAC	304	NM_001308179
IL-8	CTGAAGGAATGAGCCTGAGAAGC	TCACTTTCTTCACCCAAGGAGC	207	XM_025058206.1
TGF-β1	GATGTGACTGAGACCCTGCAGAAC	TCCCAAAAGAGACCCCAGAGAT	142	XM_008308810.3
TNF-α	TGTGAGAGCGGCCATTCATT	GGAACGACACCTGGCTGTAA	173	XM_008331806.3
IGF-I	TGGGATGTTCTCAAGAGTGCG	GGTTTGCTGAAATAAAAGCCTCTC	182	NM_001294198.1
FAS	GCTGCTCAAGCCAAACACCT	CCCTGCTCTTTGTAGCCGTCT	210	XM_008314383.3
G6PD	TGCTCCCAGACAACACCTACTTT	ACAATCACTCTGTTCCAGCCTCT	328	XM_008318150.3

TABLE 4 | Effects of dietary bile acids on hepatic function indices of tongue sole.

Group	CT	BA1	BA2
UN (pmol/mg prot)	0.97 ± 0.03^{b}	1.10 ± 0.02^{b}	1.75 ± 0.01^{a}
ALB (μg/mg prot)	0.98 ± 0.01^{b}	1.46 ± 0.01^{a}	1.42 ± 0.02^{a}
ALT (U/g prot)	191.28 ± 1.57^{a}	152.08 ± 0.89^{c}	171.58 ± 2.03^{b}
AST (U/g prot)	698.99 ± 4.00^{a}	507.74 ± 4.61^{b}	490.29 ± 2.95^{b}

Data are expressed as mean ± SE (n = 3).
CT, basal diet; BA1 and BA2, bile acids were supplemented at the level of 300 and 900 mg/kg of basal diet; UN, urea nitrogen; ALB, albumin; ALT, alanine transaminase; AST, aspartate aminotransferase.
^{a,b,c}Values with different superscript letters in the same row show significant differences ($p < 0.05$).

TABLE 5 | Effects of dietary bile acids on lipid metabolism in the liver in tongue sole.

Group	CT	BA1	BA2
TBA (μmol/g prot)	122.78 ± 0.73^{a}	96.09 ± 0.28^{b}	124.15 ± 1.73^{a}
TC (μmol/prot mg)	4.17 ± 0.04^{a}	2.24 ± 0.01^{c}	3.21 ± 0.04^{b}
TG (μmol/mg prot)	0.94 ± 0.01^{c}	1.01 ± 0.01^{b}	1.14 ± 0.02^{a}
LDL-C (μmol/mg prot)	1.46 ± 0.01^{a}	1.22 ± 0.00^{b}	1.49 ± 0.03^{a}
HDL-C (μmol/mg prot)	0.92 ± 0.00^{c}	1.36 ± 0.01^{b}	1.64 ± 0.02^{a}

Data are expressed as mean ± SE (n = 3).
CT, basal diet; BA1 and BA2, bile acids were supplemented at the level of 300 and 900 mg/kg of basal diet; TBA, total bile acid; TG, triacylglycerol; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.
^{a,b,c}Values with different superscript letters in the same row show significant differences ($p < 0.05$).

TABLE 6 | Effects of dietary bile acids on the activity levels of protease, lipase, and amylase in liver in tongue sole.

Group	CT	BA1	BA2
Protease (U/mg prot)	4.00 ± 0.05^{a}	4.83 ± 0.05^{b}	6.18 ± 0.04^{c}
Lipase (mU/g prot)	96.69 ± 1.74^{a}	120.01 ± 1.11^{b}	162.38 ± 0.68^{c}
Amylase (U/g prot)	6.71 ± 0.04^{a}	10.72 ± 0.09^{b}	15.39 ± 0.21^{c}

Data are expressed as mean ± SE (n = 3).
CT, basal diet; BA1 and BA2, bile acids were supplemented at the level of 300 and 900 mg/kg of basal diet.
^{a,b,c}Values with different superscript letters in the same row show significant differences ($p < 0.05$).

TABLE 7 | Effects of dietary bile acids on liver antioxidant capacity in tongue sole.

Group	CT	BA1	BA2
SOD (U/μg prot)	0.424 ± 0.003^{b}	0.426 ± 0.002^{b}	0.633 ± 0.007^{a}
CAT (U/mg prot)	124.45 ± 1.29^{a}	93.43 ± 0.37^{b}	84.13 ± 1.30^{c}
TAOC (ng/g prot)	283.43 ± 1.40^{a}	249.23 ± 2.04^{b}	194.48 ± 3.58^{c}
GSH-PX (mU/g prot)	289.70 ± 1.07^{b}	215.83 ± 1.04^{c}	379.61 ± 5.61^{a}
MDA (pmol/μg prot)	50.28 ± 0.87^{a}	37.28 ± 0.26^{b}	22.75 ± 0.32^{c}

Data are expressed as mean ± SE (n = 3).
CT, basal diet; BA1 and BA2, bile acids were supplemented at the level of 300 and 900 mg/kg of basal diet; SOD, superoxide dismutase; CAT, catalase; TAOC, total antioxidant capacity; GSH-PX, glutathione peroxidase; MDA, malondialdehyde.
^{a,b,c}Values with different superscript letters in the same row show significant differences ($p < 0.05$).

declined significantly ($p < 0.05$) when a low amount of BA (300 mg/kg, BA1) was added to the basal diet, while the addition level of BAs increased to 900 mg/kg basal diet (BA2), and it went up the same level of the CT group. The content of total cholesterol (TC) was significantly reduced ($p < 0.05$) in both BA supplementary groups, where it was significantly lower in the BA1 group than in the BA2 group ($p < 0.05$). The contents of TG and HDL-C in the liver were significantly gradually improved with the increase of BAs in the basal diet ($p < 0.05$). With the increase of the BA addition, the content of LDL-C in the liver showed first a significant decrease (BA1), followed by a significant rise (BA2) close to the level in the CT group.

Digestive Enzyme Activity in Liver

The activities of protease, lipase, and amylase in the liver were all gradually significantly boosted with the increase of BA inclusion levels ($p < 0.05$) (**Table 6**).

Antioxidant Status in Liver

The antioxidative parameters in the liver are listed in **Table 7**. The significantly improved superoxide dismutase (SOD) activity ($p < 0.05$) was only observed in high BA addition groups (BA2), and the improvement in the BA1 group was negligible. Catalase (CAT) activity and total antioxidant capacity (TAOC) content in the liver significantly declined when 300 mg/kg of BAs was added to the basal diet (BA1), and the values were further significantly reduced in the 600 mg/kg supplementary group (BA2) ($p < 0.05$). The activity of GSH-PX showed a significant decline in the low BA supplementation group (BA1) compared to the control group ($p < 0.05$) but underwent a marked improvement in the BA2 group compared to the other two groups ($p < 0.05$). The content of malondialdehyde (MDA) in the liver was significantly cut down with the increase of dietary BA supplementation ($p < 0.05$).

Growth and Immune-Related Gene Expression in Liver

Growth and immune gene expression levels in the liver are illustrated in **Figure 1**. As shown in **Figure 1**, the mRNA expression level of G6PD was significantly downregulated ($p < 0.05$) regardless of low

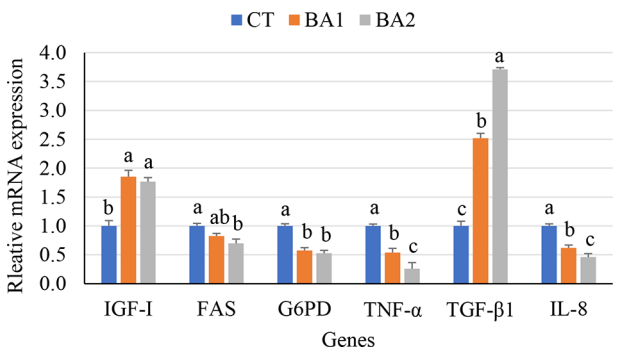

FIGURE 1 | Growth and immune-related gene expression in the liver of tongue sole fed a diet of BAs for 10 weeks. ^{a,b,c}Values with different superscript letters in the same row show significant differences ($p < 0.05$). CT, basal diet; BA1 and BA2, bile acids were supplemented at the level of 300 and 900 mg/kg of basal diet.

or high BA administration levels; the expression level of FAS was dramatically downregulated in the BA2 group ($p < 0.05$), while in the BA1 group, it showed an intermediate value ($p > 0.05$); however, dietary BAs (both the BA1 and BA2 groups) significantly increased the transcription of IGF-I ($p < 0.05$) when BAs were supplemented in diets. With the increase of supplementation of BAs in the diet, the hepatic mRNA expression levels of IL-8 and TNF-α were significantly downregulated ($p < 0.05$), while the expression level of TGF-β1 showed the opposite trend, which is significantly upregulated in both BA inclusion groups compared to the CT group ($p < 0.05$) (**Figure 1**).

DISCUSSION

Dietary Bile Acid Supplementation Is an Alternative Strategy to Boost Tongue Sole Farming

As previously mentioned, tongue sole is an important aquaculture sector of flatfish production in China. Due to its high nutritional value, tongue sole is one of the most expensive aquaculture seafood in China. However, to a large extent, the industrialization production of tongue sole was limited by its susceptibility to bacterial diseases usually resulting in a low survival rate under commercial rearing environments. Therefore, improving fish welfare to ensure high productivity by health management is of the highest priority. In this regard, genetic improvement toward disease resistance has been extensively studied by selective breeding geneticists with effort (Li et al., 2019; Hu et al., 2020; Li et al., 2020; Song et al., 2020; Lu et al., 2021). Furthermore, in the long term, improving tongue sole healthy status by functional feed additives (e.g., BAs) inclusion is a practical and effective strategy to produce fine crops.

In China, tongue sole feed has been well formulated and is commercially produced by several renowned manufacturers. In this study, to improve the formulation of tongue sole feeds and provide first-hand data to feed manufacturers, we conducted this fundamental research. Basically, these results will further boost the tongue sole aquaculture industry in China. Overall, according to our results, firstly, the growth performance of tongue sole was substantially improved, as well as the survival rate, although the experimental period is relatively short (only 10 weeks). These results were consistent with those of previous studies on other aquaculture species (Jiang et al., 2018; Zhou et al., 2018; Ding et al., 2020; Guo et al., 2020). Therefore, an extensive investigation of the beneficial effects of BAs on the hepatic health status of tongue sole is of significance for future health management.

Beneficial Effects of Bile Acid Inclusion on Hepatic Function

The studied hepatic function indices were significantly enhanced or declined with favorable trends. For example, ALT and AST as the main indicators for evaluating hepatic function showed a notable decrease, which implies an improvement status of the liver because the reasonable decline of both parameters indicates a mitigated liver degeneration and injury (Akrami et al., 2015). Further, the abnormal fluctuations of lipid metabolism indices (e.g., triacylglycerol (TG), TC, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C)) are considered underlying biomarkers of liver damage (Chrostek et al., 2013; Helkin et al., 2016). Thus, the results of assayed lipid metabolism parameters in this study may also imply the ability of BAs to improve lipid metabolism status in tongue sole. However, the increased or decreased contents of lipid metabolism parameters in this study are not exactly the same as in previous studies, which may be discussed case by case because the species and experimental designs are quite different.

Moreover, the antioxidant enzymes are of significance to protect cells and tissues from peroxidation, and they are important indicators that can reflect growth performance and the health status of the body (Mate, 2000; Jin et al., 2017). In this study, significantly enhanced activities of SOD and GSH-PX and lower contents of MDA in the liver were observed when BAs were included in the basal diet, which agrees with previous reports (Jin et al., 2019; Ding et al., 2020). On the contrary, the CAT activity and TAOC content were significantly declined when BAs were added, which are incompatible with the other three studied antioxidant parameters, whereas relevant research about the effect of dietary BAs on liver antioxidant capability is very limited in other aquaculture species. Intensive studies focused on the mechanism of liver antioxidants regulated by dietary BA inclusion are needed.

Beneficial Effects of Bile Acid Inclusion on Digestive Ability and Immune Response

As we previously mentioned in the *Introduction* section, the liver is an important organ that plays key roles in food digestion and immune response. In the present study, the activities of protease, lipase, and amylase in the liver were significantly improved in both low and high BA supplementation levels. To a large extent, these findings are in accordance with previous studies (Adhami et al., 2017; Zhou et al., 2018; Ding et al., 2020). Thus, the increased digestive enzyme activity may be a rational explanation for the healthy growth performance of tongue sole. This was corroborated by the upregulation of IGF-I with BA administration. Moreover, interestingly, the other two hepatic fatty acid regulatory enzymes (G6PD and FAS) were downregulated. Previous studies reported that the expression level of FAS was positively correlated with TC content in the body (Semenkovich, 1997; Leng et al., 2012). As indicated in this study, the hepatic TC content and FAS expression shared a consistent downtrend. The substantially decreased expression levels of FAS and G6PD may result in the decelerated accumulation of lipid in the liver and may also imply a reduced risk of fatty liver disease.

TGF-β1, TNF-α, and IL-8 are major cytokines extensively involved in pro-inflammatory reactions, innate immune responses, and wound repair processes (Secombes et al., 2001; Willuweit et al., 2001; Moldoveanu et al., 2009; Atiba et al., 2011; Cho et al., 2011), where the higher expression level of TGF-β1 means an accelerated

wound healing and reduced tissue damage, and the lower expression levels of TNF-α and IL-8 indicate the decreased inflammatory responses. In this study, the expression level of TGF-β1 in the liver was significantly upregulated, and the expression levels of TNF-α and IL-8 were significantly downregulated with dietary BA addition. These findings were consistent with those in tilapia when guanidinoacetic acid was included in the diets (Aziza et al., 2020).

CONCLUSION

This study concludes that dietary BA supplementation in tongue sole markedly improved the health status of the liver. The physiological and biochemical indices closely related to growth, lipid metabolism, immune, and antioxidants were markedly improved. The analytical results revealed well energy metabolisms and immune responses in the liver and finally explained the prominently improved growth performance and survival rate. This study provides a wider spectrum of the beneficial effects of dietary BAs on liver health in tongue sole.

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/s.

ETHICS STATEMENT

The animal study was reviewed and approved by The Care and Use of Laboratory Animals of Shandong Longchang Animal Health Product Co., Ltd.

AUTHOR CONTRIBUTIONS

SW: methodology, conceptualization, and writing—original draft preparation. WZ: methodology, data curation, visualization, validation, and writing—original draft preparation. AC: visualization and investigation. ZP: supervision, resources, investigation, and writing—reviewing and editing. TL: supervision and writing—reviewing and editing. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Effects of Soluble and Insoluble Non-Starch Polysaccharides on Growth Performance, Digestive Enzyme Activity, Antioxidant Capacity, and Intestinal Flora of Juvenile Genetic of Improvement of Farmed Tilapia (*Oreochromis niloticus*)

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This study aimed to evaluate the effects of soluble and insoluble non-starch polysaccharides (NSPs) on growth performance, digestive enzyme activity, antioxidant capacity, and intestinal flora of juvenile GIFT tilapia (*Oreochromis niloticus*). Four isonitrogenous and isolipidic experimental diets were formulated, including the FM diet (0% NSPs, FM), INSP diet (5.8% insoluble NSPs, INSP), SNSP diet (12.2% soluble NSPs, SNSP), and NSP diet (5.8% INSP and 12.2% SNSP, NSPs). Each diet was fed to triplicate groups of 40 fish (2.14 ± 0.04 g) per repetition for 10 weeks. Results showed that dietary supplementation with different types of NSPs significantly affected the digestive enzyme activity, antioxidant capacity, and intestinal flora of juvenile GIFT tilapia. Specifically, dietary supplementation with INSP significantly increased pepsin and intestinal lipase activity, while dietary supplementation with SNSP significantly decreased intestinal lipase activity and resulted in a significant decrease in plasma catalase, glutathione peroxidase, and total antioxidant capacity activity. Meanwhile, dietary supplementation with SNSP significantly increased the concentration of urea nitrogen and high-density lipoprotein cholesterol in plasma and alanine aminotransferase activity in plasma and hepatic. Moreover, LEfSe analysis showed that dietary supplementation with SNSP significantly increased the abundance of *Bacteroidales*, *Bacteroidia*, and *Cellulosilyticum*, while dietary supplementation with NSPs significantly decreased the abundance of *Mycobacteriaceae*, *Mycobacterium_neoaurum*, *Corynebacteriales*, and *Mycobacterium*. These results suggest that dietary INSP is an inert ingredient with limited effect, while dietary SNSP inhabited antioxidant capacity, induced liver damage, and altered the abundance of anaerobic/aerobic bacteria in the intestine of GIFT tilapia.

Keywords: non-starch polysaccharides, GIFT tilapia, antioxidant capacity, growth performance, intestinal flora

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INTRODUCTION

Fish meal (FM) is widely used in aquafeed production as an ideal protein source due to its good palatability and high nutrition value (FAO, 2014; Deng et al., 2017). In recent years, the limited supply of FM has made it increasingly expensive (Zhang et al., 2019), leading to a rapid increase in aquafeed production costs. Feed inputs have been reported to account for more than 50% of production costs in intensive aquaculture, which severely limits aquaculture development (Medard et al., 2018; Yannis et al., 2018). Hence, it is essential to find suitable protein sources to replace FM for the sustainable development of aquaculture (Li et al., 2006; Tacon et al., 2010; Altan and Korkut, 2011). Currently, conventional plant protein sources (PPS) have become the main alternative sources of FM with the advantages of relatively abundant supplementation, lower price, and stable quality (Deng et al., 2015; Zhang et al., 2018). However, PPS has an unbalanced amino acid profile, lacks essential nutrients, and carries anti-nutritional factors (ANFs) (Lee et al., 2010; Gao et al., 2018), making it extremely difficult to guarantee the normal growth and health of fish when FM is largely or completely replaced by PPS (Sales, 2009; Collins et al., 2013). Several studies have confirmed that ANFs disrupt intestine and liver morphology and function, upregulate inflammation-related gene expression, alter intestinal flora structure, and ultimately reduce fish feeding rate, nutrient digestibility, feed conversion rate, and growth performance (Merrifield et al., 2009; Mansfield et al., 2010; Gao et al., 2011; Król et al., 2016) Also, an excessive addition level of PPS even induced enteritis in fish (Refstie et al., 2000; He et al., 2020). Hence, ANFs are considered as the main factors restricting the use of PPS in aquafeed (Collins et al., 2013; Azeredo et al., 2017).

As the main components of plant cell walls, non-starch polysaccharides (NSPs) are highly abundant in PPS (Ma et al., 2017), which reach 10-70% of plant ingredients (varies with plant species) (Choct, 2015). Consequently, increasing the use of PPS ultimately significantly increases the content of NSPs in aquafeed (Deng et al., 2021). NSPs are indigestible carbohydrates for fish due to the scarcity or absence of endogenous NSPs-degrading enzymes in monogastric animals (including fish) (Kuz Mina, 1996), and thus NSPs are generally considered as a low- or non-nutritional feed ingredient for fish (Sinha et al., 2011). Indeed, although some components of NSPs have been observed to have beneficial effects on fish [e.g., β-glucan enhancing non-specific cellular defense mechanisms and reducing inflammation on fish (Kumar et al., 2005; Suchecka et al., 2015)], dietary NSPs are still generally considered to be one of the major anti-nutritional components of the fish diets. As previous studies reported, dietary NSPs tend to reduce the absorption of feed nutrients (Amirkolaie et al., 2005; Kraugerud et al., 2007; Glencross, 2009; Lekva et al., 2010; Glencross et al., 2012), and dietary inclusion of a high level of NSPs even causes liver lesions in fish (Ren et al., 2020). Specifically, NSPs can be divided into insoluble NSPs (INSP) and soluble NSPs (SNSP) according to solubility in neutral buffer. INSP is mainly cellulose, while SNSP consists of a range of hemicelluloses including arabinoxylan, β-glucan, mannans, and pectin, which have different physicochemical properties and thus produce different effects on fish (Dalsgaard et al., 2016; Deng et al., 2021). At present, limited studies have been conducted to evaluate the differential effects of dietary INSP and SNSP on fish (Glencross et al., 2012).

GIFT tilapia (Genetic improvement of farmed tilapia, *Oreochromis niloticus*), which is considered one of the most important aquaculture species in China, is widely cultivated due to its enormous advantages, such as fast growth, strong disease resistance and delicious meat quality (Jiang et al., 2014; Deng et al., 2020). As a typical omnivorous fish, tilapia has a high capacity to utilize dietary carbohydrates (Wilson, 1994). The digestibility of dietary NSPs ranging from 2.8% to 73% in tilapia, with an average of 24.3% (Amirkolaie et al., 2005; Haidar et al., 2016; Maas et al., 2020). Previous studies have focused on the effects of different NSP types on nutrient digestibility (Amirkolaie et al., 2005; Leenhouwers et al., 2007), while limited studies have been conducted to evaluate the effects of different NSP types on the physiology and intestinal flora of tilapia. Intestinal flora is an integral part of the organism (Bi et al., 2015), which extensively participates in the physiological processes of fish, including food digestion and absorption, nutrient metabolism, and immunity response (Dan et al., 2021). The structure and function of intestinal flora are influenced by dietary components (Du et al., 2018). NSPs are quantitatively the most important substrates for intestinal flora (Macfarlane and Macfarlane, 2002), which alters the structure and function of intestinal flora (Sinha et al., 2011). Several studies have confirmed that different dietary NSP sources have different effects on the intestinal flora of pigs, due to the different INSP/SNSP ratio of different dietary NSPs sources (Chen et al., 2013; Chen et al., 2014). This phenomenon may be associated with the inconsistent effects of dietary INSP and SNSP on the intestinal flora (Yang et al., 2020). However, studies on the effects of different types of NSPs on intestinal flora in fish are still lacking. Thus, this study evaluated the effects of different NSP types on growth performance, digestive enzyme activity, antioxidant capacity, and intestinal flora of juvenile GIFT tilapia.

MATERIALS AND METHODS

Experimental Diets

Fish meal was used as the primary protein source and soybean oil and soybean lecithin were the primary lipid sources used to prepare the basic feed (control diet, FM). By adding different types of NSPs to basic feed, four experimental diets were formulated to contain NSP levels of 0% NSPs, 5.8% INSP (cellulose), 12.2% SNSP (including 3.48% arabinoxylan, 0.82% β-glucan, 0.94% mannan and 6.96% pectin), and 18.0% NSPs (5.8% INSP and 12.2% SNSP), respectively. The inclusion levels of different types of NSPs were referred to in previous reports (Deng et al., 2021), based on their contents present in wheat bran and soybean meal as the sole protein and carbohydrate source for GIFT tilapia.

Each diet ingredient was ground into powder and passed through a 300-μm mesh (SFSP series, produced by Kunming Huaming grain, oil and feed equipment factory, Yunnan, China).

Soybean lecithin was first dissolved in soybean oil (mixed oil), then mixed with each ingredient evenly, and then 30% pure water was added to produce a stiff dough. The dough was pelleted using an extruder (KS-18, Produced by Jiangsu Jinggu rice machine Co. Ltd, Yunnan, China) through a 1.0-mm die. The moist pellets were dried at 40°C for 12 h, and then stored at -20°C until used. The formulation and proximate composition of experimental diets are shown in **Table 1** and the amino acid profiles of experimental diets are shown in **Supplementary Table 1**.

Fish, Facilities, and Breeding Trials

Juvenile GIFT tilapias were obtained from the Yunnan Xishuangbanna Aquatic Technology Promotion Station (Yunnan, China), and the experiment was completed in the aquaculture laboratory of Yunnan Agriculture University. Juvenile GIFT tilapia were placed in a net cage (0.9 m * 0.9 m * 1.0 m) and fed twice daily (08:00 and 17:00) with a commercial feed (provided by Tongwei Co. Ltd, China) for 2 weeks to acclimatize to the new cultural environment. Tilapias were fasted 24 h after 2 weeks of acclimatization, then weighed and grouped. A total of 480 healthy juvenile tilapia with uniform size (2.14 ± 0.04 g) were selected and randomly assigned to 12 net cages with 40 fish per cage (all net cages are placed in the same cement pond with a circulating water system and the culture water used was tap water with a flow rate of 100 L/min after aeration and dichlorination).The culture experiments lasted 10 weeks and were conducted under natural photoperiod (14 h light/10 h dark at the end of July and 12 h light/12 h dark at the beginning of October). The water temperature was maintained at 28 ± 1°C with NH_4^+-N < 0.02 mg/L and dissolved oxygen >5 mg/L. The test fish were fed twice daily (08:00 and 17:00) to apparent satiation and the feed consumption of each net cage was recorded during the experiment period.

Sample Collection

After the breeding experiment, fish were fasted for 24 h before collecting samples. The total number and weight of tilapia in each cage were checked and recorded to calculate weight gain rate (WGR), daily growth coefficient (DGC), metabolic body weight (MBW), feed intake (FI), feed conversion rate (FCR), and protein efficiency ratio (PER).

After counting and weighing, fish were anesthetized with eugenol (1: 12000, Macklin, Shanghai, China) and then randomly selected for sampling. Five fish were taken from each cage, stored at -20°C for measure the proximate composition of the whole-body. The dorsal muscles of another three fish were taken from each cage and placed in sealed bags, stored at -20°C immediately for amino acid profiles analysis. Blood from the tail vein of ten fish per cage was half drawn with a 1-ml pretreated syringe (moistened by heparin sodium) and the rest drawn using a normal 1-ml syringe. Blood drawn by pretreated syringe were collected in heparin sodium anti-coagulation centrifuge tubes and stored at 4°C for 3 h and centrifuged at 4000 rpm/min for

TABLE 1 | Formulation and proximate composition of the experimental diets for GIFT tilapia.

Items	FM	INSP	SNSP	NSP
Ingredients, % of the diet				
Fish meal[a]	37.00	37.00	37.00	37.00
Soybean oil	1.00	1.00	1.00	1.00
Soybean lecithin	0.50	0.50	0.50	0.50
Wheat meal[a]	28.38	28.38	28.38	28.38
Wheat bran[a]	12.00	12.00	12.00	12.00
INSP (cellulose)[b]	0.00	5.80	0.00	5.80
SNSP[c]	0.00	0.00	12.20	12.20
α-starch	18.00	12.20	5.80	0.00
$Ca(H_2PO_4)_2$	1.20	1.20	1.20	1.20
Choline chloride (50%)[a]	0.30	0.30	0.30	0.30
NaCl	0.20	0.20	0.20	0.20
Vitamin C[d]	0. 02	0. 02	0. 02	0. 02
Vitamin premix[e]	1.00	1.00	1.00	1.00
Mineral premix[f]	0.40	0.40	0.40	0.40
Proximate composition, % on a dry basis (Measured value)				
Dry matter	95.29	95.16	94.38	94.21
Crude protein	33.01	33.28	33.12	33.10
Crude lipid	4.78	4.89	5.01	4.72
Ash	9.97	9.65	9.56	9.39
Starch + sugar	39.27	34.06	28.45	23.80
Total NSPs[g]	12.97	18.12	23.86	28.99
Gross energy (KJ g^{-1})	19.85	19.89	19.99	20.05

[a]*Supplied by Kunming Tianyuan Feed Co., Ltd. (Kunming, China); fish meal, 66.3% crude protein, 15.8% crude lipid.*

[b]*Supplied by Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China).*

[c]*SNSP (soluble non-starch polysaccharide) including 3.48% arabinoxylan, 0.82% β-glucan, 0.94% mannan, and 6.96% pectin; supplied by Shanghai ZZBIO Co., Ltd. (Shanghai, China).*

[d]*L-Ascorbate-2-polyphosphate (35%), supplied by Galaxy Chemicals Co., Ltd. (Wuhan, China).*

[e]*Vitamin premix (g/kg mixture): retinyl acetate (2800000 IU/g), 2; cholecalciferol, 0.03; DL-α-tocopheryl acetate, 30; menadione, 3; thiamine hydrochloride, 8; riboflavin, 11; pyridoxine hydrochloride, 8; vitamin B_{12}, 0.02; ascorbic acid, 50; folic acid, 1; biotin, 0.1; niacin, 30; calcium D-pantothenate, 32; inositol, 25.*

[f]*Mineral premix (g/kg mixture): $MgSO_4 \bullet 7H_2O$, 180; KI, 1; $FeSO_4 \bullet H_2O$, 260; $ZnSO_4 \bullet H_2O$, 180; $CuSO_4 \bullet 5H_2O$, 25; $Na_2Se_2O_3$, 0.01; $MnSO_4 \bullet H_2O$, 180; $CoCl_2 \bullet 6H_2O$, 0.75.*

[g]*Total NSPs (%) = 100 – [crude protein + crude lipid + ash + (starch + sugar)].*

10 min to obtain plasma samples, while blood drawn by normal syringe was transferred to 1.5 mL Eppendorf tubes and stored at 4°C for 12 h, and then centrifuged at 3000 rpm/min for 10 min at 4°C to prepare serum samples. Both plasma and serum samples were stored at -80°C immediately for subsequent analysis. Another four fish per cage were dissected, and the liver, stomach, and intestine were harvested and stored at -80°C until used. Four fish were randomly selected after drawing blood and sterile dissected and the hindgut was quickly gathered to avoid pollution. Four mixed samples were prepared in each group, with each mixed sample consisting of three separate hindguts (one fish per tank in the same group was provided). The mixed samples were quickly frozen in the liquid nitrogen tank and transferred to an ultra-low-temperature refrigerator immediately, stored at -80°C for subsequent analysis.

Sample Analysis Strategy

Proximate Composition Analysis

The proximate composition of experimental diets and whole-body were analyzed by using the standard of AOAC (AOAC, 2005). The sample was dried to a constant weight at 105°C to determine moisture content and combusted at 550°C for 6 h to determine the ash content. The crude protein content was determined using the Kjeldahl method (N*6.25), the crude lipid content was estimated using the chloroform-methanol method. Starch + sugar in each experimental diets was determined by an enzymatic digestion method according to a previous study (Staessen et al., 2020).

Amino Acid Profiles of Diets and Muscles

The amino acid profiles of diets and muscles were measured by an automatic amino acid analyzer (Hitachi L-8800, Japan). All samples were freeze-dried at low temperature and the weight of each tested sample was in the range of 50 to 200 mg (accuracy is 0.01mg). The contents of amino acids were analyzed after the sample being hydrolyzed with HCl (6 mol/L) at 110°C for 24 h.

Biochemical Indicators

The content of total amino acids (TAA), total protein (TP), blood urea nitrogen (BUN), ammonia nitrogen (AN) and malondialdehyde (MDA), and the activity of γ-glutamyl transferase (GGT), alanine aminotransferase (ALT), aspartate aminotransferase (AST), superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), glutathione peroxidase (GPx), and total antioxidant capacity (TAC) in plasma were measured by commercially available kits (Nanjing Jiancheng Bioengineering Institute Co. Ltd, Nanjing, China). Hepatic GGT, ALT, AST, SOD, CAT, POD, GPx activity, and MDA content were measured following the method used in plasma. The contents of total cholesterol (TC), free cholesterol (FC), cholesterol ester (CE), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and triglyceride (TG) were also measured using commercially available kits (Nanjing Jiancheng Bioengineering Institute Co. Ltd, Nanjing, China). The protein concentration of tissue samples (stomach, intestine, liver) was also determined using commercially available kits (Nanjing Jiancheng Bioengineering Institute Co. Ltd, Nanjing, China). Sample processing, reagent preparation, and determination processes were performed in strict accordance with the methods provided in the kit instructions.

Intestinal Flora Analysis

Intestinal flora DNA was extracted using the SDS method. The DNA samples that passed the quality test were used for further amplification and sequencing. The V3-V4 region of the 16S rRNA gene was amplified using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The detection and recovery of PCR products were performed by 0.2% agarose gel electrophoresis and MinElute Gel Extraction Kit (Qiagen, Germany), respectively. The prepared library was sequenced on the Hiseq2500 PE250 pla-tform (Illumina, USA). After removing barcodes and primers, FLASH was used to splice the reads of each sample to obtain the original data tags (raw Tags). The quality control of raw tags was carried out with reference to the QIIME tags quality control standard. Raw Tags were truncated at the first low mass base site where the base number of continuous low-quality values (default quality threshold ≤ 19) reached the set length (the default length value 3). In addition, tags with consecutive high-quality bases less than 75% of the tag length were excluded from further analysis. USEARCH 7.0 software was used to remove the chimeric sequence and the final effective tags were obtained for further analysis.

All effective tags of all samples were clustered and the sequences were clustered into Operational Taxonomic Units (OTUs) with 97% consistency by default using Uparse software. Taxonomic annotation analysis was obtained and the community composition of each sample was counted at each classification level using the Mothur method and the SSUrRNA database of Silva. QIIME was used to calculate α-diversity and R software was used to perform PCA analysis, species accumulation boxplot, rarefaction curve, and difference significance analysis of inter-group community structure. In addition, the Venn diagram was drawn based on the data processed by homogenization and LEfSe analysis was used to identify species with significant differences between the groups.

Calculations and Statistical Analysis

Growth performance and feed utilization were calculated by the following formulas:

$$\mathrm{WG}\ (\%) = 100 * (W_t - W_i) / W_i;$$

$$\mathrm{DGC}(\%/\mathrm{d}) = 100 * (W_t^{\frac{1}{3}} - W_i^{\frac{1}{3}})/t;$$

$$\mathrm{MBW}\ (\mathrm{kg}) = W_t^{\frac{3}{4}} / 1000;$$

$$\mathrm{FI}\ (\mathrm{g/kg\ MBW/d}) = W_f/(\mathrm{MBW} * t);$$

$$\mathrm{FCR} = W_f / (W_t - W_i);$$

$$\mathrm{PER} = (W_t - W_i)/W_p .$$

Where W_i and W_t (g) are mean initial and final body weight of the experimental fish, respectively; W_f and W_p (W_f * crude protein content of the diets, g) are mean dry feed and protein intake of the experimental fish, respectively; t represents the duration of the feeding experiment (days). All data are shown as means ± standard error of measurement (Means ± SEM) and analyzed by SPSS (v20.0) software (Chicago, IL, USA) for one-way variance (ANOVA). $P < 0.05$ indicates a significant difference between the groups, and in this case, Tukey's HSD test was used for multiple comparison analysis.

RESULTS

Growth Performance and Feed Utilization

Dietary supplementation with different types of NSPs had no significant effect on the WGR, DGC, FI, FCR, and PER of GIFT tilapia ($P > 0.05$; **Table 2**).

Digestive Enzyme Activity

Dietary supplementation with different types of NSPs significantly increased the pepsin activity of tilapias ($P < 0.05$). In addition, dietary supplementation with INSP significantly increased intestinal lipase activity, while dietary supplementation with SNSP significantly decreased intestinal lipase activity ($P < 0.05$; **Table 3**).

Serum Lipoprotein Contents

Dietary supplementation with different types of NSPs had no significant effect on serum TC, TAG, LDL-C, FC, CE contents, and LDL-C/HDL-C, FC/TC ratio ($P > 0.05$) and dietary supplementation with SNSP significantly increased serum HDL-C content ($P < 0.05$; **Table 4**).

Plasma and Hepatic Biochemical Indicators

Dietary supplementation with different types of NSPs had no significant effect on plasma TP, TAA, AN contents, hepatic AST, and GGT activity ($P > 0.05$). Dietary supplementation with SNSP or 18.0% NSPs significantly increased plasma BUN content, plasma, and hepatic ALT activity ($P < 0.05$). Conversely, dietary supplementation with INSP significantly decreased hepatic ALT activity ($P < 0.05$; **Table 5**).

Antioxidant Capacity

Dietary supplementation with different types of NSPs had no significant effect on plasma SOD, POD activity, and MDA content ($P > 0.05$) but significantly decreased plasma CAT activity ($P < 0.05$). Dietary supplementation with SNSP significantly decreased the activity of plasma GPx, TAC and hepatic SOD, CAT, POD, GPx and TAC, but dietary supplementation with INSP significantly increased hepatic CAT activity ($P < 0.05$). Moreover, dietary supplementation with 18.0% NSPs significantly decreased hepatic SOD and GPx activity ($P < 0.05$; **Table 6**).

Whole-Body Composition

Dietary supplementation with SNSP or 18.0% NSPs significantly decreased the crude protein and lipid content of the whole-body ($P < 0.05$). Moreover, dietary supplementation with 18.0% NSPs significantly increased the ash content of the whole-body ($P < 0.05$; **Table 7**).

TABLE 2 | Growth performance and feed utilization of GIFT tilapia fed diets with different types of non-starch polysaccharides.

Items	FM	INSP	SNSP	NSP
Initial body weight (g)	2.14 ± 0.04	2.14 ± 0.04	2.14 ± 0.04	2.14 ± 0.04
Final body weight (g)	63.64 ± 1.17^{ab}	64.35 ± 0.95^{b}	59.65 ± 0.66^{a}	60.13 ± 1.03^{ab}
Weight gain rate (%)	2875.40 ± 52.63^{ab}	2910.36 ± 46.54^{b}	2689.75 ± 30.24^{a}	2713.52 ± 45.15^{ab}
DGC (%/day)	4.84 ± 0.04^{ab}	4.86 ± 0.04^{b}	4.75 ± 0.02^{a}	4.76 ± 0.04^{ab}
Feed intake (g/kg MBW/day)	11.73 ± 0.11^{ab}	11.25 ± 0.10^{a}	11.81 ± 0.15^{b}	11.75 ± 0.04^{b}
Feed conversion rate	1.28 ± 0.01^{ab}	1.33 ± 0.01^{b}	1.25 ± 0.01^{a}	1.25 ± 0.01^{a}
Protein efficiency ratio	3.88 ± 0.06^{ab}	4.02 ± 0.04^{b}	3.75 ± 0.04^{a}	3.78 ± 0.02^{a}

Values are means ± SEM (n = 3). Means in the same row with different superscripts are significantly different from each other (P < 0.05). DGC, daily growth coefficient; MBW, metabolic body weight.

TABLE 3 | Digestive enzyme activities in GIFT tilapias fed diets with different types of non-starch polysaccharides.

Items	FM	INSP	SNSP	NSP
Stomach				
Pepsin (U/mg protein)	9.57 ± 0.15^{a}	11.14 ± 0.56^{b}	12.10 ± 0.28^{b}	11.01 ± 0.44^{b}
Intestine				
Trypsin (U/mg protein)	0.97 ± 0.09	0.98 ± 0.03	1.10 ± 0.11	1.22 ± 0.07
Lipase (U/g protein)	17.23 ± 0.47^{b}	26.63 ± 0.82^{c}	12.35 ± 0.73^{a}	17.90 ± 0.75^{b}
Amylase (U/mg protein)	0.19 ± 0.01	0.19 ± 0.02	0.21 ± 0.01	0.21 ± 0.01
Lactase (U/mg protein)	4.49 ± 0.36	4.97 ± 0.38	5.63 ± 0.79	5.23 ± 0.40

Values are means ± SEM (n = 3). Means in the same row with different superscripts are significantly different from each other (P < 0.05).

TABLE 4 | Serum lipoprotein profile of GIFT tilapia fed diets with different types of non-starch polysaccharides.

Items	FM	INSP	SNSP	NSP
TC (mmol/L)	4.22 ± 0.23^{ab}	3.67 ± 0.08^{a}	4.92 ± 0.26^{b}	4.11 ± 0.19^{a}
TAG (mmol/L)	1.23 ± 0.06	1.56 ± 0.09	1.23 ± 0.10	1.30 ± 0.15
HDL-C (mmol/L)	2.53 ± 0.03^{a}	2.38 ± 0.07^{a}	2.79 ± 0.07^{b}	2.31 ± 0.08^{a}
LDL-C (mmol/L)	1.45 ± 0.20^{ab}	0.98 ± 0.08^{a}	1.89 ± 0.22^{b}	1.54 ± 0.17^{ab}
LDL-C/HDL-C	0.57 ± 0.07^{ab}	0.41 ± 0.04^{a}	0.68 ± 0.08^{b}	0.67 ± 0.07^{b}
FC (mmol/L)	0.56 ± 0.11	0.54 ± 0.05	0.67 ± 0.09	0.44 ± 0.10
CE (mmol/L)	3.67 ± 0.32^{ab}	3.13 ± 0.12^{a}	4.25 ± 0.22^{b}	3.67 ± 0.19^{ab}
FC/TC	0.14 ± 0.04	0.15 ± 0.02	0.14 ± 0.02	0.11 ± 0.03

Values are means ± SEM (n = 3). Means in the same row with different superscripts are significantly different from each other (P < 0.05). TC, total cholesterol; TAG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; FC, free cholesterol; CE, cholesterol ester.

Muscle Amino Acid Profiles

The contents of muscular histidine, methionine, phenylalanine, threonine, valine, glycine, tyrosine, proline, cysteine, and EAA/NEAA ratio were not significantly affected by the experimental diets ($P > 0.05$). Dietary supplementation with 18.0% NSPs significantly decreased the contents of muscular arginine (Arg), essential amino acids (EAA), glutamate (Glu), aspartate (Asp), serine (Ser), non-essential amino acids (NEAA), and total amino acids (TAA) ($P < 0.05$; **Table 8**).

Intestinal Flora

A total of 978,800 effective tags were obtained from 16 samples, with an average of 61,175 per sample. The species accumulation boxplot and rarefaction curve indicate that the number of samples and tags were sufficient to identify almost all microbial species (**Figure 1**). Meanwhile, the Good's coverage indices of each sample was as high as 99%, which also indicated that the depth of sequencing was sufficient (**Table 9**). The Alpha diversity of intestinal flora was calculated by the Sobs, Shannon, Simpson, Chao1, ACE, and PD-whole-tree diversity indices and these results indicated that there was no significant difference in Alpha diversity of intestinal flora between groups ($P > 0.05$, **Table 9**). A total of 3,474 OTUs were obtained in all samples, of which 1,064 OTUs were shared with all four groups. The number of unique OTUs in the FM group, INSP group, SNSP group, and NSP group were 239, 262, 314, and 159, respectively (**Figure 2**).

Intestinal flora composition analysis showed that *Actinobacteria*, *Firmicutes*, *Planctomycetes*, *Proteobacteria*, *Fusobacteria*, *Chlamydiae*, *TM6*, and *Bacteroidetes* were the dominant phyla in all groups (**Figure 3A**). In the FM group, the four dominant phyla were *Firmicutes* (16.83%), *Proteobacteria* (15.11%), *Fusobacteria* (12.99%), and *TM6* (12.42%). In the SNSP group, the four dominant phyla were *Proteobacteria* (23.53%), *Fusobacteria* (19.96%), *Actinobacteria* (19.34%), and *Firmicutes* (16.27%). In the INSP group, *Proteobacteria* (22.58%) and *Planctomycetes* (15.87%) were the most abundant phyla, followed by *Fusobacteria* (14.94%) and *Firmicutes* (14.64%). In the NSP group, *Firmicutes* (39.56%), *Proteobacteria* (17.21%), *Fusobacterium* (9.78%), and *Planctomycetes* (7.96%) were the four dominant phyla.

Intestinal flora community structure at the genus level in different groups is shown in **Figure 3B**. In the FM group, the four dominant genera were *Cetobacterium* (12.84%), *Mycobacterium* (6.78%), *Pirellula* (6.16%), and *Alpinimonas* (2.98%). While in the SNSP group, *Cetobacterium* (18.99%), *Alpinimonas* (12.49%), *Bacteroides* (5.18%), and *Cellulosilytium* (2.27%) were the most prevalent genera. In the INSP group, the four dominant genera were successively *Cetobacterium* (15.66%), *Alpinimonas* (8.14%), *Bacteroides* (6.84%), and *Pirellula* (5.54%). *Turicibacter* (15.62%), *Cetobacterium* (9.71%), *Alpinimonas* (6.00%), and *Pirellula* (4.92%) were the most abundant genera in the NSP group. Moreover, the heatmap

TABLE 5 | Protein metabolism-related parameters in GIFT tilapia fed diets with different types of non-starch polysaccharides.

Items	FM	INSP	SNSP	NSP
Plasma				
Total protein (g/L)	34.52 ± 0.67	36.38 ± 0.77	33.28 ± 1.62	32.93 ± 1.33
TAA (mmol/L)	72.92 ± 5.51	78.13 ± 5.41	81.25 ± 7.22	81.25 ± 7.22
BUN (mmol/L)	6.42 ± 0.27^{a}	6.30 ± 0.21^{a}	7.35 ± 0.27^{b}	7.28 ± 0.27^{b}
AN (mmol/L)	0.25 ± 0.01	0.22 ± 0.01	0.22 ± 0.03	0.24 ± 0.01
AST (U/ml)	26.10 ± 3.09	23.27 ± 1.86	24.03 ± 2.30	20.09 ± 1.05
ALT (U/ml)	6.25 ± 0.39^{a}	6.54 ± 0.18^{ab}	7.79 ± 0.06^{c}	7.57 ± 0.32^{bc}
GGT (U/ml)	1.24 ± 0.16	1.24 ± 0.16	1.55 ± 0.31	1.24 ± 0.16
Liver				
AST (U/g protein)	11.27 ± 0.65	10.93 ± 1.05	10.94 ± 0.70	10.05 ± 0.97
ALT (U/g protein)	39.19 ± 3.10^{b}	26.05 ± 5.23^{a}	63.69 ± 7.66^{c}	50.81 ± 6.16^{b}
GGT (U/g protein)	0.91 ± 0.03	0.90 ± 0.04	1.04 ± 0.04	1.04 ± 0.04

Values are means ± SEM (n = 3). Means in the same row with different superscripts are significantly different from each other (P < 0.05). TAA, total amino acids; BUN, blood urea nitrogen; AN, ammonia nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, γ-glutamyl transpeptidase.

TABLE 6 | Antioxidant capacity of GIFT tilapia fed diets with different types of non-starch polysaccharides.

Items	FM	INSP	SNSP	NSP
Plasma				
SOD (U/ml)	14.35 ± 1.01	11.87 ± 0.58	13.78 ± 0.76	11.52 ± 0.70
CAT (U/ml)	23.42 ± 0.95^{b}	15.42 ± 2.23^{a}	15.84 ± 1.94^{a}	15.42 ± 1.49^{a}
POD (U/ml)	74.67 ± 2.23	79.70 ± 4.18	80.59 ± 1.57	81.48 ± 6.12
GPx (U/μl)	0.44 ± 0.01^{b}	0.47 ± 0.02^{b}	0.35 ± 0.01^{a}	0.42 ± 0.02^{ab}
TAC (U/ml)	8.02 ± 0.63^{b}	6.63 ± 0.55^{ab}	4.96 ± 0.43^{a}	6.31 ± 0.32^{ab}
MDA (nmol/L)	4.03 ± 0.37	3.95 ± 0.22	3.96 ± 0.21	4.03 ± 0.70
Liver				
SOD (U/mg protein)	4.48 ± 0.07^{b}	4.67 ± 0.09^{b}	3.83 ± 0.06^{a}	3.79 ± 0.11^{a}
CAT (U/mg protein)	12.20 ± 0.06^{b}	14.44 ± 0.07^{c}	9.41 ± 0.64^{a}	10.71 ± 0.25^{ab}
POD (U/mg protein)	1.34 ± 0.07^{bc}	1.46 ± 0.06^{c}	0.78 ± 0.10^{a}	1.10 ± 0.05^{ab}
GPx (U/mg protein)	0.29 ± 0.01^{c}	0.29 ± 0.01^{c}	0.16 ± 0.01^{a}	0.23 ± 0.01^{b}
TAC (U/mg protein)	0.48 ± 0.06^{b}	0.45 ± 0.02^{b}	0.29 ± 0.01^{a}	0.45 ± 0.03^{ab}
MDA (μmol/g protein)	0.34 ± 0.02^{a}	0.38 ± 0.02^{a}	0.57 ± 0.02^{b}	0.40 ± 0.03^{a}

Values are means ± SEM (n = 3). Means in the same row with different superscripts are significantly different from each other (P < 0.05). SOD, superoxide dismutase; CAT, catalase; POD, peroxidase; GPx, glutathione peroxidase; TAC, total antioxidant capacity; MDA, malondialdehyde.

presented the flora distribution at the genus level in different groups more intuitively (**Supplementary Figure 1**).

Anosim inter-group difference analysis showed that there were significant differences in the community structure between the FM group and the SNSP group ($P < 0.05$, **Table 10**). LEfSe analysis results showed that the abundance of *Bacteroidales*, *Bacteroidia*, and *Cellulosilyticum* in the SNSP group were significantly higher than that in the FM group ($P < 0.05$, **Figure 4A**), and the abundance of *Mycobacteriaceae*, *Mycobacterium_neoaurum*, *Corynebacteriales*, and *Mycobacterium* in the FM group were significantly higher than that in the NSP groups ($P < 0.05$, **Figure 4B**).

DISCUSSION

Fish have limited tolerance to dietary fiber, which at a low level (30-50 g/kg), improves fish growth (Zhou et al., 2005; Altan and Korkut, 2011) but excessive dietary fiber content inhibits fish digestion and absorption of feed nutrients (Watanabe et al., 1994). In addition, the effect of dietary fiber on fish is related to the fiber type (Adorian et al., 2016; Goulart et al., 2017). (Amirkolaie et al., 2005) reported that dietary supplementation with 8% INSP (cellulose) had non negative effects on Nile tilapia (*Oreochromis niloticus L*), while dietary supplementation with 8% SNSP (guar gum) significantly reduced the growth and feed conversion rate on Nile tilapia. However, dietary supplementation with different types of NSPs (5.8% INSP, 12.2% SNSP, and 18.0% NSPs) had no significant effects on the growth and FCR of GIFT tilapia in this study. These results indicate that the antinutritional effect of dietary SNSP is closely related to its composition.

Digestive enzymes could effectively metabolize proteins, lipids, and carbohydrates (Zokaeifar et al., 2012) and their activities are closely related to the characteristic and quantity of feed nutrients (Zhang et al., 2021). The significant increase of pepsin and intestinal lipase activity in the INSP group may be related to the physicochemical properties (e.g., insolubility, water absorption) of INSP and dietary INSP expanded the digesta, thereby increasing the contact area between digestive enzymes and substrates, leading to an increase in digestive activity (Li et al., 2017). While dietary SNSP form a high-viscosity environment in the digestive tract and provide a high-quality reproduction condition for microorganisms, these microorganisms produce a large amount of short-chain fatty acid through a fermentation process that lowers the pH of the digestive tract (Engberg et al., 2009; Niba et al., 2009), and thus participated in the regulation of digestive enzyme activity, which may explain the pepsin activity significant increase in SNSP and NSP groups. However, digestive enzymes activity may also be decreased by complexation with NSPs or by the encapsulation of substrates by NSPs (Sinha et al., 2011), which may explain the significant decrease in intestinal lipase activity in the SNSP group. Similarly, dietary supplementation with 16.8% SNSP significantly decreased the intestinal trypsin activity of rainbow trout (Deng et al., 2021). Moreover, considering that dietary supplementation with 18.0% NSPs (5.8% INSP + 12.2% SNSP) had no significant effect on intestinal lipase activity of GIFT

TABLE 7 | The whole-body composition of GIFT tilapia fed diets with different types of non-starch polysaccharides.

Items	FM	INSP	SNSP	NSP
Moisture (%)	71.75 ± 0.08	71.19 ± 0.20	72.05 ± 0.21	72.22 ± 0.34
Crude protein (%)	15.86 ± 0.07^{c}	15.90 ± 0.07^{c}	15.33 ± 0.13^{b}	14.58 ± 0.18^{a}
Crude lipid (%)	7.45 ± 0.17^{c}	6.98 ± 0.16^{bc}	6.43 ± 0.06^{ab}	6.27 ± 0.15^{a}
Ash (%)	4.48 ± 0.01^{a}	4.47 ± 0.04^{a}	4.60 ± 0.04^{a}	4.83 ± 0.03^{b}

Values are means ± SEM (n = 3). Means in the same row with different superscripts are significantly different from each other (P < 0.05).

TABLE 8 | Amino acids profiles (% dry matter) in the whole-body of GIFT tilapia fed diets with different types of non-starch polysaccharides.

Treatments	FM	INSP	SNSP	NSP
Arginine	4.64 ± 0.02^{c}	4.62 ± 0.02^{bc}	4.43 ± 0.05^{ab}	4.34 ± 0.06^{a}
Histidine	1.35 ± 0.01	1.35 ± 0.03	1.36 ± 0.02	1.29 ± 0.02
Isoleucine	2.03 ± 0.01^{a}	2.09 ± 0.01^{b}	2.10 ± 0.01^{b}	2.02 ± 0.02^{a}
Leucine	3.66 ± 0.01^{ab}	3.69 ± 0.03^{b}	3.73 ± 0.04^{b}	3.54 ± 0.04^{a}
Lysine	4.60 ± 0.19^{ab}	4.41 ± 0.04^{ab}	4.77 ± 0.11^{b}	4.22 ± 0.05^{a}
Methionine	1.30 ± 0.01	1.31 ± 0.01	1.31 ± 0.02	1.25 ± 0.03
Phenylalanine	1.74 ± 0.01	1.78 ± 0.02	1.74 ± 0.04	1.72 ± 0.03
Threonine	2.27 ± 0.05	2.31 ± 0.04	2.31 ± 0.06	2.23 ± 0.03
Valine	2.31 ± 0.01	2.37 ± 0.01	2.39 ± 0.01	2.28 ± 0.05
∑EAA	23.91 ± 0.17^{b}	23.93 ± 0.14^{b}	24.15 ± 0.14^{b}	22.80 ± 0.27^{a}
Glutamate	8.30 ± 0.06^{b}	8.12 ± 0.03^{b}	8.08 ± 0.03^{b}	7.60 ± 0.10^{a}
Glycine	2.59 ± 0.03	2.59 ± 0.04	2.55 ± 0.04	2.54 ± 0.04
Alanine	4.04 ± 0.01^{ab}	4.06 ± 0.05^{ab}	4.07 ± 0.02^{b}	3.93 ± 0.02^{a}
Tyrosine	1.31 ± 0.04	1.34 ± 0.01	1.40 ± 0.01	1.39 ± 0.04
Aspartate	6.24 ± 0.06^{b}	6.18 ± 0.01^{b}	6.14 ± 0.05^{b}	5.68 ± 0.09^{a}
Serine	1.79 ± 0.01^{b}	1.78 ± 0.01^{b}	1.77 ± 0.03^{b}	1.70 ± 0.01^{a}
Proline	3.10 ± 0.04	3.15 ± 0.05	3.13 ± 0.01	3.09 ± 0.06
Cysteine	0.35 ± 0.01	0.36 ± 0.03	0.35 ± 0.02	0.38 ± 0.02
∑NEAA	27.42 ± 0.06^{b}	27.27 ± 0.16^{b}	27.19 ± 0.10^{b}	26.02 ± 0.13^{a}
∑TAA	51.34 ± 0.11^{b}	51.20 ± 0.30^{b}	51.34 ± 0.19^{b}	48.82 ± 0.30^{a}
EAA/NEAA	0.87 ± 0.01	0.88 ± 0.01	0.89 ± 0.01	0.88 ± 0.01

Values are means ± SEM (n = 3). Means in the same row with different superscripts are significantly different from each other (P < 0.05). EAA, essential amino acids; NEAA, non-essential amino acids; TAA, total amino acids.

tilapia in this study, this phenomenon also indicated that dietary INSP was able to mitigate the adverse effects on digestive enzymes activities of dietary SNSP.

ALT, AST, and GGT are enzymes involved in amino acid metabolism which are mainly distributed in hepatocytes and released into the blood when hepatocytes are damaged. Therefore, the activity of ALT, AST, and GGT in plasma can effectively reflect the status of liver function (Habte-Tsion et al., 2015; Hanim et al., 2015; Talaveron et al., 2019). In this study, dietary supplementation with SNSP significantly increased plasma ALT activity, indicating that dietary SNSP induced liver function impairment in GIFT tilapia. Similarly, largemouth bass (*Micropterus salmoides*) suffered severe liver damage with dietary supplementation of 6% SNSP (carboxymethyl cellulose) (Shi et al., 2019), and dietary supplementation of 15% SNSP (pectin) induced liver damage in yellow catfish (*Pelteobagrus fulvidraco*) (Cai et al., 2019).

The significant increase of plasma BUN level and hepatic ALT activity of GIFT tilapia in the SNSP group suggests that dietary SNSP may enhance protein catabolism, thus causing a decrease in the whole-body crude protein and amino acid content in both the SNSP and NSP groups. The activation of protein catabolism may be associated with the reduced intake of lipids and carbohydrates, with protein being used more for catabolism to

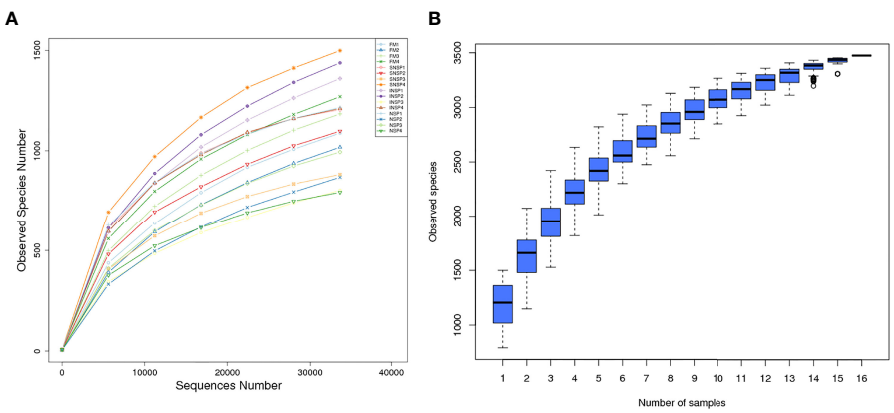

FIGURE 1 | Determine whether the number of tags and samples in this study were sufficient for subsequent data analysis. **(A)** Rarefaction curves were constructed based on the amount of sequencing data extracted in this study and the corresponding number of species. **(B)** Species accumulation boxplot was used to describe the increasing trend of species diversity as the number of samples increased.

TABLE 9 | Effects of experimental diets on Alpha diversity of intestinal flora in GIFT tilapia.

Parameters	FM	INSP	SNSP	NSP	*P*-value
Sobs	1140.00 ± 55.09	1203.00 ± 141.40	1171.00 ± 128.32	967.75 ± 92.56	0.185
Shannon	5.19 ± 0.54	5.87 ± 0.16	5.58 ± 0.59	5.03 ± 0.69	0.323
Simpson	0.89 ± 0.03	0.93 ± 0.01	0.89 ± 0.04	0.88 ± 0.03	0.301
Chao 1	1635.43 ± 52.42	1634.83 ± 204.62	1422.96 ± 176.63	1284.43 ± 112.51	0.147
ACE	1714.51 ± 57.50	1701.28 ± 20.75	1478.26 ± 173.45	1359.49 ± 117.09	0.143
PD-whole-tree	93.14 ± 6.42	95.44 ± 8.76	96.87 ± 7.58	76.28 ± 4.50	0.077
Coverage	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00	0.104

The data are present as the means ± SEM (n=4), P-values were determined using Welch's t-test.

supply energy as dietary SNSP interferes with the absorption of carbohydrates and lipids (Choct et al., 1996), which may also explain the significant decrease in the content of some amino acids in muscle. Moreover, dietary fiber has been reported to stimulate mucus secretion from the digestive tract and accelerate the loss of endogenous nitrogen (Larsen et al., 1993; Sales, 2009), while Ser, Thr, Gly, Glu, and Asp account for more than 50% of the total amino acid content of mucin (Sales, 2009), which means that these amino acids are easily lost with the excretion of mucin. Additionally, Ala, Arg, Ile, Lys, Ser, Glu, and Asp are all important glucogenic amino acids that can be converted into glucose by the gluconeogenic pathway when animals are deficient in carbohydrates. This may explain the significant decrease in Ala, Arg, Ile, Lys, Ser, Glu, and Asp of the muscles in GIFT tilapia with the dietary supplementation of 18.0% NSP in this study.

Previous studies have shown that dietary SNSP can further influence lipid metabolism, as Hossain et al. (Hossain et al., 2001; Hossain et al., 2003) reported that dietary supplementation with SNSP (sesbania endosperm, *Sesbania aculeate*) significantly reduced serum TC concentrations in carp and tilapia; (Liu et al., 2021) also reported that soluble fiber had the ability to reduce serum TC concentrations. However, in contrast to these above studies, dietary supplementation with SNSP had no significant effect on serum TC concentrations but significantly increased serum HDL concentrations in this study. The difference in these results suggest that the impact of dietary SNSP on serum TC concentrations may vary with the test species and the composition of dietary SNSP. The hypolipidemic effect of dietary SNSP may be related to the viscosity change of chyme, and (Pasquier et al., 1996) suggested that high viscosity conditions would inhibit the fat emulsification process and

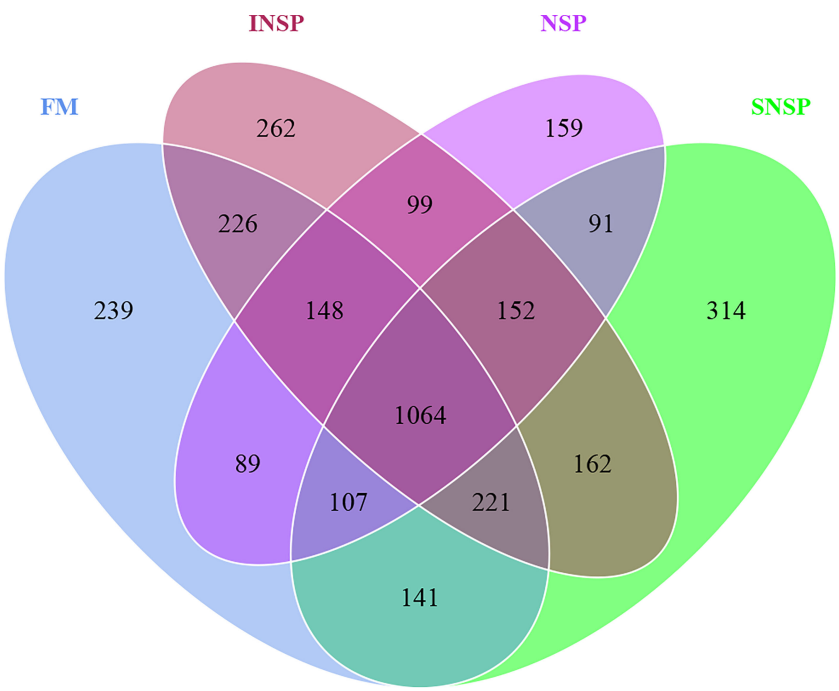

FIGURE 2 | Venn diagram of OTUs in each group. Venn graph was drawn by analyzing the common and unique OTUs among different groups according to the results of OTU clustered analysis with 97% identification based on 16S rRNA sequencing data.

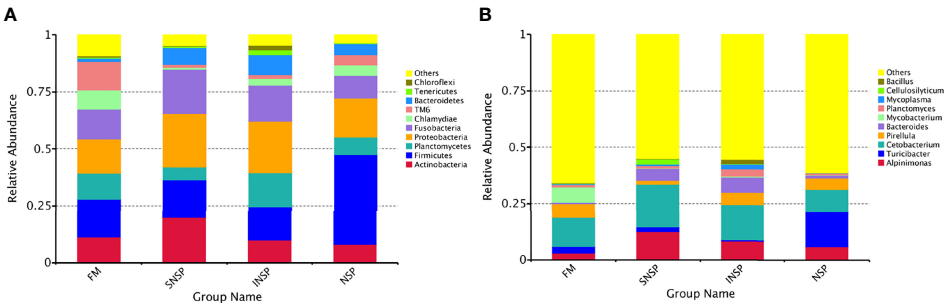

FIGURE 3 | Microbial community structure of different groups. **(A)** Microbial community structure at the phylum level in different groups of FM, SNSP, INSP and NSP. **(B)** Microbial community structure at the genera level in different groups of FM, SNSP, INSP and NSP.

lipolysis. This may explain the significant decrease in whole-body crude lipid content in the SNSP and NSP groups in this study. Similarly, dietary supplementation with SNSP (endosperm, separated from *Sesbania* seeds) significantly decreased the whole-body crude protein and lipid contents in common carp (Hossain et al., 2001), and the whole-body lipid content of largemouth bass also significantly decreased with dietary supplementation of 6% SNSP (carboxymethyl cellulose) or 6% INSP (microcrystalline cellulose) (Shi et al., 2019). However, dietary supplementation with either INSP or SNSP had no significant effect on the body composition of Chinese mitten crab (*Eriocheir sinensis*) (Wu et al., 2015). The differences in these results may be related to the differences in digestive physiology between fish and crustaceans, which requires further study.

The antioxidant capacity of plasma and the liver significantly decreased in the SNSP group, indicating dietary SNSP induced oxidative stress in GIFT tilapia. Similarly, dietary supplementation with SNSP also induced oxidative stress in largemouth bass and rainbow trout (Shi et al., 2019; Deng et al., 2021). Conversely, (Enes et al., 2012) suggested that dietary supplementation with low doses of SNSP (4% guar gum) may have a preventive effect on intestinal oxidative stress in white seabream (*Diplodus sargus*). Moreover, several recent studies have shown that dietary SNSP from natural resources possess strong reducing power and antioxidant properties (Amamou et al., 2020), which may promote antioxidant enzyme activity or modulate antioxidant signaling pathways by scavenging free radicals (Chen et al., 2021). These results suggest that the effect of dietary SNSP on the antioxidant capacity of fish was type- and dose-dependent.

A recent study reported that dietary SNSP (xylan and pectin) had no significant effects on the Alpha diversity of intestinal flora in yellow catfish (*Pelteobagrus fulvidraco*) (Zhang, 2014). In this study, diets containing different types of NSPs also had no significant effect on the Alpha diversity of intestinal flora in GIFT tilapia. Contrary to our results, dietary soluble xylan significantly increased the Alpha diversity and richness of intestinal flora in Chinese mitten crab (*Eriocheir sinensis*) (Wu, 2015). These different results may be caused by the types of dietary NSPs and the differences of digestive physiology between fish and crustaceans, which needs to be further studied. Noteworthy, different diets significantly changed the composition of dominant bacteria in GIFT tilapia.

At the phylum level, although the abundance of dominant phylum in each group was different, the species of dominant phylum were relatively consistent and all groups have *Firmicutes*, *Proteobacteria*, *Fusobacteria*, *Actinobacteria*, *Planctomycetes*, and *Bacteroidetes* as the core flora. This result was consistent with the intestinal flora of the cichlid (Baldo et al., 2015) and partially similar with the wild and cultured Nile tilapia (Zheng et al., 2018; Beneberu, 2020), which proved that our results were reliable.

At the genus level, *Cetobacterium* was the dominant genus in the intestine of GIFT tilapia in all groups in this study. *Cetobacterium* is the core flora in the intestinal tract of fish (Ni et al., 2012), and it has been identified in carp (*Cyprinus carpio*) (Kessel et al., 2011), Nile tilapia (Yun et al., 2018), giant amazonian fish (*Arapaima gigas*) (Ramírez et al., 2018) and some other freshwater fish. The metabolism of *Cetobacterium* in the intestine of freshwater fish can produce vitamin B_{12} (VB_{12}) and acetic acid (Sugita et al., 1991; Lin et al., 2019) and these metabolites have important functions. The VB_{12} participates in the regulation of intestinal ecology and promotes intestinal health (Degnan et al., 2014), while acetic acid can promote sugar and fat metabolism or protein synthesis and can synthesize proteins, carbohydrates, and fats for the host's use (Lin et al., 2019). Host species are a key factor affecting the composition of the intestinal flora (Egerton et al., 2018), *Cetobacterium* can be the dominant intestinal microbiota of

TABLE 10 | Inter-group differences of community structure based on Anosim method.

Group	R-value	*P*-value
FM-INSP	0.031	0.450
FM-SNSP	0.250	0.028
FM-NSP	−0.052	0.546

The R-value is range from −1 to 1. When the R-value is greater than 0, indicating that there is significant difference between groups, and when R-value is less than 0, indicating that the difference within the group is greater than that between the groups. The reliability of statistical analysis is expressed by P-value, and P < 0.05 indicates statistical significance.

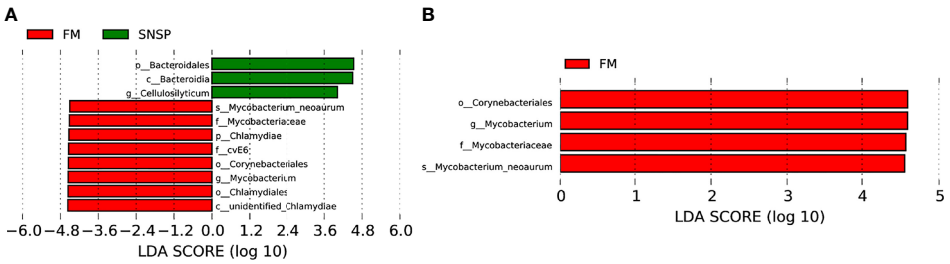

FIGURE 4 | LEfSe analysis of intestine microbial compositions among different groups. (FM, SNSP, INSP and NSP). Significant differences (LDA score ≥ 2, $P < 0.05$) between FM and SNSP **(A)**, FM and NSP, **(B)**. There was no significant difference between the other groups ($P > 0.05$).

many freshwater fish, which may be due to its important physiological functions. *Mycobacterium* has been proven to be associated with pathogenic bacteria (Novotny et al., 2004; Shanmugham and Pan, 2013) and can even cause the infection of fish (Hashish et al., 2018). *Mycobacterium* is a kind of aerobic bacteria, while the dietary SNSP decreases the oxygen tension of the intestine (Sinha et al., 2011), and this may explain why the abundance of *Mycobacterium* was significantly decreased in the SNSP and NSP groups. This phenomenon suggests that dietary SNSP possesses a potential mechanisms to reduce the aboudance of aerobic bacteria in the intestine of fish, which needs to be further studied.

Early studies have shown that the abundance of *Bacteroides* increases when inflammatory bowel disease occurs (Prindiville et al., 2000; Bloom et al., 2011) and so *Bacteroides* may serve as a marker bacteria for the development of intestinal diseases. Moreover, *Bacteroides* is one of the main bacteria involved in the fermentation process of intestinal contents and its metabolic production of short-chain fatty acids plays an important role in resisting host enteritis (Besten et al., 2013). Increasing the abundance of *Bacteroides* may be one of the strategies for host resistance to enteritis but this hypothesis needs to be further explored. *Bacteroides* is a kind of anaerobic bacteria and its relative abundance increased significantly in the SNSP group, which may be associated with the reduction of oxygen tension in the intestine *via* the SNSP fermentation process.

CONCLUSION

In summary, this study showed that dietary INSP had limited effects on the growth and intestinal flora of GIFT tilapia, whereas dietary SNSP inhibited intestinal lipase activity, reduced antioxidant capacity, and induced liver damage of GIFT tilapia. Additionally, dietary SNSP reduced intestinal aerobic microbial abundance but elevated anaerobic microbial abundance.

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: NCBI [accession: PRJNA806406].

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

JD and BT directed the completion of the experimental design, and YL completed the breeding of test fish, sample collection and measurement, data analysis and manuscript writing. SX and WZ directed the manuscript revision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.872577/full#supplementary-material

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Effects of Dietary Nano-Curcumin Supplementation on Growth Performance, Glucose Metabolism, and Endoplasmic Reticulum Stress in Juvenile Largemouth Bass, *Micropterus salmoides*

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This study evaluated the effects of nano-curcumin on growth performance, mucosal immunity, antioxidant response, glucose metabolism, and endoplasmic reticulum stress in largemouth bass. Three groups (three replicates/group) of 270 fish with the mean body weight of 7.00 ± 0.02 g were fed with diets containing 0% (control), 0.1%, and 0.2% nano-curcumin for 60 days. The results showed that dietary supplementation of nano-curcumin had no significant effects on the growth performance, body composition, lysozyme (LYZ), and alkaline phosphatase (AKP) in skin mucus, superoxide dismutase (SOD), and catalase (CAT) activity in serum of largemouth bass. However, dietary supplementation with 0.2% nano-curcumin significantly increased peroxidase (POD) activity in skin mucus and decreased the serum MDA activity compared with the control group. Moreover, dietary supplementation of 0.2% nano-curcumin significantly decreased the levels of serum glucose and liver glycogen, which may be mainly related to the increased gene expression of glucose transporter 2 (*GLUT2*), glucokinase (*GK*), phosphofructokinase (*PFK*), and pyruvate kinase (*PK*). At the same time, the hepatic gene expression of glucose-regulated protein78 (*GRP78*), activating transcription factor6 (*ATF6*), inositol-requiring enzyme 1 (*IRE*), and eukaryotic initiation factor 2α (*eIF2*α) in the 0.2% nano-curcumin group were rapidly suppressed, indicating that adding 0.2% nano-curcumin to the diet can alleviate endoplasmic reticulum stress (ERS) in fish. In conclusion, diets supplemented with 0.2% nano-curcumin effectively improved mucosal immunity, antioxidant properties, and glucose metabolism and alleviated ERS induced by long-term carbohydrate intake in largemouth bass.

Keywords: nano-curcumin, immunity, antioxidant, glucose, endoplasmic reticulum stress, largemouth bass

INTRODUCTION

Curcumin, known as diferuloylmethane, is a water-insoluble phenolic compound extracted from the rhizome of *Curcuma longa*. Consistently, curcumin has attracted extensive attention in aquaculture, because of its wide range of pharmacological properties, such as anti-inflammatory, antioxidant, antitumor, antivirus, hypoglycemic, and lipid-lowering effects (Nm et al., 2018; Alagawany et al., 2021; Ji et al., 2021). In recent years, Mahmoud et al. (2017) and Ajani et al. (2020) have revealed that the supplementation of curcumin in feed could enhance the growth performance and disease resistance of *Oreochromis niloticus* (Mahmoud et al., 2017; Ajani et al., 2020). Yonar et al. (2019) showed that the addition of curcumin in the feed could improve the growth, immunity, and antioxidant capacity of *Oncorhynchus mykiss* (Yonar et al., 2019). Zhao et al. (2021b) reported that curcumin could reduce chlorpyrifos-induced apoptosis and liver damage in largemouth bass (Zhao et al., 2021b). However, curcumin has limited clinical potential, due to its extremely low oral bioavailability due to its poor aqueous solubility and its chemical instability, having an oral bioavailability of only 1% in rats (Yang et al., 2007; Hewlings and Kalman, 2017). To overcome the solubility issues of curcumin and facilitate its intracellular delivery, several approaches have been developed to increase its bioavailability, including liposomal curcumin (Feng et al., 2017), curcumin nanoparticles (Bisht et al., 2007), and curcumin-phospholipid complexes (Maiti et al., 2007). Recent studies showed that nano-curcumin could potentially improve glucose uptake and insulin sensitivity in diabetic mice (Afifi et al., 2020; Shehata et al., 2020). Marchiori et al. (2019) showed that the addition of 200 mg/kg curcumin/nanocurcumin can effectively improve the growth performance and liver health of broiler chickens reared under cold stress conditions (Marchiori et al., 2019). Yu et al. (2016; 2018) found that Zn(II)-curcumin has a protective effect on the liver on *Litopenaeus vannamei* (Yu et al., 2016; Yu et al., 2018). However, there is still a paucity of research on nano-curcumin in carnivorous fish, and further studies are needed.

Largemouth bass (*Micropterus salmoides*) is a carnivorous species, which has become one of the most commercially important species in China, and the total production of largemouth bass in 2020 reached more than 600,000 t (Ministry of Agriculture and Rural Affairs of China, 2021). As a carnivorous fish species, largemouth bass is considered to be a typical model to investigate glucose-induced liver disease due to its poor utilization of carbohydrates; it would exhibit pronounced hyperglycemia after consuming carbohydrates for long periods of time (Guo et al., 2020, Chen P. et al., 2021). Long-term carbohydrate intake would lead to liver damage and glycogen accumulation, as well as increase the occurrence of oxidative stress and endoplasmic reticulum stress (Liu et al., 2017; Guo et al., 2020). Taking into account the beneficial effects of curcumin, the purpose of this study was to evaluate the effects of nano-curcumin on the growth performance, hepatoprotective potential, antioxidant response, glucose metabolism, and endoplasmic reticulum stress of largemouth bass. This will be helpful for providing a theoretical basis for reasonable supplementation of nano-curcumin in largemouth bass feed.

MATERIALS AND METHODS

Diet Preparation

Formulation of the experimental diets (control (without nano-curcumin), 0.1% nano-curcumin, and 0.2% nano-curcumin) is listed in **Table 1**. The method of diet preparation was carried out with reference to the previous literature report (Xie S. W. et al., 2020). In brief, all the powdered ingredients were precisely weighed and thoroughly mixed, then the lipids and water were added. The twin-screw cooking extruder (HQ, Zhaoqing, China) was used to extrude the wet mixture into 2.5-mm-diameter pellets, and the pellets were air-dried to a moisture content of about 10%, and then stored in a refrigerator at -20°C until use.

Fish and Experimental Conditions

Largemouth bass were provided by a local hatchery (Foshan, China). The fish were adapted to an indoor recirculating aquaculture system and fed with commercial diet for 2 weeks. After the acclimation period, 270 fish of similar body weight (7.00 ± 0.02 g) were randomly divided into three groups; each group had three replicates (30 fish/tank). During the 60-day breeding experiment, fish were hand fed twice a day to visual satiation at 8:30 and 16:30, and feed consumption was recorded daily. Throughout the experimental period, the water temperature was 25°C–31°C, dissolved oxygen > 6.0 mg/l, pH 7.0 ± 0.5, and NH4+-N 0.15–0.20 mg/l.

Sample Collection and Chemical Analyses

At the end of the feeding trial, all fish were fasted for 24 h and then anesthetized with MS-222 (tricaine methanesulfonate, Sigma, USA). After being counted and weighed, skin mucus of the fish was collected using the method of Hoseinifar et al. (2014) with slight modifications. Briefly, 10 fish from each tank were randomly collected and transferred to a polyethylene bag containing 100 ml of 50 mM NaCl. The bag was gently shaken for 2 min to collect skin mucus. The mucus samples were immediately transferred to 50-ml sterile centrifuge tubes and centrifuged at 1,500 rmp/min under 4°C for 10 min to obtain the sample supernatant, and then the supernatant was stored at -80° C for further analysis (Hoseinifar et al., 2014).

Blood samples from nine fish were collected from the caudal vein with disposable medical syringes. Three fish were dissected for liver samples, which were used for histological analysis and gene expression analysis or biochemical index; all the samples were immediately placed into liquid nitrogen and then stored at −80°C before the analysis. Blood samples were centrifuged (4,000 rmp/min, 15 min) at 4°C, then supernatants from six fish were mixed and used to measure hemolymph biochemical indices and glucose metabolic indices; all samples were immediately frozen and stored at −80°C until analysis. The muscle of six fish was collected for glycogen analysis and nutrient composition determination.

TABLE 1 | Ingredients and proximate composition of the experimental diets.

Ingredient (%)	Dietary effective nano-curcumin contents (%)		
	0	0.1%	0.2%
Fish meal	40	40	40
Soybean meal	20	20	20
Peanut meal	10	10	10
Gluten	7	7	7
Wheat flour	11	10.5	10
Fish oil	3	3	3
Soy lecithin	1	1	1
Beer yeast	3	3	3
Calcium dihydrogen phosphate	1.5	1.5	1.5
Choline chloride	0.5	0.5	0.5
Premix[a]	3	3	3
Nano-curcumin (20%)[b]	0	0.5	1
Total	100	100	100
Proximate analysis (% dry matter)			
Moisture (%)	7.67	8.58	8.41
Crude protein (%)	48.51	48.72	48.68
Crude lipid (%)	16.21	14.91	15.25
Ash (%)	13.04	12.75	12.91

[a]*Premix: vitamin mixture: (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, 8,000 mg; calcium pantothenate, 1,250 mg; nicotinic acid, 2,000 mg; choline chloride, 8,000 mg; vitamin D3, 45,000 IU; vitamin C, 7,000 mg. Mineral mix (kg^{-1} of diet): $ZnSO_4$ « 7 H_2O, 0.04 g; $CaCO_3$, 37.9 g; KCl, 5.3 g; KI, 0.04 g; NaCl, 2.6 g; $CuSO_4$ « 5 H_2O, 0.02 g; $CoSO_4$ « 7 H_2O, 0.02 g; $FeSO_4$ « 7 H_2O, 0.9 g; $MnSO_4$ « H_2O, 0.03 g; $MgSO_4$ « 7H_2O, 3.5 g; $Ca(HPO_4)_2$ « 2H_2O, 9.8 g. Manufactured by the Guangdong Chengyi Co., Ltd.*

[b]*Nano-curcumin (20%): carrier: liposome, size = 70 nm, encapsulation rate > 95%, drug loading = 20%, manufactured by the Shanghai Ehoo Biotechnology., Ltd.*

Moisture, crude protein, crude fat, and crude ash of experimental diet, whole body, and muscle were determined by the method of AOAC (AOAC, 2003). The moisture content was determined by the constant temperature drying weight loss method at 105°C. The crude protein content was determined by the Kjeldahl method. The crude fat content was determined by the Soxhlet extraction method. The crude ash content was determined by the muffle furnace ashing method.

Serum Glucose, Liver Glycogen, and Muscle Glycogen

The glucose oxidase-peroxidase (GOD-POD) method was used to determine the serum glucose of experimental animals. The content of glucose in the sample can be calculated by measuring the absorbance of the blue substance, which was generated by the hydrogen peroxide and o-toluidine generated by the decomposition of glucose under the action of peroxidase.

Determination of glycogen in the liver and muscle was done using the anthraquinone method (Liu et al., 2017).

Activities of LYZ, POD, and ALP in Skin Mucosal of Largemouth Bass

Lysozyme (LYZ) hydrolyzed peptidoglycan on the bacterial wall so that it increased the light transmission of the turbid bacterial solution. Therefore, the content of lysozyme could be speculated according to the change in turbidity, which was determined by the absorbance at 520 nm. The determination of peroxidase (POD) was based on the principle that peroxidase catalyzes hydrogen peroxide reaction, and its enzyme activity was obtained by measuring the change of absorbance at 420 nm. Alkaline phosphatase (AKP) was determined by using its decomposable disodium diphenyl phosphate to produce free phenol, which reacted with 4-aminoantipyrine in alkaline solution to produce red quinone derivatives. The enzyme activity could be determined according to the depth of red. All kits were purchased from Nanjing Jiancheng Institute of Biological Engineering.

Antioxidant Indicators Measurement

The activities of superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) in serum were performed as described by Chen et al. (Chen Y. K. et al., 2021).

Liver Histological Examination

Liver histological examination was performed as described by Yu et al. In brief, liver tissue samples were fixed in Bouin solution and embedded in paraffin wax. The embedded liver was cut into 5-μm sections with a microtome and finally stained with hematoxylin and eosin to make the liver tissue sections (Yu et al., 2018).

RNA Extraction and Gene Expression Analysis

Total RNA from liver was extracted using TransZol Up Plus RNA Kit, and RNA was reversely transcribed to cDNA using EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, China). Real-time quantitative PCR (RT-qPCR) was performed using the SYBR® Premix Ex Taq™ System in a QuantStudio3 machine (Applied Biosystems, Foster City, CA, USA) with the following program: 94°C for 30 s; 40 cycles of 94°C for 5 s, 60°C for 30 s, 95°C for 15 s, 60°C for 60

s, and 95°C for 1 s. Gene expression was determined using 2-ΔΔCt. All primers used in this study are listed in **Table 2**.

Statistical Analysis

The parameters in this study were calculated as per the following formula:

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

$$\text{Specific growth rate}\left(\text{SGR, \% da y}^{-1}\right) = 100 \times [\text{Ln(final body weight)} - \text{Ln(initial body weight)}]/t$$

$$\text{Survival(\%)} = 100 \times (\text{final number of fish})/(\text{initial number of fish})$$

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

where it is the experimental duration in days.

The data are presented as means ± SEM. All experimental data are used with SPSS 22.0 (SPSS, Chicago, IL, USA) for one-way analysis of variance (one-way ANOVA); when the difference was significant, Duncan's multiple-comparison test was performed, and the significance level was set to $P < 0.05$.

RESULTS

Growth Performance

Addition of nano-curcumin in the feed had no significant effect on the survival rate of juvenile largemouth bass. The survival rate of each group was above 99% ($P > 0.05$) (**Table 3**). No significant difference was observed in WGR, SGR, and FCR among the fish in all groups ($P > 0.05$).

Effect of Dietary Nano-Curcumin on Whole-Body and Muscle Composition of Largemouth Bass

Nano-curcumin had no significant effect on the approximate composition of the whole body and muscles of fish, including crude protein and crude fat ($P > 0.05$) (**Table 4**).

Effects of Dietary Nano-Curcumin on Serum Glucose and Tissue Glycogen of Largemouth Bass

As set out in **Figure 1**, the serum glucose levels and liver glycogen in the 0.2% nano-curcumin group were significantly lower than those in the control group and 0.1% nano-curcumin group ($P < 0.05$). There was no significant difference in muscle glycogen levels among groups ($P > 0.05$).

TABLE 2 | Primer sequences.

Genes	Forward sequence (5′–3′)	Reverse sequence (5′–3′)	Reference
GLUT2	GTGTTTGCTGTGCTGCTCCT	GCTCCGTATCGTCTTTGGG	(Zhang et al., 2020)
GK	GGGTTTTACCTTCTCCTTTC	GGTGGCTACTGTGTCATTCA	(Li et al., 2020)
PFK	CTGGCTGAGCTCGTAAAG	GTGCCGCAGAAGTCGTTG	(Li et al., 2020)
PK	CTCTTTCATCCGCAAAGC	AATTCCCAGGTCACCACG	(Li et al., 2020)
G6pase	TGTTTACTATTTTCTTCCACCCA	GCTGACACAGTTATTCTTTGCTC	(Zhang et al., 2020)
Fbp1	GCGATTGGCGAATTTATC	ACTCTGTGACGGCGGGTT	(Li et al., 2020)
Pepck	ATTCCCTTCAGTATGGGTCCT	CCACTCCTTCAGCCAGTTTC	(Zhang et al., 2020)
GRP78	TTGCCGATGACGACGAAA	CAATCAGACGCTCACCCT	(Zhao et al., 2021a)
ATF6	CAGGACGAAGTGCTTAGAGTT	AGAGTAATGGACGGTCACAAT	(Zhao et al., 2021a)
IRE1	ACGGACCAATCGTGAGAC	CGGGAGGTGAAGTAGGAG	(Zhao et al., 2021a)
eIF2α	CCTCGTTTGTCCGTCTGTATC	GCTGACTCTGTCGGCCTTG	(Zhao et al., 2021a)
β-actin	AAAGGGAAATCGTGCGTGAC	AAGGAAGGCTGGAAGAGGG	(Li et al., 2020)

GK, glucokinase; PFK, phosphofructokinase; PK, pyruvate kinase; GLUT2, glucose transporter 2; G6pase, glucose-6-phosphatase catalytic subunit; fbp1, fructose-bisphosphatase1; Pepck, phosphoenolpyruvate carboxykinase; GRP78, glucose-regulated protein78; ATF6, activating transcription factor6; IRE1, inositol-requiring enzyme 1; eIF2α: eukaryotic initiation factor 2α.

TABLE 3 | Growth performance, feed efficiency, and survival rate of largemouth bass fed different experimental diets for 60 days.

Items/groups	0%	0.1%	0.2%
Initial weight/g	7.00 ± 0.02	7.00 ± 0.02	7.00 ± 0.02
Final weight/g	52.78 ± 0.87	52.97 ± 0.66	53.93 ± 1.93
WGR/%	653.44 ± 12.82	656.16 ± 8.62	669.98 ± 26.75
SGR %/day	3.37 ± 0.03	3.37 ± 0.02	3.40 ± 0.06
FCR	0.84 ± 0.02	0.83 ± 0.02	0.84 ± 0.01
Survival/%	100 ± 0.00	99.00 ± 1.00	99.00 ± 1.00

The values (n = 30) are expressed as mean ± standard error. The mean values in same line with different superscripts are significantly different (p < 0.05).

TABLE 4 | Whole-body and muscle composition of largemouth bass fed different experimental diets for 60 days.

Items/groups	0%	0.1%	0.2%
Whole fish body			
Crude protein (%)	58.05 ± 0.72	59.47 ± 0.77	59.24 ± 0.38
Crude lipid (%)	23.51 ± 1.59	23.94 ± 0.73	24.06 ± 0.10
Muscle			
Crude protein (%)	89.86 ± 0.78	90.05 ± 0.18	92.11 ± 1.98
Crude lipid (%)	11.81 ± 1.81	12.41 ± 0.79	12.91 ± 1.38

The values (n = 6) are expressed as mean ± standard error. The mean values in same line with different superscripts are significantly different (p < 0.05).

Effects of Dietary Nano-Curcumin on LYZ, POD, and AKP in Skin Mucosal of Largemouth Bass

As is shown in **Figure 2**, the activities of LYZ and AKP in the skin mucus of largemouth bass in the 0.2% nano-curcumin group were higher than those in the control group, but there were no significant differences between the groups ($P > 0.05$). Meanwhile, the POD content of the nano-curcumin group was significantly higher than that of the control group ($P < 0.05$).

Effects of Dietary Nano-Curcumin on Antioxidant Enzymes in Serum of Largemouth Bass

As is presented in **Table 5**, treatments of nano-curcumin did not affect the activity of SOD and CAT in serum ($P > 0.05$), and with the increase in nano-curcumin content, the activity of MDA significantly decreased in the 0.2% nano-curcumin group ($P < 0.05$).

Liver Histology

The HE-stained paraffin sections of the liver from fish in all groups are shown in **Figure 3**. The results of the hematoxylin–eosin staining section showed that the fish in the control group had obvious inflammatory cell infiltration compared with the fish in nano-curcumin treatment groups. In addition, the morphology of hepatocytes in the control group and 0.1% nano-curcumin group was similar, with hypertrophy, vacuolization, and loss of nucleus of hepatocytes.

Expression of Genes Involved in Glucose Metabolism and Endoplasmic Reticulum Stress in Liver of Largemouth Bass

The effect of nano-curcumin on the expression of genes related to glucose metabolism in the liver of largemouth bass is shown in **Figure 4**. The results showed that with the supplementation of nano-curcumin, the mRNA expression of *GK*, *PFK*, and *PK* in fish fed 0.2% nano-curcumin significantly increased compared with the control group and 0.1% nano-curcumin group ($P < 0.05$). The mRNA expression of *G6Pase* and *Fbp1* did not show a significant change among the three groups ($P > 0.05$), while the mRNA expression of *PECPK* and *GLUT2* was significantly increased in the 0.1% nano-curcumin group compared with the control group ($P < 0.05$).

The relative expression of endoplasmic reticulum stress-related genes in the liver of largemouth bass is shown in **Figure 5**. Compared with the control group, the relative expression of *GRP78*, *ATF6*, *IRE*, and *eIF2α* in the 0.2% nano-curcumin group was significantly downregulated ($P < 0.05$).

DISCUSSION

In the present study, dietary nano-curcumin did not affect the growth performance of largemouth bass, which is consistent with Pirani et al. (2021) who found that dietary curcumin nanomicelles also had no effect on the growth performance of *Cyprinus carpio* (Pirani et al., 2021). However, the improvement of growth performance was observed in other animals with dietary nano-curcumin supplementation, such as mice, chicken, and white shrimp (Yu et al., 2016; Yu et al., 2018; Marchiori et al., 2019; Afifi et al., 2020; Bhoopathy et al., 2021). There is no consensus on plausible explanations for this discrepancy, which may be related to the size, species, feed composition, culture environment, or difference of nanocarriers.

It is well known that medicinal plants and their extracts can enhance the mucosal immunity of fish (Doan et al., 2020). As the first layer of the innate immune system, fish skin mucus contains

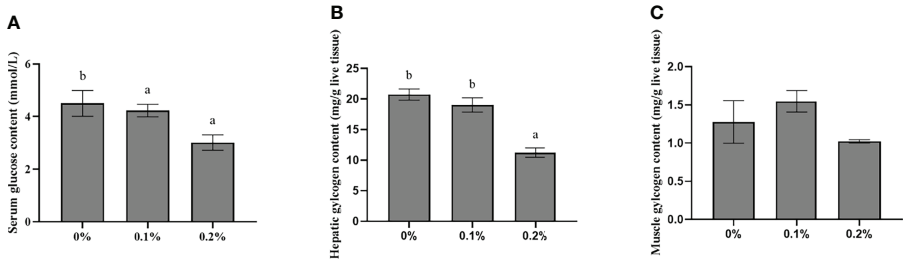

FIGURE 1 | Effects of dietary nano-curcumin on serum glucose **(A)**, liver glycogen **(B)**, and muscle glycogen **(C)** in largemouth bass. Values (mean ± standard error of the mean, SEM) in bars that have the same letter are not significantly different (P > 0.05; Duncan's test) between treatments (N = 6).

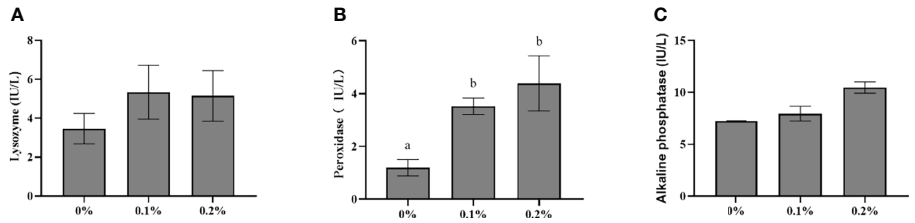

FIGURE 2 | Effect of dietary nano-curcumin on lysozyme **(A)**, peroxidase **(B)**, and alkaline phosphatase **(C)** in the liver of largemouth bass. Values (mean ± standard error of the mean, SEM) in bars that have the same letter are not significantly different (P > 0.05; Duncan's test) between treatments (N = 6).

different innate immune components, such as lysozyme, protease, alkaline phosphatase, lectin, catalase, and complement (Lazado and Caipang, 2015). In the present study, dietary supplementation of nano-curcumin could increase the activities of LYZ, POD, and AKP in skin mucus of largemouth bass. The same results were found in carp (Giri et al., 2019), *Larimichthys crocea* (Yu et al., 2015), and *Labeo rohita* (Sukumaran et al., 2016), indicating that nano-curcumin, as well as curcumin, could improve the mucosal immunity of fish, which might be related to its immune stimulation characteristics, but the exact mechanism of stimulating mucosal immunity of fish still needs to be further explored.

Superoxide dismutase (SOD) and catalase (CAT) are the evaluation indicators of the body's antioxidant capacity, which are widely present in animals and can protect cells and prevent peroxidation (Lesser, 2006). Malondialdehyde (MDA) is a breakdown product of lipid peroxidation; it can indirectly reflect the degree of cell damage (Koruk et al., 2004). In this study, compared with the control group, the 0.2% nano-curcumin group did not significantly increase the activity of SOD and CAT in serum but significantly decreased the activity of MDA, which echoed the results of liver histopathology, indicating that the appropriate amount of nano-curcumin can improve the antioxidant ability of largemouth bass. The results were in accordance with the previous study, which confirmed that curcumin could improve the antioxidant ability of fish, such as grass carp (Jiang et al., 2016), rainbow trout (Yonar et al., 2019), and Nile tilapia (Mahmoud et al., 2017). This may be due to the molecular structure of curcumin which contains phenolic hydroxyl with strong ability to remove free radicals, and curcumin also significantly increased the activity of antioxidant enzymes, such as SOD and CAT (Ramassamy, 2006; Hewlings and Kalman, 2017; Moniruzzaman and Min, 2020). Moreover, previous studies have shown that nanoencapsulation can enhance the scavenging ability of curcumin on free radicals (Coradini et al., 2014), which may be one of the reasons why nano-curcumin improves the antioxidant capacity of largemouth bass.

As a facilitative glucose transporter, glucose transporter 2 (GLUT2) could promote the bidirectional transfer of glucose across the cell membrane. Together with sodium-glucose cotransporter 2, GLUT2 completes the absorption and transport of glucose, which is the first step in glucose metabolism (Klover and Mooney, 2004). In the present study, dietary addition of 0.1% nano-curcumin significantly upregulated the relative expression of *GLUT2* in the liver of fish, indicating that glucose transporters transported glucose to hepatocytes for glycolysis. As the only metabolic pathway leading to glucose catabolism in all organisms, including fish, the important rate-limiting enzymes of glycolysis included GK, PFK, and PK (Seiliez et al., 2013; Ren et al., 2021). Our study found that the relative gene expressions of *GK*, *PFK*, and *PK* were significantly increased in the 0.2% nano-curcumin group compared with the control group, demonstrating that nano-curcumin could reduce serum glucose levels by promoting the expression of key rate-limiting enzyme genes in glycolysis. Similarly, previous studies have also reported that nano-curcumin upregulated the mRNA expression of *GK* and *GLUT2* in diabetic mice (Afifi et al., 2020). Dietary supplementation of curcumin significantly reduced blood glucose levels and improved the liver glycogen content in diabetic db/db mice (Seo et al., 2008). In addition, the change in liver glycogen content is related to gluconeogenesis, which refers to the conversion of non-sugar substances into glucose or glycogen, and G6Pase, Fbp1, and PEPCK are important rate-limiting enzymes in the gluconeogenesis process (Cowey et al., 1977). In the present study, the addition of 0.2% nano-curcumin to the diet did not significantly reduce the relative expression of

TABLE 5 | Antioxidant enzymes in serum of largemouth bass fed different experimental diets for 60 days.

Items/groups	0%	0.1%	0.2%
SOD (U mg prot^{-1})	33.79 ± 2.46	33.06 ± 3.83	38.38 ± 1.31
CAT (U mg prot^{-1})	76.97 ± 4.10	72.08 ± 6.56	81.43 ± 3.04
MDA (nmol mg prot^{-1})	12.41 ± 2.17 ^{a}	7.47 ± 1.05 ^{ab}	5.80 ± 1.68 ^{b}

The values (n = 6) are expressed as mean ± standard error. The mean values in same line with different superscripts are significantly different (p < 0.05).

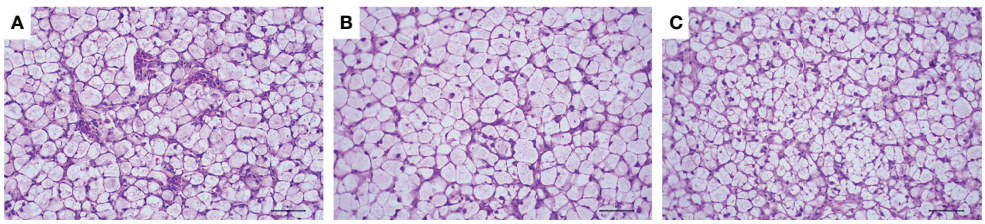

FIGURE 3 | Effect of dietary nano-curcumin on the liver histology (hematoxylin and eosin staining, original magnification ×400) of largemouth bass after 60 days. **(A)** Liver from fish fed diet 0% nano-curcumin. **(B)** Liver from fish fed diet 0.1% nano-curcumin. **(C)** Liver from fish fed diet 0.2% nano-curcumin.

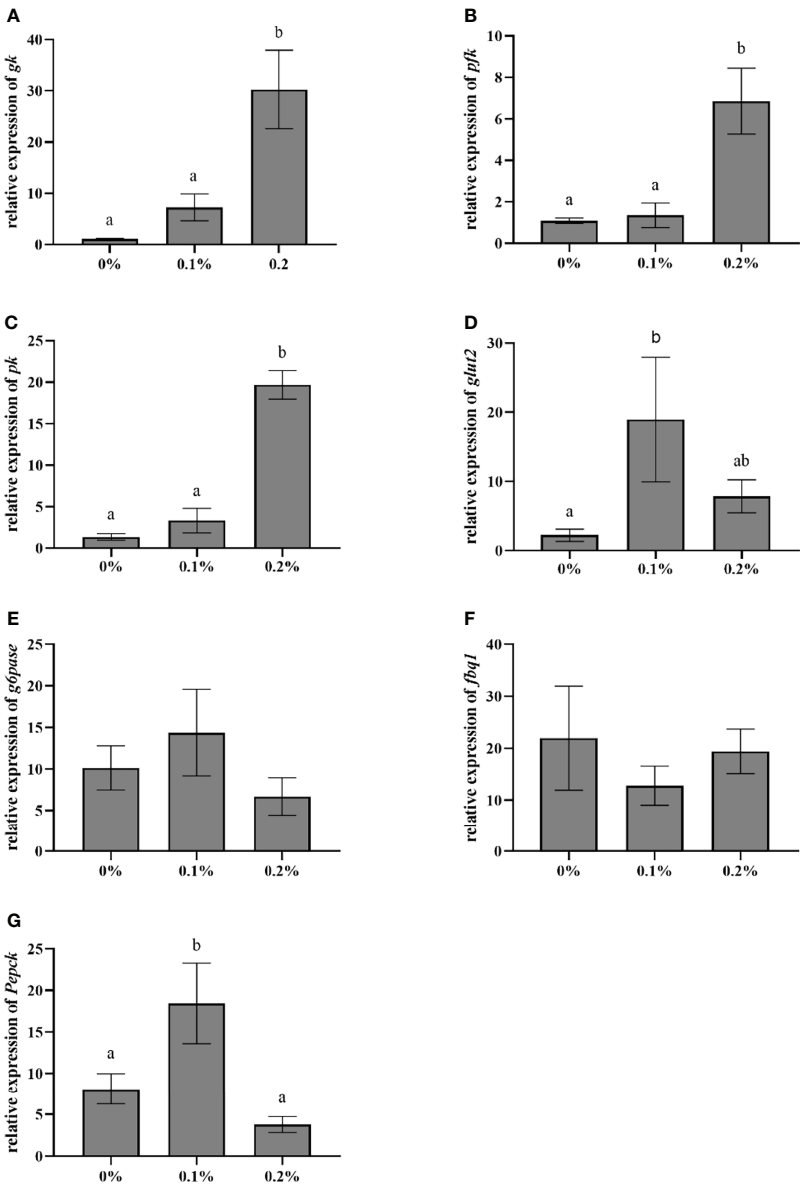

FIGURE 4 | Effect of dietary nano-curcumin on glucose transporter 2 (*glut2*) **(A)**, glucokinase (*gk*) **(B)**, phosphofructokinase liver type (*pfk*) **(C)**, pyruvate kinase (*pk*) **(D)**, glucose-6-phosphatase catalytic subunit (*G6pase*) **(E)**, fructose-bisphosphatase1 (*fbp1*) **(F)**, and phosphoenolpyruvate carboxykinase (*Pepck*) **(G)** related gene mRNA levels in the liver of largemouth bass. Values (mean ± standard error of the mean, SEM) in bars that have the same letter are not significantly different ($P > 0.05$; Duncan's test) between treatments ($N = 9$).

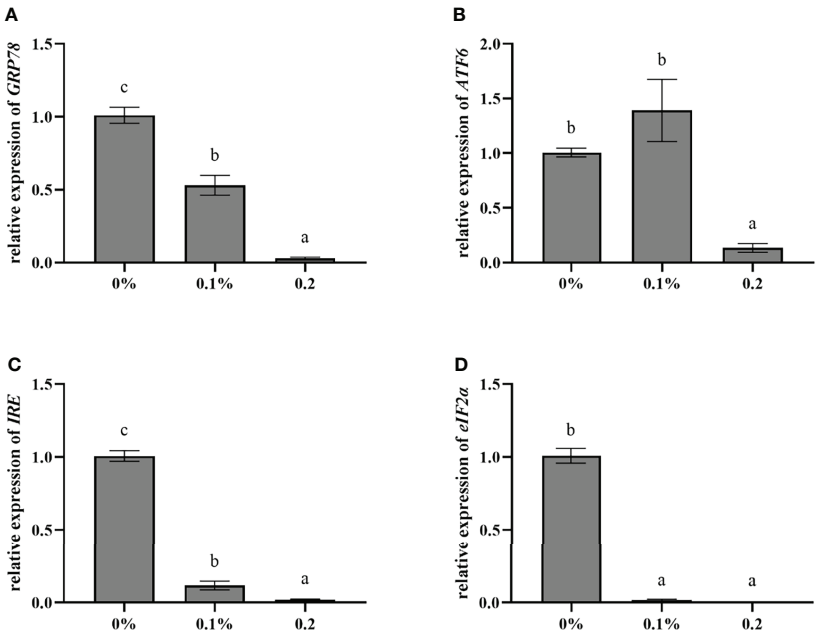

FIGURE 5 | Effect of dietary nano-curcumin on glucose-regulated protein78 (*GRP78*) **(A)**, activating transcription Factor6 (*ATF6*) **(B)**, inositol-requiring enzyme (*IRE*) **(C)**, and eukaryotic initiation factor 2α (*eIF2α*) **(D)** related gene mRNA levels in the liver of largemouth bass. Values (mean ± standard error of the mean, SEM) in bars that have the same letter are not significantly different (P > 0.05; Duncan's test) between treatments (N = 9).

G6Pase, *Fbp1*, and *PEPCK* genes but significantly reduced the content of liver glycogen compared with the control group. These results were partially consistent with previous studies that curcumin could downregulate *G6Pase* and *PEPCK* gene expression in diabetic mice and inhibit gluconeogenesis in the liver (Kim et al., 2009; Zhao et al., 2017). Additional studies demonstrated that curcumin could inhibit glucose production in isolated mouse hepatocytes (Fujiwara et al., 2008). Based on the above results, we speculated that nano-curcumin could promote glucose transport and glycolysis, which may be the principal reason for the decrease in serum glucose and liver glycogen content.

Previous studies have shown that excessive carbohydrate intake can induce endoplasmic reticulum stress (ERS) in largemouth bass, which is caused by the accumulation of a large number of unfolded or misfolded proteins (Zhao et al., 2021a). GRP78 is considered to be the marker protein of ERS (Jiang et al., 2017). When the body is in the ERS state, the overexpression of GRP78 can promote the dissociation and activation of ATF6, IRE1, and protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), while the dissociated and activated PERK can promote the phosphorylation of eIF2α (Bánhegyi et al., 2007). In mice and cell models, curcumin has been proven to markedly alleviate ERS induced by multiple factors, such as lipopolysaccharide (Zhang et al., 2018, Xie X. et al., 2020), diabetes (Fan et al., 2013; Rashid et al., 2017), and alcohol (Mohan et al., 2019; Wang et al., 2019). Similarly, our study showed that dietary supplementation of 0.2% nano-curcumin significantly reduced the relative gene expression of *GRP78*, *IRE*, *eIF2α*, and *ATF6* in the liver of largemouth bass, suggesting that nano-curcumin may alleviate endoplasmic reticulum stress, which is induced by long-term intake of carbohydrates. Due to the low number of studies on nano-curcumin, further research is needed to confirm our findings.

CONCLUSIONS

In conclusion, the study showed that dietary nano-curcumin had no significant effect on the growth performance and approximate components of largemouth bass, but the non-specific immunity was improved by increasing the activities of mucosal immune indexes. Adding 0.2% nano-curcumin to the diet can alleviate oxidative stress and endoplasmic reticulum stress induced by long-term carbohydrate intake diet, and at the same time, it can reduce serum glucose levels by increasing the expression of liver glucose transport and glycolysis genes, and improve glucose homeostasis.

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 study was approved by the animal research committees of Foshan University Animal Ethics Committee (approval number: 2020056).

AUTHOR CONTRIBUTIONS

XB and YyY designed the experiments. XB executed the experiments, analyzed the data, and wrote the manuscript. MC, YhY, and HL contributed to the sampling. YyY, HY, YY, and ND revised the manuscript. All authors contributed to the article and approved the submitted version.

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Effects of Soybean Lecithin on Growth Performance, Intestine Morphology, and Liver Tissue Metabolism in Rock Bream (*Oplegnathus fasciatus*) Larvae

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Investigations have demonstrated a strong and positive association between dietary intact phospholipid (PL) inclusion and aquatic larval growth, nevertheless, the precise molecular mechanism underlying PL inclusion on growth performance has not been well elucidated. This study aimed to investigate the effects of dietary soybean lecithin (SL) inclusion on growth performance, liver metabolism, resistance to hypoxia stress, and potential molecular mechanisms in rock bream (*Oplegnathus fasciatus*) larvae. Four types of equal-protein and equal-lipid content microdiets (MDs) were formulated with graded levels of SL to achieve phospholipid levels of (PLs, dry matter) 3.84% (SL0), 6.71% (SL4), 9.38% (SL8), and 12.21% (SL12). Rock bream larvae (25 days post-hatching) were fed the respective MDs for 30 days with three replicates. We found that dietary SL inclusion promoted growth performance, survival rate, and stress resistance to hypoxia stress. The increased dietary SL inclusion improved intestinal structure, as shown by the increased perimeter ratio, muscular thickness, and mucosal fold height of the mid-intestinal tissue. Moreover, a high SL inclusion diet (SL12) increased the activity of the key lipolysis-related enzyme (lipase [LP]) in liver tissue but decreased the activity of amino acid catabolism-related enzymes (aspartate aminotransferase [AST] and alanine aminotransferase [ALT]). RNA sequencing results in liver tissue revealed that the SL12 diet increased the transcriptional level of fatty acid activation-related genes (*acsl6* and *acsbg2*), phospholipid catabolism-related genes (*acat2*, *lpin2*, and *crls*), and amino acid synthesis-related genes (*gs*, *csb*, *aldh18a1*, and *oct*), but decreased the expression of amino acid catabolism-related gene *gprt2*. Notably, the SL12 diet significantly increased the expression of ribosome biogenesis-related genes (*pes1*, *nop56*, *nop58*, and *rpf2*) in liver tissue. The ribosome protein-related pathways were the most enriched pathways mapped in the GO database. Collectively, this study demonstrated the necessity of dietary SL for survival, growth performance, promotion of mid-intestinal morphology, and hypoxia stress during the rock bream larval stage. The SL-induced growth performance promotion was likely

attributed to increasing nutrient acquisition by intestinal morphology improvement and to increasing SL catabolism and thereby sparing amino acids for protein synthesis.

Keywords: soybean lecithin, larvae, growth performance, intestinal morphology, liver tissue, fatty acid metabolism

INTRODUCTION

Larval rearing is a bottleneck for aquaculture development. To obtain a large quantity and high-quality larvae, a microdiet (MD) with rich and comprehensive nutrients is indispensable for larval rearing. With numerous studies focused on MD phospholipids (PLs), the inclusion of intact PLs has been recognized to improve culture performance, survival, and stress resistance as well as reduce the occurrence of deformities (Tocher et al., 2008).

Progress has been made to elucidate the limited ability for endogenous *de novo* biosynthesis of PLs during the early life stage of marine fish larvae (Carmona-Antoñanzas et al., 2015). Nevertheless, the precise molecular mechanism underlying PL inclusion on growth performance in the early life stage of fish has not been well elucidated. A series of proposed plausible hypotheses have been proposed to explain the possible mechanism. One of the possible explanations for the requirement for PLs is that PL inclusion increases lipid and fatty acid transportation from enterocytes to the rest of the body through lipoprotein synthesis, as PLs are essential for lipoprotein assembly (Tocher et al., 2008). The beneficial effects of PL inclusion were generally believed to act on intestinal tissue (De Santis et al., 2015). The mid-intestine is the main tissue for nutrient digestion and absorption. A well-developed intestinal structure is important for nutrient digestion, absorption, and subsequent nutrient acquisition. However, there has been a debate about the effects of intact PL inclusion on intestinal morphology at the fish larval stage. Feeding with a PL-deficient diet led to intestinal lumen epithelial damage, whereas high PL inclusion increased goblet cell quantity and therefore aided digestion in Arctic char (*Salvelinus alpinus* L.) larvae (Olsen et al., 1999). However, dietary PL source and level showed no significant effect on the mid-intestinal enterocyte height of Atlantic salmon (*Salmo salar*) larvae (Taylor et al., 2015). Our recent study discovered that the high inclusion level of dietary SL led to intestinal morphology development in yellow drum (*Nibea albiflora*) larvae (Tan et al., 2022). Further studies are warranted to confirm whether dietary PL inclusion can improve intestinal morphology.

After exogenous PLs are absorbed, their biochemical metabolism primarily occurs in liver tissue. Liver tissue plays a central role in the synthesis, metabolism, and redistribution of carbohydrates, proteins, and lipids. Controversial results have been obtained across aquatic species in response to dietary PLs. A previous study on large yellow croaker (*Larimichthys crocea*) noted that increased PL content significantly increased the activity of lipase, the key enzyme for lipid catabolism (Cai et al., 2016). Further studies have elucidated that PLs decrease excessive lipid accumulation by inhibiting fatty acid uptake and lipid synthesis, together with promoting lipid export at the transcriptional level (Cai et al., 2017). Consistently, PL inclusion decreased hepatic lipid content in blunt snout (*Megalobrama amblycephala*) fingerlings (Li et al., 2015) and juvenile hybrid snakehead (*Channa argus*×*Channa maculata*) (Lin et al., 2018). However, a recent study on hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂) reported that dietary PL inclusion increased hepatic crude lipid and triglyceride contents in a dose-dependent manner, possibly by regulating the synthesis and hydrolysis of triglycerides (Huang et al., 2021). Despite this being the case, most of the studies in the field of PLs have only focused on lipid metabolism but fail to explain the growth promotion effect of dietary PL inclusion.

The over-exploitation of wild fisheries decreased the availability of phospholipid from fish meal and fish oil. The sustainable, alternative to marine products is plant-based, but these contain very little phospholipid in comparison with marine products. Crude soybean oil contains around 1.5-3.1% phospholipids that are removed to become the by-product, SL (Tocher et al., 2008). SL is widely used in diet manufacture and the supplementation of SL in larvae MD has attracted much attention. Individual growth, more precisely protein retention, is closely related to lipid metabolism (Francis and Turchini, 2017). Based on the finding that dietary PLs can serve as a preferential source of energy in the larval stage, the larvae could decrease the amino acid flux into the tricarboxylic acid (TCA) cycle and thereby spare precious amino acids for body constitution. We propose a hypothesis that the growth promotion effect of high dietary SL inclusion was partially attributed to the protein-sparing effect. To our knowledge, there is no relevant research focusing on protein or amino acid metabolism regarding dietary SL inclusion in fish larvae. The mechanistic investigation and systematic understanding of how dietary SL level affects growth performance and liver tissue metabolism in fish larvae are still in the preliminary stage.

Unique molecular identifiers (UMIs) RNA sequencing has provided a method to accurately qualify mRNA duplicates because they have identical alignment coordinates and identical UMI sequences (Alejo and Tafalla, 2011). Rock bream (*Oplegnathus fasciatus*) is a vital commercial species in the East China Sea. Due to overexploitation and environmental pressures, rock bream stocks have experienced a drastic decline in recent years. To date, the strategy for improving their survival and growth is still unclear. This has hindered the industrialization and commercialization of this species. Therefore, the overall purpose of the present study was to investigate the effects of dietary SL inclusion on growth performance and the underlying molecular mechanism using UMI-RNA sequencing. The present study could contribute to the development of the rock bream larval MD. Moreover, this study could provide a better understanding of how dietary SL affects growth performance at the larval stage.

MATERIALS AND METHODS

All animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals formulated by the Ministry of Science and Technology of China. The study was approved by the Committee on Ethics of Animal Experiments of Zhejiang Ocean University and was performed in compliance with the ethical principles and standards of Frontiers in Marine Science. Prior to sampling, the larvae were anesthetized with 10 mg L^{-1} tricaine methanesulfonate (MS-222, Sigma, USA) until the respiratory opercula stopped moving.

Chemical Analysis

The crude protein, lipid, moisture, and ash contents of the ingredients, larval body, and MDs were determined according to the methods of the Association of Official Analytical Chemists (AOAC, 2016). The MDs were placed in an oven at 105°C for 7 hours to a constant weight. Crude protein content was determined using the Kjeldahl method with an automatic digester (KjelFlex K-360, BUCHI, Switzerland). Ether extraction was used to determine crude lipids using a Soxtec System HT (Soxtec 2055, FOSS Tecator, Sweden). Ash content was determined by burning off the organic matter in a muffle furnace at 550°C for 6 hours.

Total lipid isolation and purification procedures were the same as described previously description (Folch et al., 1957). Briefly, the lipids were extracted by homogenizing the freeze-dried samples with 2:1 chloroform-methanol (v/v) and filtering the homogenate. Afterward, the filtrate was freed from these substances by being placed in contact with over 5-fold its volume of water. The lipid solvent was evaporated under nitrogen gas and desiccated in a vacuum for at least 16 hours. The fatty acid methyl esters (FAMEs) were prepared using the procedures in a previous description (Zuo et al., 2012). Fatty acids were eventually reported as mol % of total identified fatty acids. The amino acid content of MDs and the larval body was analyzed as described previously (Wu et al., 2019).

The PL content of MDs and the larval body was determined using the molybdenum blue colorimetry method. Briefly, total lipids were extracted as described in the fatty acid profile analysis. Dietary PLs from the extracted lipids were burned to phosphorus pentoxide and further oxidized to phosphoric acid by hydrochloric acid. Phosphoric acid and sodium molybdate formed sodium phosphomolybdate, which was further reduced to molybdenum blue by hydrazine sulfate. A colorimetric analysis was performed using a visible spectrophotometer at 650 nm wavelength (Biochrom Ultrospec 2100 pro, England). The number of larval bodies was not sufficient to be analyzed for each individual tank; therefore, the PL content determination was pooled together for each treatment.

Microdiet Preparation

The MD formulation was primarily based on our previous study by Tan et al. (2022). Fish meal, low-temperature krill meal, low-temperature squid meal, and casein meal were the main protein sources. Fish oil, soybean oil, and SL served as the main lipid sources (**Table 1**). MDs were incorporated with increasing SL content (Cargill, Germany): 0% (SL0), 4% (SL4), 8% (SL8), and 12% (SL12). The diet formulation was as follows: protein sources, yeast, hydrolyzed fish paste, α-starch, sodium alginate, vitamin premix, mineral premix, ascorbyl polyphosphate, attractant, and mold inhibitor were mashed into a fine powder and passed through a 125-μm mesh. These ingredients were thoroughly mixed according to the proportion and then mixed with the remaining ingredients, including fish oil, soybean oil, SL, ethoxyquinine, and chloride choline solution, to make a dough. Strips of 1.0 mm diameter were processed in an F-26-type screw plodder (South China University of Technology, China) and then ripened for approximately 3 hours in an oven at 60°C. The strips were then smashed in a granulating machine (South China University of Technology, China) and further sifted into three different particle sizes: over 178 μm, 125 to 178 μm, and under 125 μm. The prepared MDs were packed and stored at −20°C until use.

The PL contents (dry matter) were 3.84% (SL0), 6.71% (SL4), 9.38% (SL8), and 12.21% (SL12). The decrease in soybean oil content in MDs was compensated for by SL inclusion (**Table 1**). Composition analyses of the dietary lipid sources and MDs indicated only a trace amount of docosahexaenoic acid (DHA, C22:6n-3) and eicosapentaenoic acid (EPA, C20:5n-3) in SL, with similar fatty acid profiles obtained among the MDs (**Table 2**).

Larval Rearing

The larvae were reared in a 40-m^3 pool after hatching on Xixuan Island (Zhoushan, Zhejiang, China). The larvae were fed rotifers (*Brachionus rotundiformis*) from 3 days post-hatching (dph) (mouth opening) to 20 dph and a mix of rotifers with *Artemia* nauplii from 20 to 22 dph. To acclimatize the experimental larvae, 1800 larvae (150 larvae per tank, 25 dph) were transferred to a flowing water system with water temperatures of 20-23°C, a salinity of 28 ± 1 ‰, and dissolved oxygen of over 6.0 mg per liter of seawater. To wean the larvae onto an MD, they were fed a mix of *Artemia* nauplii and MD for an additional 3 days prior to the feeding trial (22-24 dph). During the acclimation period, dead larvae were siphoned out, and fresh individuals were added to maintain a fixed quantity of larvae in each tank. During the feeding trial, larvae (initial body weight [IBW] 15.1 mg; initial body length [IBL] 1.01 cm) were hand-fed with the MDs every 2 hours from 06:00 to 20:00 (7 times a day). The amount of water volume in each tank was doubled every day (flow rate ~0.5 L/min). Using the siphon method, the tank was cleaned twice daily to remove feces, residual bait, and corpses. The larval corpses were carefully identified and recorded. From 0 to 10 days of larval rearing, the larvae were fed MD particles under 125 μm in size. Then, the larvae were fed with MDs of larger particle sizes.

Sample Collection

Prior to the beginning of the feeding trial, 50 larvae were randomly collected, drained of seawater, and weighed to obtain

the total weight, and then the total length of each larva was measured and recorded. In the middle of the feeding trial (15-day rearing, larvae 40-dph), seven larvae per tank were randomly collected to measure the whole length (WL, length from the front of the mouth to the end of the caudal fin), body length (BL, length from the front of the mandible to the root of the caudal fin), and body weight (BW).

At the end of the feeding trial (larvae 55-dph), the mid-intestinal tissues of 2 larvae pre-tank were randomly collected under a dissecting microscope and stored in a 4% paraformaldehyde (PFA) solution for hematoxylin and eosin (H&E) section analysis. Twenty larvae were reserved per tank for the air exposure challenge, and the WL, BL, and BW of the remaining larvae were measured and recorded individually. The liver tissue of ten larvae per tank was randomly collected and pooled together in 1.5 mL microtubes (Biotech, China). These hepatic samples were immediately frozen in liquid nitrogen and stored at −80°C before enzyme activity analysis. After washing with phosphate-buffered saline (PBS), the liver tissue of three larvae per tank from larvae fed SL0 and SL12 were randomly collected and pooled (n=3 for each treatment), and tissues were stored in RNAlater (Thermo Fisher Scientific, USA) at −20°C until total RNA extraction. The remaining sampled larval bodies were collected by tank and stored at −20°C until proximate composition analysis and fatty acid and amino acid profile analysis.

Hypoxia Stress Challenge

After the sampling was conducted, the rest of the fish (20 larvae per tank) were fed with corresponding MDs for an additional two days. The primary experiments were conducted to determine the appropriate time for the hypoxia stress challenge. At the beginning of the hypoxia stress challenge, 20 larvae from each tank were captured by a mesh net. The larvae were exposed to air for an accurate 5 minutes and then returned to their original tank. Over the next 24 hours, the dead fish were collected, and body parameters were recorded. After completing the aforementioned steps, all live larval bodies were weighed (BW), measured (WL and BL), collected, and stored at −20°C for the analysis of larval body PL content.

TABLE 1 | Formulation and nutrient analysis of microdiets (MDs) (%, dry weight).

Ingredients	Microdiets (phospholipid level,% dry weight)			
	SL0 (3.84)[1]	SL4 (6.71)[2]	SL8 (9.38)[3]	SL12 (12.21)[4]
Fish meal[5]	21.00	21.00	21.00	21.00
Low-temperature krill meal[6]	14.00	14.00	14.00	14.00
Low-temperature squid meal[7]	12.00	12.00	12.00	12.00
Casein[8]	20.00	20.00	20.00	20.00
Yeast[9]	3.00	3.00	3.00	3.00
Hydrolyzed fish paste[10]	4.00	4.00	4.00	4.00
α-starch[11]	4.00	4.00	4.00	4.00
Sodium alginate	1.50	1.50	1.50	1.50
Vitamin premix[12]	1.50	1.50	1.50	1.50
Mineral premix[13]	1.50	1.50	1.50	1.50
Ascorbyl polyphosphate	0.20	0.20	0.20	0.20
Attractant[14]	2.00	2.00	2.00	2.00
Mold inhibitor[15]	0.05	0.05	0.05	0.05
Ethoxyquinine	0.05	0.05	0.05	0.05
Chloride choline	0.20	0.20	0.20	0.20
Fish oil	3.00	3.00	3.00	3.00
Soybean oil	12.00	8.00	4.00	0.00
SL[16]	0.00	4.00	8.00	12.00
Total	100.00	100.00	100.00	100.00
Proximate nutrients (mean values, % dry weight)[17]				
Phospholipids	3.84	6.71	9.38	12.21
Crude protein	57.25	57.58	60.17	58.95
Crude lipid	21.03	20.96	20.08	20.89
Ash	9.31	9.57	9.07	9.22

[1-4]0%, 4%, 8%, and 12% SL was added to the microdiets.

[5-11]Fish meal: crude protein, 72.47%, crude lipid, 9.64%; low-temperature krill meal: crude protein, 63.65%, crude lipid, 7.55%; low-temperature squid meal: crude protein, 66.81%, crude lipid, 13.35%; casein: crude protein, 93.62%, crude lipid, 0.76%; yeast: crude protein, 42.6%, crude lipid, 1.00%; hydrolyzed fish paste: 76.92% crude protein, 0.51% crude lipid; α-starch: crude protein, 0.38%, crude lipid, 0.25%.

[12]Vitamin premix (mg or g kg^{-1} diet): carotene, 0.10 g; vitamin D, 0.05 g; tocopherol, 0.38 g vitamin B_1, 0.06 g; vitaminB_2, 0.19 g; vitamin B_6, 0.05 g; cyanocobalamin, 0.1 mg; biotin, 0.01 g; inositol, 3.85 g; niacin acid, 0.77 g; pantothenic acid, 0.27 g; folic acid, 0.01 g; chloride choline, 7.87 g; and cellulose, 1.92 g.

[13]Mineral premix (mg or g kg^{-1} diet): NaF, 2 mg; KI, 0.8 mg; $CoCl_2{\cdot}6H_2O$, 50 mg; $CuSO_4{\cdot}5H_2O$, 10 mg; $FeSO_4{\cdot}H_2O$, 80 mg; $ZnSO_4{\cdot}H_2O$, 50 mg; $MnSO_4{\cdot}H_2O$, 60 mg; $MgSO_4{\cdot}7H_2O$, 1200 mg; $Ca(H_2PO_4)_2{\cdot}H_2O$, 3000 mg; NaCl, 100 mg; and zoelite powder, 15.45 g.

[14-16]Attractant: glycine:betaine = 1:3; mold inhibitor: fumaric acid:calcium propionate = 1:1; SL: Cargill Co., Ltd, Germany, EPIKURON 100G, containing approximately 630 g phospholipids/kg, including 230 g/kg phosphatidylcholine, 190 g/kg phosphatidylethanolamine, 70 g/kg phosphatidylic acid, and 140 g/kg phosphatidylserine.

[17]Proximate nutrients were presented as the mean to two replicates.

Intestinal Structure by H&E Section Analysis

Mid-intestinal samples were sliced transversely into 6 μm sections and stained with H&E according to a previously described method (Baeverfjord and Krogdahl, 1996; Dimitroglou et al., 2009). A Panoramic 250 FLASH digital pathology system (3DHISTECH, Hungary) was adopted to image the slices. The mucosal fold height, muscular thickness, and perimeter ratio of the intestinal tissue slices were further analyzed according to previously described methods (Penn et al., 2011). The mucosal fold height, perimeter ratio, and muscular thickness were determined from 24 slices (6 slices for each treatment).

Enzyme Activity Analysis

To analyze hepatic metabolism enzyme activities, samples were homogenized with cold PBS (0.1 M, pH 7.0, 4°C) at a proportion of 1:9 (m/v). Afterward, the homogenate was centrifuged at 4°C at 3000 ×*g* for 10 minutes. The sediment was discarded, and the supernatant was stored at – 80°C until analysis. Using commercial kits (a total protein quantitative assay kit, alanine aminotransferase [ALT] kit, aspartate aminotransferase [AST] kit, and lipase [LP] assay kit, Nanjing Jiancheng Bioengineering Institute, China), the total protein content and ALT, AST, and LP enzyme activities were analyzed following the manufacturer's instructions. To

TABLE 2 | Lipid sources, and MD fatty acid composition (%, identified fatty acids).

Fatty acids	Lipid sources		Microdiets (phospholipid level, % dry weight)			
	SL	SO	SL0 (3.84)	SL4 (6.71)	SL8 (9.38)	SL12 (12.21)
C12:0	ND [8]	ND	0.05	0.05	0.05	0.06
C14:0	0.11	0.08	3.02	3.04	3.06	3.06
C15:0	0.06	0.01	0.25	0.24	0.26	0.28
C16:0	18.55	10.68	12.63	14.49	16.58	18.74
C17:0	0.15	0.09	0.30	0.34	0.28	0.42
C18:0	4.25	3.99	3.89	4.04	4.09	4.14
C20:0	0.22	ND	0.38	0.38	0.35	0.30
C21:0	ND	0.05	ND	ND	ND	ND
C22:0	0.42	ND	0.30	0.28	0.25	0.27
C24:0	0.25	ND	0.10	0.12	0.16	0.17
∑SFAs[1]	24.00	14.90	20.92	22.98	25.08	27.44
C14:1	ND	ND	0.04	0.07	0.06	0.02
C16:1	0.49	0.09	2.63	2.99	3.24	3.49
C17:1	0.02	0.05	0.21	0.23	0.24	0.28
C18:1n-9t[2]	0.01	0.04	0.09	0.10	0.14	0.15
C18:1n-9c[3]	12.01	25.49	22.86	19.24	16.66	13.79
C20:1n-9	0.08	0.56	0.44	0.32	0.20	0.12
C22:1n-9	ND	0.04	0.12	0.09	0.09	0.09
C24:1n-9	0.03	ND	0.13	0.16	0.21	0.24
∑MUFAs[4]	12.64	26.28	26.52	23.20	20.84	18.18
C18:2n-6t	ND	0.34	0.04	0.03	0.04	ND
C18:2n-6c	55.35	51.36	35.16	37.64	38.98	40.19
C18:3n-6	0.06	0.03	0.25	0.26	0.28	0.30
C20:3n-6	ND	0.04	1.02	1.07	1.06	0.99
C20:4n-6	0.06	ND	0.33	0.36	0.38	0.41
∑n-6 PUFAs[5]	55.48	51.77	36.80	39.37	40.74	41.84
C20:2	0.13	0.42	1.07	1.06	0.85	0.64
C22:2	ND	0.14	0.20	0.24	0.26	0.30
C18:3n-3	7.15	6.50	3.72	3.98	4.10	4.22
C20:3n-3	0.15	ND	ND	0.12	0.12	0.18
C20:5n-3	ND	ND	0.42	0.47	0.56	0.59
EPA	0.41	ND	3.04	3.16	3.26	3.36
DHA	0.03	ND	3.58	3.60	3.62	3.66
∑n-3 PUFAs[6]	7.75	6.50	10.76	11.33	11.66	12.01
∑n-3 LC-PUFAs[7]	0.59	ND	7.04	7.35	7.55	7.79
DHA/EPA	0.07	–	1.17	1.14	1.11	1.09

Values are the means of two replicates.

[1]SFAs,saturated fatty acids.

[2]t: trans-fatty acids.

[3]c: cis-fatty acids.

[4]MUFAs,monounsaturated fatty acids.

[5]n-6 PUFAs:n-6 polyunsaturated fatty acids.

[6]n-3 PUFAs:n-3 polyunsaturated fatty acids.

[7]n-3 LC-PUFAs:n-3 long-chain polyunsaturated fatty acids.

[8]ND,not detected.

normalize the enzyme activity, the tissue protein concentration was utilized.

UMI-RNA-Seq and Bioinformatics Analysis

The total RNA of the liver tissue from larvae fed a PL-insufficient diet (SL0) and PL-sufficient diet (SL12) was extracted using TRIzol reagent (Invitrogen, USA). RNA degradation was monitored in a 1% agarose gel, and RNA purity was measured using a NanoPhotometer® spectrophotometer (Implen, USA). The RNA concentration was determined using a Qubit® RNA Assay Kit with Qubit® 2.0 Fluorometer (Life Technologies, USA). RNA integrity was measured using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, USA). Clustering and sequencing were performed with the help of the Experimental Department of Novogene Co., Ltd. (Beijing, China). All libraries were sequenced using the Illumina HiSeq 2500 sequencing platform (Illumina, USA). The library preparation for RNA-seq, sequence quality assessment and assembly, annotation, and differential expression gene (DEG) analyses were as described in detail in our previous publication with some differences (Xu et al., 2018). In the synthesis of second-strand cDNA, a UMI label and sequencing adaptor were added to the cDNA duplicates. After RNA sequencing, UMI reads were mapped to the genome sequences of the rock bream. The DEG cutoff was set as an adjusted P (q) value < 0.05 and fragments per kilobase per million (FPKM) fold change > 2. Detailed information on the sequencing data and mapping are summarized in **Table S1**.

DEG Validation by Real-Time Quantitative PCR

Total RNA was extracted from identical samples used for Illumina sequencing. Reversed cDNA was synthesized using a Prime Script RT Reagent Kit with a gDNA eraser (Takara, Japan) following the manufacturer's instructions. The gene expression profiles of the 12 target genes were validated by real-time quantitative PCR (qRT–PCR). The primers used are summarized in **Table S2**. PCR was performed using an ABI Real-Time PCR machine (ABI Step One Plus, ABI, USA). The program settings used were the same as those described in our previous study (Wu et al., 2019). Briefly, SYBR® Premix Ex Taq (TaKaRa, Japan) was used for RT-qPCR. Each PCR reaction system consisted of 10.0 μl SYBR® Premix Ex Taq, 0.4 μl of each forward and reverse primer (10 μM), 0.4 μl ROX Reference Dye, 2 μl cDNA, and 6.8 μl dH_2O in a total volume of 20 μl. Then, RT-qPCR was performed on a StepOnePlus™ Real-Time PCR machine (Applied Biosystems, USA). The standard procedure for amplification was as follows: 95°C for 30 s and 40 cycles of 95°C for 5 s, 57°C for 30 s. β-actin and *g6pdh* were used as internal controls for the normalization of gene expression levels. Expression levels were calculated using the comparative CT method (Livak & Schmittgen, 2001). The relative expression level of target genes was calculated by dividing the copy number of target genes by the geometric mean copy number of the two reference genes (Vandesompele et al., 2002).

Calculations and Data Statistics

The following variables were calculated:

Final body weight (FBW, g) = W_t/N_t

Survival rate (%) = $100 \times N_t/N_0$

Survival rate after hypoxia stress (%) = 100 × larvae survival quantity/number of larvae for the stress test

Specific growth rate (SGR, %/d) = $100 \times [\text{Ln}(W_t/N_t) - \text{Ln}(W_0/N_0)]/t$

Weight gain rate (WGR, %) = $100 \times (W_t - W_0)/W_0$

Condition factor (CF, %) = $100 \times (\text{BW, g})/(\text{BL, cm}^3)$

where W_t and W_0 are the sums of the final and initial larval BWs, respectively; N_t and N_0 are the final and initial larval quantities in each tank, respectively; and t is the experimental duration in days. All data were processed using GraphPad version 9.0.0 for Windows (USA, www.graphpad.com) and are presented as the mean ± standard error of the mean (S.E.M.). The normality and homogeneity of variances of the data were confirmed using the Levene test. The Kruskal–Wallis test was performed when the data were not homogeneous. Otherwise, one-way ANOVA with Tukey's HSD test was used to assess significant differences among treatments at a significance level of $P < 0.05$. The DEG association networks were predicted by String (string-db.org) and further subjected to Cytoscape software (www.cytoscape.org) (Shannon et al., 2003). We performed a multidimensional correlation analysis using the R packages ggplot2, linkET, and dplyr. A schematic representation of the effects of the PL-rich diet (SL12) on hepatic metabolic and physiological responses was created with "BioRender.com".

RESULTS

Larval Growth Performance

At the end of 15 days of rearing (larvae 40-dph), the statistical results showed that the WL, BL, BW, WGR, and SGR of the larvae fed the SL4 and SL8 diets were significantly higher than those of larvae fed the SL0 diet ($P < 0.05$). The larvae fed the SL12 diet showed significantly higher WL, BL, and SGR than those fed the SL0 diet ($P < 0.05$). There was no significant difference in CF among treatments ($P > 0.05$) (**Table 3**).

At the end of the feeding trial (larvae 55-dph), the larvae fed the SL12 diet showed significantly higher WL, BL, BW, WGR, and SGR than those fed the SL0 diet ($P < 0.05$). The WL and BL were significantly higher in the larvae fed the SL8 diet than in the larvae fed the SL0 diet ($P < 0.05$). There was no significant difference in the aforementioned parameters between the larvae fed the SL4 and SL0 diets ($P > 0.05$). There was also no significant difference in CF among treatments ($P > 0.05$) (**Table 3**).

Larval Body Proximate Nutrient, Fatty Acid, and Amino Acid Profiles

The increased dietary SL inclusion significantly increased larval whole-body ash and protein contents ($P < 0.05$). The whole-body

lipid content was significantly higher in the larvae fed the SL4 diet than in the rest of the groups ($P < 0.05$). The larval whole-body PL content reached comparable values among groups (**Table 4**).

The dietary fatty acid composition was roughly reflected by the composition of larval bodies. Significantly increased saturated fatty acids (SFAs) and decreased monosaturated fatty acids (MUFAs) proportions were observed in the larvae with increasing dietary SL inclusion ($P < 0.05$). No significant differences in n-6 polyunsaturated fatty acids (PUFAs), n-3 PUFAs, or n-3 long-chain polyunsaturated fatty acids (LC-PUFAs) were observed among the groups ($P > 0.05$) (**Table 5**).

The larval whole-body amino acid content showed an increasing trend with dietary PL content increasing from 3.84% to 12.21% (**Table 6**). The essential amino acids (EAAs) and nonessential amino acids (NEAAs) contents of the larval body were found to be significantly higher in the SL12 and SL8 groups than in the SL4 group ($P < 0.05$) (**Table 6**).

Larval Survival, Resistance to Hypoxia Stress, and Hepatic Metabolism-Related Enzyme Activities

The siphon method was adopted to collect corpses, and the corpse quantity was carefully recorded twice daily (**Figure 1A**). At the end of the feeding trial, the survival rates were significantly higher in larvae fed the SL8 ($P <0.01$) and SL12 ($P <0.05$) diets than in the larvae fed the SL0 diet. Similarly, the resistance to hypoxia test showed that the high dietary inclusion of SL (SL8 and SL12 groups) increased the survival rate ($P<0.05$) (**Figure 1B**).

Results of metabolism-related enzyme activity in liver tissue indicated that the SL12 and SL8 diets significantly decreased hepatic ALT activity (**Figure 1C**) ($P<0.05$). Moreover, feeding with the SL12 diet significantly decreased the hepatic AST activity compared with that of the larvae fed the SL0 or SL4 diet (**Figure 1D**) ($P<0.05$). In contrast, the larvae fed the SL12 diet showed significantly higher hepatic LP activity than the larvae fed the SL0 diet (**Figure 1E**) ($P <0.05$).

Larval Mid-Intestinal Structure Analysis

Mid-intestinal histological sections in 55-dph larvae revealed improved intestinal structure with increased SL inclusion (**Figure 2**). Statistical analysis of the mid-intestinal structure of 55-dph larvae demonstrated that insufficient inclusion of dietary SL (SL0) significantly decreased the mucosal fold height, muscular thickness, and perimeter ratio ($P < 0.05$). The SL12 group showed the best intestinal structure among the groups.

Liver Tissue RNA-Seq Quality Control, Mapping, and Bioinformatics Analysis

Clean reads were mapped on the annotated genome of rock bream, and an average of 96.88% of the UMI reads could be successfully mapped, of which an average of 89.42% were uniquely mapped (**Table S1**).

Based on the FPKM method, a total of 427 significant DEGs, including 126 up- and 301 downregulated DEGs, were detected in the SL0 versus SL12 groups (**Figure 3A**). Among the DEGs, some protein metabolism-related genes (*gpt2*, *gs*, and *pro1*) as well as lipid metabolism-related genes (*acsbg2*, *acsl6*, *crls*, *lpin2*, *acsbg2*, *pemt*, and *mgll*), were observed. The heatmap (**Figure 3B**) presented special selected DEGs involved in metabolism.

To validate the DEGs identified by RNA-seq analysis, we performed qRT–PCR on 12 selected genes (**Figure 3C**). The qRT–PCR analysis revealed that all genes shared the same expression tendencies as those from the transcriptome data (ΔΔCt versus $\log_2$ fold change). Pearson's correction analysis indicated that the r-value was 0.9403 between these two methods, and the *P*-value was below 0.0001. The qRT–PCR results confirmed the credibility of the UMI-RNA-seq data in this study.

All DEGs were subjected to Gene Ontology (GO) analysis, and the results showed strong enrichment of DEGs in two main categories: biological processes and molecular functions (**Figure 3D**). Among them, ribonucleoprotein complex biogenesis, metabolic process, and glutamine metabolic process were the most enriched GO pathways. The top 18

TABLE 3 | Growth performance and body indices of 40-dph and 55-dph rock bream larvae.

	Parameters	Microdiets (phospholipid level,% dry weight)			
		SL0 (3.84)	SL4 (6.71)	SL8 (9.38)	SL12 (12.21)
40 dph	WL (cm)	1.71 ± 0.09^{c}	2.14 ± 0.03^{ab}	2.24 ± 0.04^{a}	1.96 ± 0.07^{b}
	BL (cm)	1.36 ± 0.07^{b}	1.70 ± 0.03^{a}	1.78 ± 0.04^{a}	1.60 ± 0.05^{a}
	BW (g)	0.08 ± 0.01^{b}	0.14 ± 0.01^{a}	0.17 ± 0.02^{a}	0.12 ± 0.01^{ab}
	WGR (%)	414.4 ± 68.6^{b}	851.8 ± 68.0^{a}	1011.3 ± 117.9^{a}	736.3 ± 79.7^{ab}
	SGR (%/d)	11.56 ± 1.01^{b}	16.06 ± 0.50^{a}	17.12 ± 0.75^{a}	15.10 ± 0.72^{a}
	CF	0.031 ± 0.001	0.029 ± 0.001	0.029 ± 0.001	0.031 ± 0.001
55 dph	WL (cm)	3.38 ± 0.20^{b}	4.05 ± 0.24^{ab}	4.20 ± 0.05^{a}	4.54 ± 0.16^{a}
	BL (cm)	2.76 ± 0.18^{c}	3.20 ± 0.13^{bc}	3.44 ± 0.01^{ab}	3.75 ± 0.20^{a}
	BW (g)	0.72 ± 0.18^{b}	1.04 ± 0.11^{ab}	1. ± 0.02^{ab}	1.48 ± 0.09^{a}
	WGR (%)	4671.1 ± 1180.9^{b}	6803.5 ± 697.8^{ab}	7525.1 ± 112.8^{ab}	9732.2 ± 585.7^{a}
	SGR (%/d)	12.70 ± 1.32^{b}	14.08 ± 0.62^{ab}	14.45 ± 0.10^{ab}	15.28 ± 0.20^{a}
	CF	0.033 ± 0.002	0.032 ± 0.001	0.028 ± 0.001	0.028 ± 0.001

WL, whole length; BL, body length; BW, body weight; WGR, weight gain rate; SGR, specific growth rate; CF, condition factor. Values (mean ± S.E.M., n=3) within a row sharing the same superscript letter or without a superscript are not significantly different from the other experimental treatments (P > 0.05).

TABLE 4 | Larval body proximate composition of 55-dph rock bream larvae.

Parameters	Microdiets (phospholipid level, % dry weight)			
	SL0 (3.84)	**SL4 (6.71)**	**SL8 (9.38)**	**SL12 (12.21)**
Moisture (%)	76.92 ± 1.04[a]	76.46 ± 0.81[ab]	76.71 ± 0.63[ab]	75.98 ± 1.05[b]
Ash (%)	3.20 ± 0.01[c]	3.29 ± 0.03[b]	3.36 ± 0.03[b]	3.72 ± 0.07[a]
Lipid (%)	3.98 ± 0.01[b]	4.54 ± 0.02[a]	3.94 ± 0.04[b]	3.98 ± 0.02[b]
Protein (%)	14.95 ± 0.05[b]	14.78 ± 0.01[b]	14.78 ± 0.04[b]	15.27 ± 0.12[a]
PLs content (%)	3.97	4.53	3.94	3.97

PL content, total phospholipid content. All larvae in the same group were pooled together to analyze the total phospholipid content because the sample was insufficient for individual analysis. Values (mean ± S.E.M., n=3) within a row sharing the same superscript letter or without a superscript are not significantly different from the other experimental treatments (P > 0.05).

TABLE 5 | Fatty acid composition (%, identified fatty acids) of 55-dph rock bream larvae.

Fatty acids	Microdiets (phospholipid level, % dry weight)			
	SL0 (3.84)	**SL4 (6.71)**	**SL8 (9.38)**	**SL12 (12.21)**
C12:0	0.10 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.49 ± 0.66
C14:0	4.35 ± 0.47[b]	5.11 ± 0.30[ab]	4.88 ± 0.83[ab]	5.57 ± 0.45[a]
C15:0	0.54 ± 0.06[b]	0.66 ± 0.05[b]	0.65 ± 0.13[b]	0.83 ± 0.08[a]
C16:0	25.21 ± 3.11[b]	29.18 ± 0.97[ab]	27.56 ± 3.79[ab]	31.08 ± 1.82[a]
C17:0	0.59 ± 0.19	0.73 ± 0.22	0.72 ± 0.30	0.84 ± 0.27
C18:0	9.75 ± 0.84	11.04 ± 0.37	9.99 ± 1.44	10.47 ± 0.84
C20:0	0.54 ± 0.01	0.59 ± 0.03	0.59 ± 0.02	0.51 ± 0.03
C21:0	0.01 ± 0.01	0.31 ± 0.36	0.32 ± 0.46	0.42 ± 0.45
C22:0	0.27 ± 0.01	0.30 ± 0.03	0.27 ± 0.05	0.27 ± 0.03
C23:0	ND	0.03 ± 0.05	ND	0.02 ± 0.04
C24:0	0.16 ± 0.02	0.18 ± 0.03	0.18 ± 0.04	0.21 ± 0.04
SFAs	41.53 ± 4.30[b]	48.25 ± 1.73[ab]	45.29 ± 7.03[ab]	50.73 ± 4.09[a]
C14:1	0.06 ± 0.00	0.05 ± 0.04	0.08 ± 0.01	0.08 ± 0.02
C15:1	ND	ND	ND	ND
C16:1	4.12 ± 0.58	3.23 ± 2.68	2.66 ± 3.57	5.47 ± 0.71
C17:1	0.16 ± 0.13	0.14 ± 0.14	0.12 ± 0.05	0.12 ± 0.03
C18:1n9t	0.28 ± 0.07	0.13 ± 0.10	0.30 ± 0.02	0.26 ± 0.03
C18:1n9c	34.28 ± 3.84[a]	33.41 ± 0.46[a]	28.03 ± 2.20[b]	24.97 ± 1.97[b]
C20:1n9	0.22 ± 0.01[b]	0.25 ± 0.02[ab]	0.28 ± 0.02[a]	0.28 ± 0.00[a]
C22:1n9	0.18 ± 0.04	0.16 ± 0.04	0.24 ± 0.08	0.20 ± 0.04
C24:1n9	0.24 ± 0.12	0.27 ± 0.06	0.23 ± 0.18	0.34 ± 0.17
MUFAs	39.56 ± 4.46[a]	37.64 ± 2.68[ab]	31.92 ± 5.83[b]	31.72 ± 2.78[b]
C18:2n6t	0.16 ± 0.05	0.11 ± 0.05	0.17 ± 0.09	0.12 ± 0.02
C18:2n6c	14.09 ± 6.07	10.31 ± 3.21	15.95 ± 9.17	12.67 ± 5.00
C18:3n6	0.18 ± 0.03	0.25 ± 0.01	0.26 ± 0.02	0.30 ± 0.02
C20:3n6	0.58 ± 0.18[b]	0.69 ± 0.25[ab]	0.63 ± 0.48[ab]	1.06 ± 0.05[a]
C20:4n6	0.11 ± 0.10	0.09 ± 0.06	0.22 ± 0.15	0.16 ± 0.07
n-6 PUFA	15.12 ± 6.35	11.45 ± 3.04	17.24 ± 8.91	14.31 ± 5.01
C18:3n3	0.88 ± 0.66	0.39 ± 0.45	1.08 ± 1.31	0.63 ± 0.82
C20:3n3	0.08 ± 0.07	0.07 ± 0.06	0.15 ± 0.09	0.17 ± 0.03
C20:5n3	0.98 ± 0.53	0.77 ± 0.51	1.64 ± 0.73	0.74 ± 0.51
C22:6n3	0.71 ± 0.63	0.44 ± 0.39	1.57 ± 1.71	1.04 ± 0.78
DPA	0.16 ± 0.14	0.10 ± 0.10	0.31 ± 0.43	0.21 ± 0.17
n-3 PUFA	2.81 ± 2.00	1.77 ± 0.52	4.74 ± 4.27	2.80 ± 1.34
n-3 LC-PUFA	1.84 ± 1.27	1.38 ± 0.27	3.36 ± 2.96	2.17 ± 0.77
C20:2	0.65 ± 0.28	0.70 ± 0.28	0.38 ± 0.43	0.48 ± 0.38
C22:2	0.34 ± 0.15	0.32 ± 0.05	0.40 ± 0.07	0.31 ± 0.05

Values (mean ± S.E.M., n=3) within a row sharing the same superscript letter or without a superscript are not significantly different from the other experimental treatments (P > 0.05).

[1]SFAs,saturated fatty acids.

[2]t:trans-fatty acids.

[3]c:cis-fatty acids.

[4]MUFAs,monounsaturated fatty acids.

[5]n-6 PUFAs: n-6 polyunsaturated fatty acids.

[6]n-3 PUFAs: n-3 polyunsaturated fatty acids.

[7]n-3 LC-PUFAs: n-3 long-chain polyunsaturated fatty acids.

[8]ND,not detected.

TABLE 6 | Amino acid composition of the MD and 55-dph rock bream larvae (mg/g, dry matter).

Amino acids	MD	Microdiets (phospholipid level,% dry weight)			
		SL0 (3.84)	SL4 (6.71)	SL8 (9.38)	SL12 (12.21)
Arginine	2.97	3.55 ± 0.19^{ab}	3.30 ± 0.26^{b}	3.76 ± 0.02^{a}	3.75 ± 0.05^{a}
Histidine	0.96	1.23 ± 0.08^{b}	1.19 ± 0.04^{b}	1.44 ± 0.07^{a}	1.39 ± 0.08^{a}
Isoleucine	1.99	2.17 ± 0.13^{ab}	2.05 ± 0.16^{b}	2.30 ± 0.08^{a}	2.31 ± 0.04^{a}
Leucine	2.95	3.88 ± 0.25^{ab}	3.63 ± 0.31^{b}	4.11 ± 0.09^{a}	4.13 ± 0.08^{a}
Lysine	3.25	4.15 ± 0.27^{ab}	3.91 ± 0.39^{b}	4.40 ± 0.15^{a}	4.49 ± 0.02^{a}
Methionine	0.85	1.47 ± 0.22	1.39 ± 0.23	1.40 ± 0.10	1.44 ± 0.17
Phenylalanine	1.98	2.30 ± 0.12^{ab}	2.14 ± 0.18^{b}	2.39 ± 0.10^{a}	2.40 ± 0.06^{a}
Threonine	1.85	2.48 ± 0.13^{ab}	2.30 ± 0.23^{b}	2.66 ± 0.08^{a}	2.63 ± 0.08^{a}
Valine	2.17	2.60 ± 0.15^{ab}	2.45 ± 0.18^{b}	2.78 ± 0.06^{a}	2.77 ± 0.07^{a}
EAAs [1]	18.95	23.83 ± 1.51^{ab}	22.36 ± 1.97^{b}	25.24 ± 0.75^{a}	25.31 ± 0.31^{a}
Alanine	2.19	3.65 ± 0.15^{ab}	3.46 ± 0.24^{b}	3.89 ± 0.19^{a}	3.95 ± 0.15^{a}
Asparagine	3.29	5.18 ± 0.31^{ab}	4.82 ± 0.45^{b}	5.43 ± 0.19^{a}	5.45 ± 0.10^{a}
Glutamic acid	4.97	7.67 ± 0.43^{ab}	7.17 ± 0.71^{b}	8.07 ± 0.25^{a}	8.12 ± 0.23^{a}
Glycine	1.93	4.38 ± 0.28^{ab}	4.10 ± 0.24^{b}	4.60 ± 0.21^{a}	4.68 ± 0.23^{a}
Proline	2.11	2.65 ± 0.16^{b}	2.47 ± 0.15^{b}	3.30 ± 0.62^{a}	2.82 ± 0.13^{b}
Serine	2.22	2.47 ± 0.14^{ab}	2.26 ± 0.25^{b}	2.61 ± 0.04^{a}	2.59 ± 0.04^{a}
Tyrosine	1.83	1.84 ± 0.13^{ab}	1.73 ± 0.13^{b}	1.95 ± 0.06^{a}	1.94 ± 0.04^{a}
NEAAs [2]	18.53	27.84 ± 1.39^{ab}	26.00 ± 2.09^{b}	29.83 ± 0.31^{a}	29.55 ± 0.91^{a}
TAAs [3]	38.04	51.67 ± 2.82	48.36 ± 4.06	55.07 ± 1.06	54.86 ± 1.21

Data for the MD are the mean of two replicates. Values (mean ± S.E.M., n=3) within a row sharing the same superscript letter or without a superscript are not significantly different from the other experimental treatments (P > 0.05).

[1] EAAs, essential amino acids.

[2] NEAAs, nonessential amino acids.

[3] TAAs, total amino acids.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways are shown in **Figure 3E**. Ribosome biogenesis in eukaryotes was the KEGG pathway with the highest rich factor, with the involvement of protein metabolism-related pathways (in blue) and lipid metabolism-related pathways (purplish-red character), and some other pathways (in black).

The DEG association network was predicted using STRING with high confidence, and the interaction network was constructed by Cytoscape software. **Figure 3F** left panel shows a cluster illustrating some lipid metabolism-related gene interactions, such as *dhcr24* and *aacs*, as well as some protein metabolism-related gene interactions, such as *lap3* and *pro1*. The middle panel primarily highlights a clustering illustrating ribosome protein biosynthesis-related gene interactions and their interactions with nucleotide metabolism. The genes *aprt*, *gart*, *atic*, and *umps* translate key proteins that are involved in adenosine synthesis, while the genes *pes1*, *nop56*, *nop58*, and *rpf2* translate key proteins for ribosome biogenesis, maturation, and assembly. The upright panel shows a clustering illustrating lipid metabolism gene interactions, such as *acadvl*, *mgll*, and *acsl6*. The bottom right panel shows a clustering illustrating genes encoding mitochondrial ribosome proteins (*tufm*, *mrpl2*, *mrSL12*, and *mrpl16*) interacting with genes encoding mitochondrial inner membrane proteins (*tim8*, *tim10*, and *tim13*).

Multidimensional Correlation

As shown in **Figure 4**, the correlation analysis between gene expression and nutritional parameters showed that the survival rate was significantly positively correlated with growth performance (WGR) and body ash content ($P < 0.05$). The whole-body ash content was positively correlated with the whole-body protein content ($P<0.001$) and TAA content ($P<0.01$) but negatively correlated with the MUFAs proportion ($P<0.05$), and lipid and fatty acid metabolism-related genes, as well as protein and amino acid metabolism-related genes, were significantly correlated with the whole-body ash content (Mantel's r≥0.9, $P<0.05$). Notably, the whole-body protein content was significantly negatively correlated with the MUFAs proportion, and protein and amino acid metabolism-related genes were significantly correlated with the whole-body protein content (Mantel's r≥0.4, $P<0.01$). Ribosome biogenesis-related gene expression was closely related to the whole-body EAA (Mantel's $P<0.01$) and TAA (Mantel's r≥0.9, $P<0.01$) contents.

DISCUSSION

Numerous investigations have demonstrated a strong and positive association between dietary intact PL inclusion and aquatic larval growth performance as well as stress resistance (Zhao et al., 2013; Gao et al., 2014; Zhang et al., 2022). Consistent with these results, larvae fed the high SL content MDs showed significantly higher growth performance and hypoxia stress resistance than those fed the SL0 MD. To investigate the potential mechanism underlying the growth promotion effect of dietary SL inclusion, mid-intestinal morphology, body nutrient composition, the activity of metabolism-related enzymes, and RNA sequencing in liver tissues were conducted. All DEGs have been listed as a supplemental table (**Table S3**). A schematic representation of the effects of the PL-enriched (SL12) diet on liver tissue metabolic and physiological responses is presented in **Figure 5**.

Dietary Phospholipids Improved Intestinal Morphology

Studies have revealed the beneficial effects of PLs in diet emulsification (Koven et al., 1993), fatty acid absorption (Fontagné et al., 2000; Hadas et al., 2003), digestive enzyme activities (Gisbert et al., 2005), intestinal lipid transportation (Lin et al., 2018) and microbiota composition (Wang et al., 2021). However, no consensus has been reached about the relationship between dietary intact PLs and intestinal morphology at the fish larval stage. A previous study on Atlantic salmon larvae argued that there was no significant difference in mid-intestinal enterocyte height among dietary treatments (Taylor et al., 2015). Moreover, the height of enterocytes was higher in carp (*Cyprinus carpio* L.) larvae that were fed the PL-deficient diet than in larvae fed a phosphatidylcholine-enriched diet (Fontagné et al., 1998). In contrast, the PL-deficient diet was reported to cause epithelial damage in the intestinal lumen of Arctic char larvae (Olsen et al., 1999). In line with that, our recent study in yellow drum larvae found that the high inclusion of dietary PLs induced increased fatty acid β-oxidation, which provided energy for the proliferation of enterocytes and eventually improved intestinal morphology (Tan et al., 2022). The rapid proliferation of enterocytes requires a considerable amount of energy, and dietary PL inclusion can

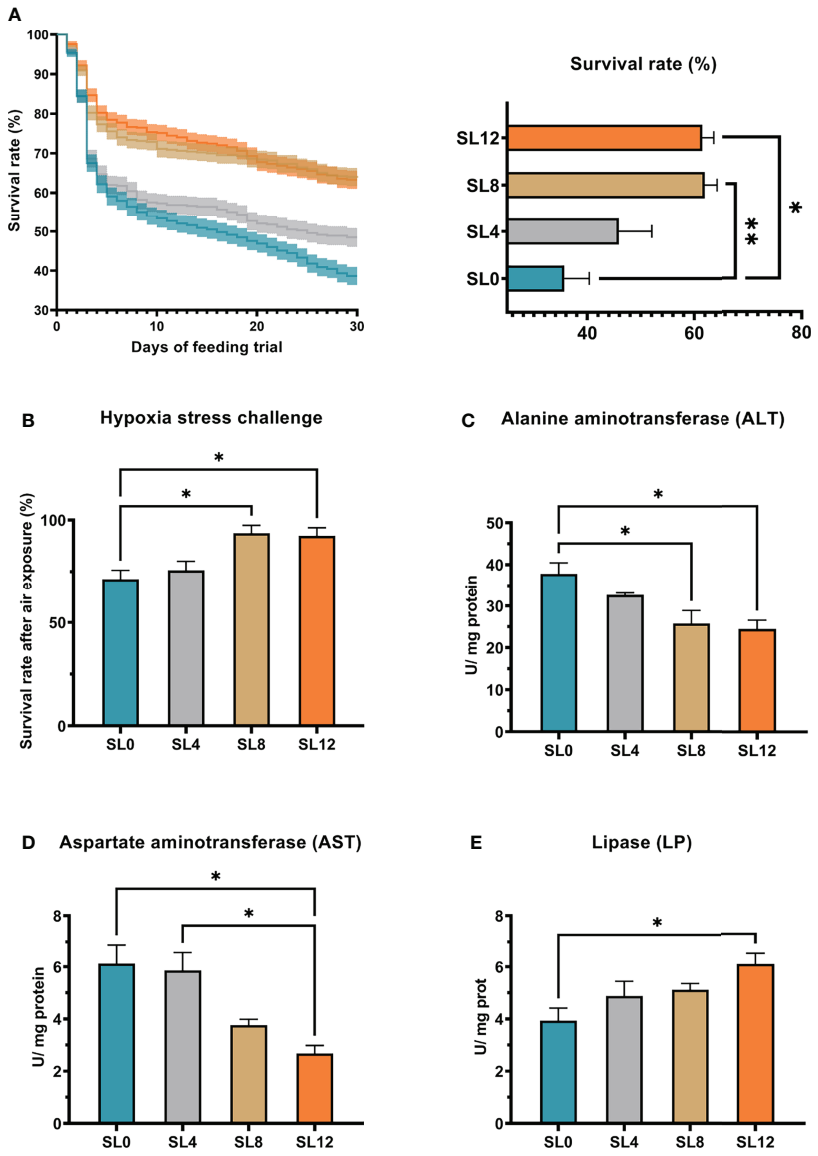

FIGURE 1 | Rock bream larval survival rate, stress resistance to hypoxia stress, and liver tissue metabolism-related enzyme activities. **(A)** The rock bream larval survival rate was recorded during the feeding trial daily, and final statistical analyses are presented in the right panel. **(B)** Larval stress resistance to air exposure for 5 minutes. **(C-E)** Larval liver tissue alanine aminotransferase (ALT) Aspartate aminotransferase (AST) and lipase (LP) enzyme activities correspond to dietary soybean lection inclusion levels. *P < 0.05, **P < 0.01, otherwise, no significant difference (P > 0.05).

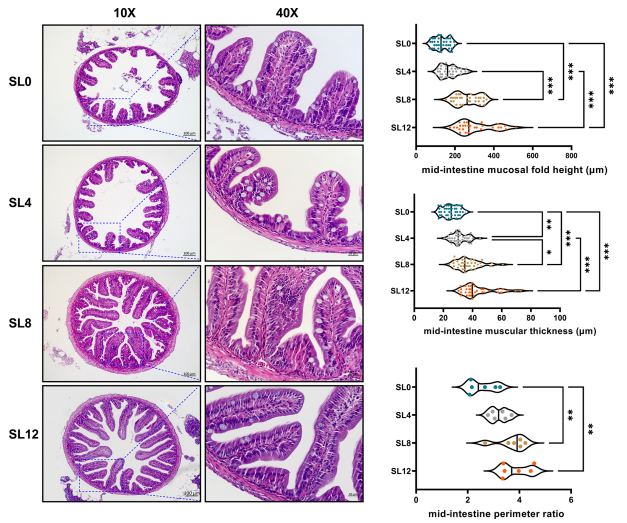

FIGURE 2 | Longitudinal paraffin section of the mid-intestine. A total of 24 slices with 6 slices per treatment were observed. The perimeter ratio, intestinal muscular thickness, and mucosal fold height are presented by a violin plot. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; otherwise, no significant difference($P > 0.05$)

serve as a preferential energy source during larval growth and development (Penn et al., 2011). In addition, fatty acids and choline from PL catabolism were indispensable for the synthesis of new cellular structures (Sargent et al., 1999). In the present study, the mid-intestine perimeter ratio, muscular thickness, and mucosal fold height were observed to significantly increase with dietary soybean lection inclusion. Therefore, the improved mid-intestine morphology could probably contribute to exogenous nutrient acquisition in larvae fed diets with high SL contents. The possible molecular mechanism was likely caused by the significantly increased mitochondrial fatty acid oxidation and energy production, as mitochondrial fatty acid β-oxidation key genes (*ppara* and *cpt1*) in larvae fed a high soybean lectin inclusion diet were significantly enhanced (Carmona-Antoñanzas et al., 2015; Cai et al., 2016; Tan et al., 2022).

SL-Enriched Diets Increased Phospholipid Catabolism

In this study, the activity of LP, a key lipolysis enzyme in the liver was significantly enhanced by the high inclusion of SLs; in addition, the expression level of fatty acid activation-related genes (*acsl6* and *acsbg2*) was significantly increased. These results were in accordance with the findings in some other aquatic species that dietary SL inclusion increased lipid catabolism (Cahu et al., 2003; Niu et al., 2008; Azarm et al., 2013; Cai et al., 2016; Zhang et al., 2022). Larvae fed a high PL diet have increased energy production because PLs can serve as a preferential source of energy in the larval stage (Tocher et al., 2008). ACSBG2 and ACSL6 can both catalyze the conversion of long-chain fatty acids and activate diverse SFAs, MUFAs, and PUFAs to their active form acyl-CoA for degradation *via* β-oxidation (Pei et al., 2006; Nakahara et al., 2012). The increased gene expression of *ascl6* and *acsbg2* in the liver of the SL12 group indicated the increased activation of fatty acid conversion and thereby increased fatty acid flux to β-oxidation. Corresponding to the increase in fatty acid β-oxidation, the expression levels of mitochondrial biogenesis-related genes (*mrpl2*, *mrSL12*, and *mrpl16*), as well as mitochondrial intermembrane chaperone-related genes (*tim8*, *tim10*, and *tim13*), were significantly upregulated. These mitochondrial biogenesis-related genes encoding 39S ribosomal proteins are essential for mitochondrial construction (Amunts et al., 2015). At a high confidence level, DEG interactions (**Figure 3F**) indicated that genes encoding mitochondrial biogenesis and mitochondrial intermembrane chaperones interacted with *tufm*, which was required for mitochondrial protein biosynthesis and maintenance of mitochondrial DNA (Christian and Spremulli, 2012).

The increased dietary SL content seems to have little effect on the whole-body PL content. Extra dietary SLs could lead to a crowding-out effect, characterized as increasing PL catabolism and increasing the biosynthesis of other lipids. Correspondingly, RNA sequencing results indicate that the SL12 diet significantly increased the expression of some other lipid anabolism-related genes, such as cholesterol synthesis-related *acat2* (An et al., 2006) and *dhcr24* (Waterham et al., 2001), diacylglycerol synthesis-related *lpin2* (Donkor et al., 2007), LC-PUFA synthesis-related *elov5* (Monroig et al., 2022), and diphosphatidylglycerol (cardiolipin) synthesis-related *crls* (Lu et al., 2006). These results were in accordance with the findings in hybrid grouper, in which dietary PL inclusion increased hepatic crude lipid and triglyceride by regulating the synthesis and hydrolysis of triglyceride (Huang et al., 2021). Moreover, upregulated *mgll* expression in the SL12 group indicated that an SL-enriched diet enhances the conversion of monoacylglycerides to free fatty acids and glycerol (Taschler et al., 2011) and thereby increases the fatty acids supply for lipid biosynthesis. Notably, high dietary SL content significantly decreased the expression of *pemt*. PEMT is the key enzyme that catalyzes the three sequential steps of the methylation pathway of phosphatidylcholine biosynthesis (Tocher et al., 2008). This result acts in cooperation with the whole-body PL content, and the mechanism could be the negative feedback loop in response to high dietary PL content. In summary, a high soybean lection content improves fatty acid flux to energy production and increases PL catabolism to other lipids, such as cholesterol, diacylglycerol, LC-PUFAs, and diphosphatidylglycerol.

SL-Enriched Diets Decreased Amino Acid Catabolism

AST and ALT are the key enzymes mediating amino acid catabolism through the transamination of amino acids into TCA cycle precursors (Bibiano Melo et al., 2006). A previous study demonstrated that the activities of ALT and AST were negatively correlated with protein efficiency (He et al., 2010).

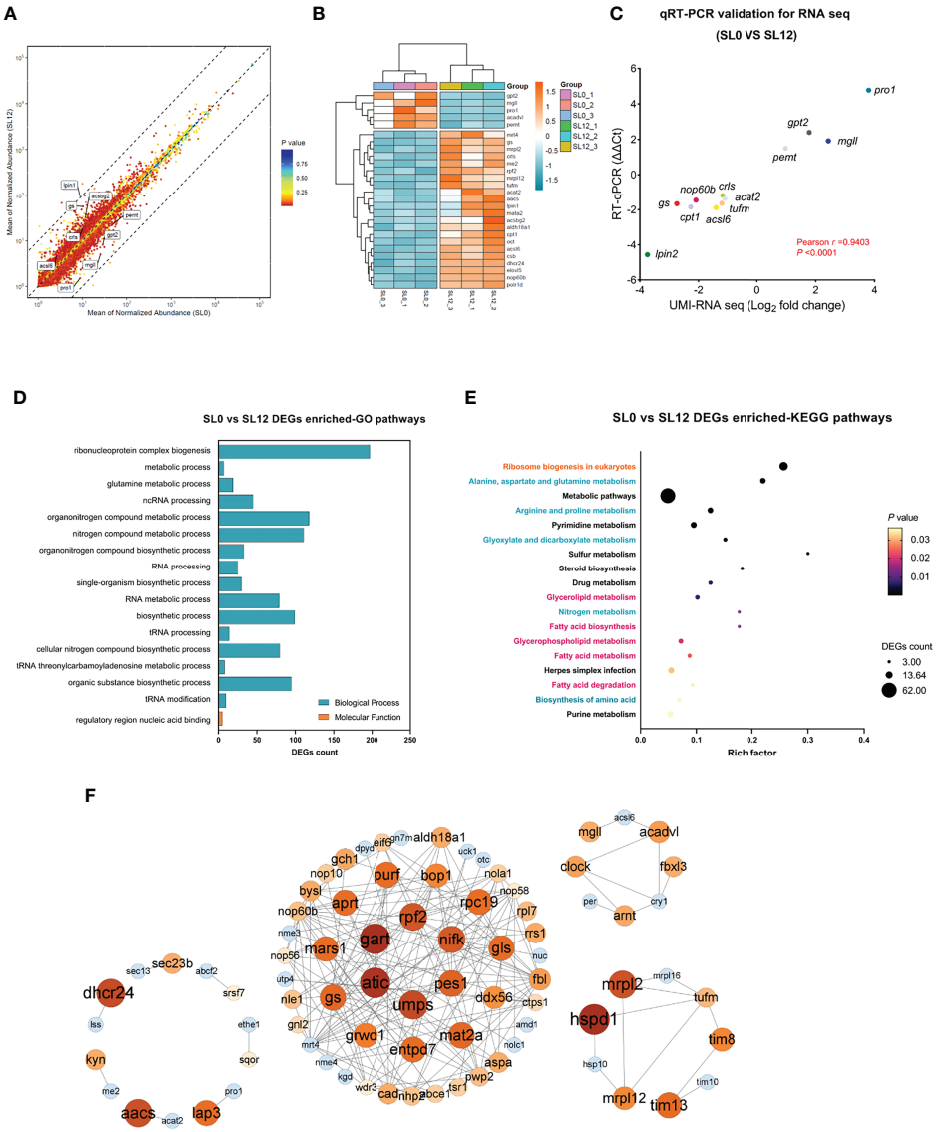

FIGURE 3 | Transcriptome analysis of larval liver tissue corresponding to dietary phospholipid levels. **(A)** Volcano plot of detected gene expression levels between the SL0 and SL12 groups. A total of 427 DEGs were detected. **(B)** Heatmap presentation for partial DEGs between treatments. The block color diversity characterizes the gene expression levels. **(C)** UMI-RNA-seq DEG validation by qRT–PCR. **(D, E)** DEG-enriched GO and KEGG pathways between groups. **(F)** Potential association network for predicted DEGs constructed using Cytoscape software with high confidence.

A study in largemouth bass (*Micropterus salmoides*) larvae reported that the inclusion of marine protein hydrolysates significantly decreased the activities of AST and ALT accompanied by a growth promotion effect (Dai et al., 2020). In the present study, the activities of the AST and ALT enzymes in liver tissue were observed to be significantly higher in the larvae fed the SL0 diet than in the larvae fed the SL12 diet. A possible explanation for this result could be that the increased of AST and ALT activities enhanced the flux of amino acids into the TCA cycle for ATP production in the SL0 group. In accordance with the decreased aminotransferase enzyme activities, the RNA sequencing results showed that the *gpt2* (encoding alanine aminotransferase 2) expression level in the liver tissue of larvae fed the SL12 diet was significantly decreased. Interestingly, the expression of amino acid synthesis-related genes was significantly increased. These genes include glutamine synthesis-related *gs* (Eelen et al., 2018), cystathionine synthesis-related *csb* (Casique et al., 2013), proline synthesis-related *aldh18a1* (Baumgartner et al., 2000), S-adenosylmethionine synthesis-related *mata2* and L-citrulline-synthesis-related *oct* (Couchet et al., 2021). As a consequence, the increased amino acid synthesis and decreased amino acid catabolism in the SL12 group could be attributed to the increased whole-body protein and amino acid

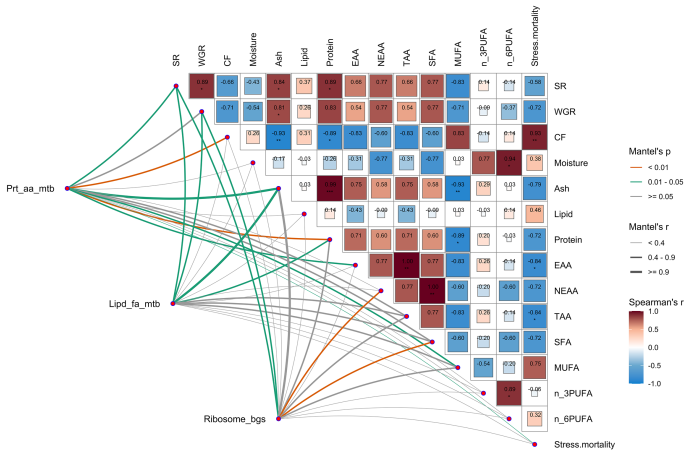

FIGURE 4 | Multidimensional correlation analysis between gene expression and larval nutrition parameters. Nutritional parameters: survival rate (SR), weight gain rate (WGR), condition factor (CF), larval body moisture, ash, lipid, protein, essential amino acid content (EAA), nonessential amino acid content (NEAAs), total amino acid content (TAAs), saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), n-3 polyunsaturated fatty acids (n_3PUFAs), n-6 polyunsaturated fatty acids (n_6PUFAs) and stress resistance to hypoxia stress (stress mortality). Gene expression: hepatic protein and amino acid metabolism-related gene expression, including *pro1*, *mata2*, *gs*, *oct*, *aldh18a1*, *csb*, and *gpt2* (Prt_aa_mtb); hepatic lipid and fatty acid metabolism-related gene expression, including *acat2*, *crls*, *me*, *aacs*, *lpin2*, *elovl5*, *mgll*, *acsbg2*, *acsl6*, and *pemt* (Lipd_fa_mtb); hepatic ribosome biogenesis-related gene expression, including *rpf2*, *polr1d*, *nop60b*, *mrpl2*, *mrSL12*, *mrpl16*, *nop56*, *nop58*, *bop1* and *mrt4* (Ribosome_bgs). Pairwise comparisons of nutritional parameters are presented, with a color gradient and color block size denoting Pearson's correlation coefficients. Related gene expression levels were related to the characterized parameters by Mantel's tests. The edge width and color correspond to Mantel's r statistic and Mantel's p statistic for the corresponding distance correlations, respectively.

contents in larvae. The multidimensional correlation analysis indicated that the whole-body protein content was significantly negatively correlated with the whole-body MUFAs proportion, while protein and amino acid metabolism-related genes were significantly correlated with the whole-body protein content (Mantel's r≥0.4, P<0.01). The multidimensional correlation analysis supports our hypothesis that larval whole-body protein content was regulated by fatty acid and amino acid metabolism processes.

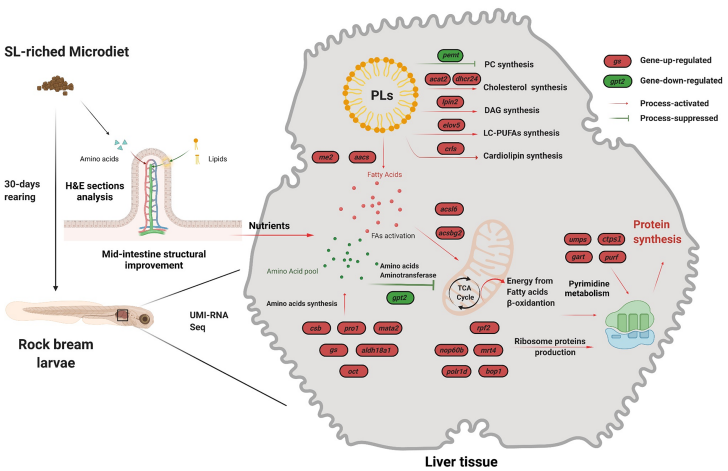

FIGURE 5 | Schematic representation of the effects of the PL-rich (SL12) diet on intestine morphology and liver tissue molecular response. Transcriptome analysis was used to elucidate the potential molecular mechanisms underlying the observed results. The red ovals indicate upregulated genes, and the green ovals indicate downregulated genes. The SL12 diet improved the mid-intestine structure and thereby increased nutrient acquisition. The UMI-RNA sequencing results indicated that the SL12 diet could increase the *de novo* synthesis of lipids other than phosphatidylcholine. The SL12 diet increased energy production from fatty acid β-oxidation by fatty acid activation but decreased amino acid aminotransferase activity in the TCA cycle. The SL12 diet increased amino acid synthesis and ribosome production-related gene expression.

SL-Enriched Diets Increased Ribosome Biogenesis

Individual growth, more precisely protein retention, primarily occurs in the ribosome. The ribosome is the essential unit of all living organisms that executes protein synthesis and therefore fuels cell growth and cell proliferation. The biogenesis of ribosomes requires abundant nutrients and ATP; therefore, the synthesis of ribosomes is tightly controlled (Piazzi et al., 2019). In this study, the SL12 diet significantly increased ribosome biogenesis-related gene expression in the liver tissue, characterized as *rpf2*, *pes1*, *nop56*, *nop58*, *nop60b*, *mrt4*, *polr1d*, and *bop1*. The proteins RPF2, PES1, NOP56, NOP58, NOP60B, MRT4, and BOP1 are essential elements for ribosome biogenesis, maturation, and assembly processes (Hayano et al., 2003; Rohrmoser et al., 2007; Michalec et al., 2010; Sloan et al., 2013). The DEG-enriched GO and KEGG pathways revealed that ribosome protein synthesis-related pathways were the most enriched pathways. These results indicated that the SL12 diet significantly increased ribosome biogenesis, which was in accordance with the increased whole-body protein content. It was inferred that a high content of amino acids together with abundant ribosomes led to increased protein synthesis in larvae fed the SL12. In line with this, the multidimensional correlation analysis indicated that the whole-body EAA and TAA contents were closely related to ribosome biogenesis-related gene expression.

Surprisingly, the protein-to-protein interaction analysis by Cytoscape software indicated that nucleotide metabolism-related genes (*aprt*, *ctps1*, *gart*, *atic*, and *umps*), especially pyrimidine and purine metabolism-related genes, closely interacted with ribosome protein biosynthesis-related }gene expression. The protein synthesis process not only requires amino acids and ribosomes but also requires templates for that process. Nucleotide metabolism plays a role in supplying messenger RNAs, transfer RNA and ribosomal RNA, as well as precursor substances for energy carriers (ATP and GTP) (Lane & Fan, 2015). Meanwhile, nucleotide metabolism is closely related to amino acid metabolism, and nucleotides and amino acids can be transformed into each other. Further study is warranted to reveal the role of nucleotide metabolism in the process.

CONCLUSIONS

Feeding trials and subsequent transcriptome analysis revealed the effects of dietary SL content on survival rate, growth performance, intestinal structure in rock bream larvae, as well as the underlying molecular mechanism. The conclusions are summarized as follows:

(1) The PL-enriched diet (SL12) increased the growth performance and resistance to hypoxia in rock bream at the larval stage.
(2) Dietary SL improved mid-intestine morphology.
(3) The PL-enriched diet could increase phospholipid catabolism and amino acid synthesis-related gene expression but decreased amino acid catabolism-related gene expression.
(4) The PL-enriched diet increased ribosome biogenesis-related gene expression.

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/bioproject/PRJNA848209

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Research and Ethics Committees of Ocean University of Zhejaing Province.

AUTHOR CONTRIBUTIONS

PT: conceptualization, data curation, formal analysis, funding acquisition, project administration, supervision, software, validation, roles/writing – original draft, and writing – review and editing. PZ: data curation. LZ: data curation. WZ: data curation. LW: data curation. RC: data curation. QZ: formal analysis. DX: funding acquisition, project administration, supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at:
https://www.frontiersin.org/articles/10.3389/fmars.2022.942259/full#supplementary-material

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GLOSSARY

acadvl	Very-long-chain specific acyl-CoA dehydrogenase, mitochondrial
acat2	acetyl-CoA acetyltransferase, cytosolic
acsbg2	long-chain-fatty-acid-CoA ligase ACSBG2
acsl6	long-chain fatty acid CoA ligase 6
aldh18a1	glutamate dehydrogenase, mitochondrial
aprt	adenine phosphoribosyltransferase
atic	bifunctional purine biosynthesis protein PURH
bop1	ribosome biogenesis protein bop1
cpt1	carnitine o-palmitoyltransferase 1
crls	cardiolipin synthase; csb, cystathionine beta-synthase
ctps1	CTP synthase 1
dhcr24	delta(24)-sterol reductase
elov5	elongation of very-long-chain fatty acid protein 5
g6pdh	glucose-6-phosphate 1-dehydrogenase
gart	trifunctional purine biosynthetic protein adenosine 3
gpt2	alanine aminotransferase 2
gs	glutamine synthetase
lap3	cytosol aminopeptidase
lpin2	phosphatidate phosphatase LPIN2
mata2	S-adenosylmethionine synthase isoform type-2
mgll	monoglyceride lipase
mrpl2	39S ribosomal protein L2
mrSL12	9S ribosomal protein L12
mrpl16	39S ribosomal protein L2
mrt4	mRNA turnover protein 4 homolog; nop56, nucleolar protein 56
nop58	nucleolar protein 58
nop60b	H/ACA ribonucleoprotein complex subunit 4
oct	ornithine carbamoyltransferase, mitochondrial; pemt, phosphatidylethanolamine N-methyltransferase;
pes1	pesc_salsa pescadillo homolog; polr1d, DNA-directed RNA polymerases I and III subunit RPAC2
ppara	peroxisome proliferator-activated receptor a
pro1	proline dehydrogenase 1, mitochondrial
rpf2	ribosome production factor 2 homolog
tim8	mitochondrial import inner membrane translocase subunit Tim8
tim10	mitochondrial import inner membrane translocase subunit Tim10
tim13	mitochondrial import inner membrane translocase subunit Tim13
tufm	elongation factor Tu, mitochondrial; umps, uridine 5'-monophosphate synthase

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REVIEW
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β-Glucan: Mode of Action and Its Uses in Fish Immunomodulation

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β-glucan is considered as an effective immunostimulant because of its binding capacity to different receptors on leukocytes leading to the stimulation of immune responses including bactericidal activity, cytokine productivity, and survival fit ability at cellular levels. In response to immune cell surface receptors, β-glucan stimulates to release cytokines and chemokines. It has been found that these signaling proteins eventually stimulate the immunocompetent cells in fish such as monocytes, macrophages, and neutrophils for killing pathogens by phagocytosis, oxidative burst, and cytotoxic killing activities. They also procreate immunological memories and specific antibodies through activation of T and B lymphocytes. Researchers have proved that β-glucan can modulate some important biochemical (serum hemoglobin, serum protein, and total hemocyte count) and immunological (lysozyme activity, phagocytic activity, oxidative burst activity, and phenoloxidase activity) properties providing more competent immune profile for treating fish and aquatic organisms. β-glucan-supplemented fish showed limited sensitivity of genes involved in acute inflammatory reactions. Findings have shown that β-glucan exerts a positive impact on fish and aquatic organisms' immunity, enhancing their disease resistance by increasing functional and decreasing deleterious responses. This review focuses on the basic bump of β-glucan on fish and shellfish immunity and recent information on the uses of β-glucan in progressive aquaculture.

Keywords: beta-glucan, mechanism of action, fish growth, fish health, immunomodulation, aquaculture

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INTRODUCTION

Different studies in fishes have proven β-glucan as a potent immunostimulant for improving the immunity of cultured fish against disease and stress (Figueras et al., 1998; Kawakami et al., 1998; Meena et al., 2013; Yamamoto et al., 2018a; Yamamoto et al., 2018b; Yamamoto et al., 2020). The immunostimulatory properties of β-glucans were first identified in mammals resulting in an increased resistance to infectious pathogens (Di Luzio, 1985). However, in recent years, special attention has been given on the use of β-glucan in stimulating the recovery potential of immunocompromised fish and aquatic organisms. Many studies have proven the significant effect of β-glucan on fish growth and survival (Cook et al., 2003; Misra et al., 2006), protective resistance to specific pathogen (Welker et al., 2007; Sealey et al., 2008), and adjuvant effect on increased antibody production (Selvaraj et al., 2005; Kamilya et al., 2006). The introductory section of this article aims to provide an elaborate

discussion on β-glucan sources, their distinguishing properties, and the scope of using it in aquaculture.

Many fungi possess β-glucans in their cell wall, which has important immunomodulatory properties (Brown et al., 2003). The cell walls of many cereal plants (barley, oat, etc.) are accordingly flourished with β-glucans (Volman et al., 2008). The yeast species *Saccharomyces cerevisiae* cell wall comprises 50%–55% β (1/3) glucan and 10%–15% β-(1/6) glucan of the total polysaccharides (**Figure 1**) (Lesage and Bussey, 2006). Mushroom species *Lentinus edodes*, *Grifola frondosa*, and *Ganoderma lucidum* are reliable sources of good-quality β-glucans having therapeutic significance (Wasser and Weis, 1999). *Laminaria* sp., the members of brown seaweed, may also be better sources of β-glucan. They are extensively cultivated for commercial extraction of laminaran, fucoidan, alginate, etc. (Rioux et al., 2007). The pathogenic fungus *Pneumocystis carinii* holds cell wall β-glucan responsible for the secretion of pro-inflammatory signaling proteins (cytokines and chemokines) (Lebron et al., 2003). Cell wall composition of *Cryptococcus neoformans* demonstrates β-glucan as an essential structural element of this morbific mold (Reese et al., 2007). The ascomycetes member *Sclerotinia sclerotiorum* retains reactive β-glucan in their cell wall components (Borchers et al., 2004). The cell walls of some non-pathogenic bacteria of the Rhizobiaceae family are also composed of β-glucan (McIntosh et al., 2005).

β-glucan is an indigestible constituent (fiber substance) that makes up the cell wall of cereal plants (**Figure 2**) (e.g., oat, barley, etc.) DeVries, J. W. (2003). β-glucan is a starch-less polysaccharide with recurrent units of glucose monomers united by (1/3)-β-D-glycosidic linkages and very often arranged with β (1/6)-linked side chains of non-identical presentation (Buckeridge et al., 2004). The most convenient forms of β-glucans are those composed of glucose monomers linked by β (1/3) glycosidic bonds (Goodridge et al., 2009; Meena et al., 2013). Glucans may be branched in several ways depending on their sources. The yeast and fungal glucans share β (1/3, 1/6) glycosidic bonds that are usually highly branched (**Figure 1**). Yeast's β-glucans consist of β (1/3)-linked backbones and β (1/6)-linked side chains (Zlatkovic et al., 2003). Algal β-glucan laminaran has a skeleton of β (1/3) glucopyranosyl units with β (1/6) branching (Zvyagintseva et al., 1999). In nature, cereals' (oat, barley, and rye) β-glucans contain β (1/3, 1/4)-joined links (**Figure 2**), whereas mushrooms contain higher amounts of β (1/3, 1/6)-joined links (Volman et al., 2008). Some oat β-glucans are linear, unbranched polysaccharides, furnished with 70% 1/4-O-linked bondages and 30% 1/3-O-linked bondages between their β-D-glucopyranosyl units (Butt et al., 2008). On the contrary, bacterial β-glucan (curdlan) is unbranched with only (1/3)-β-D-linkages between its glycopyranosyl molecules (Johansson et al., 2008).

Larger (molecular weight > 5 kDa and < 200 kDa) sizes of β-glucans have regulatory effects on the host immune system (Bagni et al., 2005). They boost up the host's non-specific defense mechanism and instigate leukocytes triggering their phagocytic and anti-pathogenic reactions through production of pro-active oxygen species (superoxide anions, hydrogen peroxide, hydroxyl radical, hypochlorous acid, etc.) and nitrogen intermediates (nitrite, amides, and nitrogen dioxide) (Lee et al., 2002). The variations in molecular weight, shape, and structure of β-glucans have an impact on their immune performances (Akramiene et al., 2007; Meena et al., 2013; Chu, 2014). Lines of evidence demonstrate that specific physicochemical properties, for example, molecular structure, solution strength, molecular weight, and net charge of β-glucan, play a vital role in determining the magnitude of β-glucan binding to macrophage receptor(s) and how it modulates the immune responses (Mueller et al., 2000). Researchers have found that insoluble (1/3, 1/6) β-glucans have higher biological involvement than that of its soluble β (1/3, 1/4) counterparts (Ooi and Liu, 2000). Indigestible β-glucans may lead to an alteration in the population of gut microbiota (Swennen et al., 2006). β-glucans are capable of modulating biological immune responses (Miura et al., 1996). β-glucan modulates immune cells but never overstimulates, which is key to the safety of this product (Gatlin and Li, 2004).

The provision of β-glucans, either dietary or supplementary injection, stimulates the recovery potential of immunosuppressed cells in teleost fishes (e.g., Atlantic salmon and rainbow trout) against infectious diseases under ordinary habitat condition (Petit and Wiegertjes, 2016). Most of the traditional antibiotics

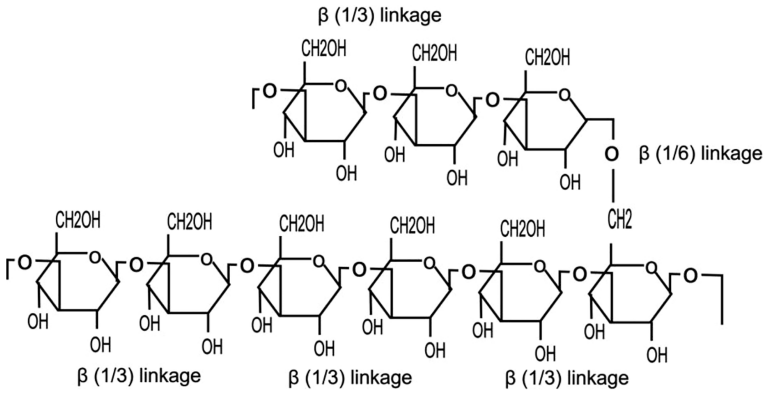

FIGURE 1 | Molecular structure of a typical yeast β-glucan

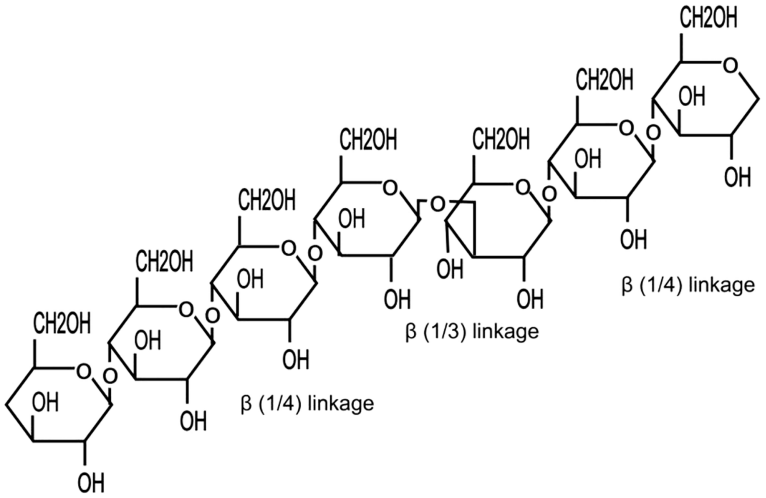

FIGURE 2 | Molecular structure of a typical cereal β-glucan.

are banned due to the potential exposure of antibiotic-resistant bacteria, their residual effects on aquatic habitat, and suppressing repercussion on aquatic organisms' immune system (FAO, 2002). Moreover, antibiotics may disrupt growth and feed efficiency by declining gut microbes and reducing amino acid utilization by the host animal (Rawles et al., 1997). These adverse effects can successfully be overcome by exploring the prebiotic nature of β-glucan (Meena et al., 2013). Thus, the immunomodulating effects of β-glucan can be characterized into (1) prebiotic effects of β-glucan as indirect immunomodulation in terms of fermentation of β-glucan by naïve bacteria and changes in microbial composition as well as the shift in the production of short chain fatty acid (SCFA) metabolites in the gastrointestinal tract (GIT) of fish, and (2) immunity enhancement of host by β-glucan as the direct immunomodulation in terms of receptor-mediated recognition in GIT of fish (Petit et al., 2022).

The production of fish fry is often hampered up to 10% in the aquaculture sector by high mortality rates due to infectious diseases. The delivery of β-glucan as a dietary supplement to larval fish can have a considerable effect in improving the animals' innate defenses (Bricknell and Dalmo, 2005). A considerable amount of protective resistance has been achieved with the β-glucan adjuvanted vaccine in rainbow trout (*Onchorynchus mykiss*) (Siwicki et al., 2004), catla (*Catla catla*) (Kamilya et al., 2006), and sea bass (*Dicentrarchus labrax*) (Bonaldo et al., 2007). Thus, the potential of adjuvanticity of β-glucan can also be explored for vaccination of fish in commercial aquaculture.

IMMUNITY IMPROVING MECHANISMS OF β-GLUCAN

The innate immune system comprises different integrants including physical barriers (skin, epithelial cell surfaces, and mucus itself), phagocytic cells (monocytes, macrophages, and neutrophils), antibacterial enzymes and peptides [lysozymes, phospholipase (A2), defensins, cathelicidins, transferrin, etc.], inflammation responsive serum proteins (complement, C-reactive protein, lectins, and ficolins), cells that produce cytokines and inflammatory mediators (macrophages, mast cells, and natural-cytotoxic cells), and their surface receptors [toll-like receptors (TLRs) and other pattern recognition receptors (PRRs)]. These components initiate the enzyme cascade system to establish the first-line defense system by eliminating or destroying pathogens (Kumagai and Akira, 2010). The first line of defense identifies pathogens or immunostimulants by decoding the generic properties of their macromolecules (carbohydrates, lipids, nucleic acids, and proteins), the pathogen-associated molecular patterns (PAMPs) (Akira and Hemmi, 2003). This first line of defense has evolved receptors, PRRs, capable of pointing out and encountering pathogens *via* their PAMPs (Brown and Gordon, 2003). Microorganisms or their toxins or bioactive ingredients are identified by PRRs when first encountering the immune cells and mechanisms of the baseline defense (Medzhitov, 2007). The innate immune defenses are non-specific, predominantly respond to pathogens in a customary way, and destroy them by an inclusive manner (Alberts et al., 2002). Thus, these non-specific defenses can also be triggered by damage-associated (injured or abnormal cells' mediated) molecular patterns (DAMPs) (Matzinger, 2002). Following the initial responses to a specific pathogenic infection, the invading organism acquires an immunological memory (pathogen-specific receptor) and leads to a more specific reaction (specific antibody production through genetic recombination) to succeeding the infection by the previous one (**Figure 3**). This secondary response is known as adaptive immunity. Like the inherent immune defense, the acquired immunity comprises both humoral immunity complements and cell-mediated immunity complements (Alberts et al., 2002). Humoral components are soluble proteins of the plasma and body fluids. The molecules include transferrin, interferons, lytic enzymes, macroglobulin, natural antibodies, proteins of the classical and alternative pathways, and proteins under the group cytokines

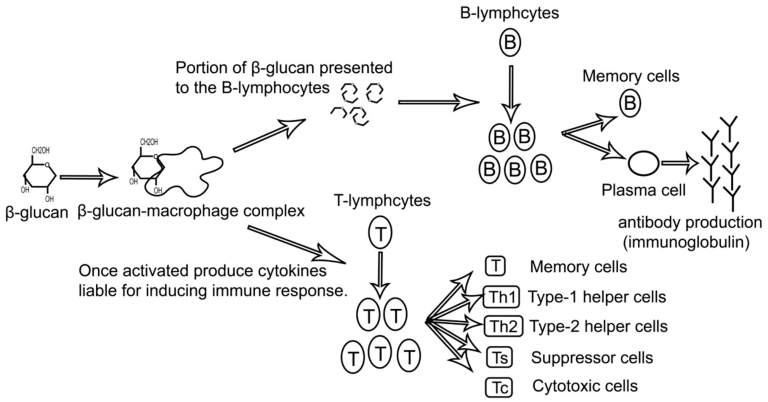

FIGURE 3 | Mechanism of modulating the fish immune system by β-glucan.

and chemokines (Rodrigues et al., 2020). A study in seabream (*Sparus aurata*) fed a diet supplemented with yeast cell showed humoral immune response (antibody production and specific defense) (Cuesta et al., 2004). Teleostean B cells are reported to produce immunoglobulin M (IgM), immunoglobulin D (IgD), and immunoglobulin T (IgT) (Danilova et al., 2005; Solem and Stenvik, 2006). Glucans are generally believed to be associated with a first-line defense mechanism by binding to specific receptors on major phagocytic cells and other components of the inherent immune system (Mueller et al., 2000). Different cell surface receptors contain lectins, scavenger receptors (SRs), transmembrane proteins on monocytes/macrophages, natural cytotoxic cells (NCC), and other lymphocyte subcomponents binding various types of β-glucan (Brown and Gordon, 2003). A variety of glucan binding sites on macrophages have been identified, but their mechanism of modulating an innate immune system is not fully clear.

The first step of β-glucan–macrophage complex formation is binding to specific receptors present on the immune cell surface (**Figure 3**). Complement receptor type 3 (CR3) (Vetvicka et al., 1996), lactosylceramide moiety (Zimmerman et al., 1998), dectin-1 (C-type lectin) (Rice et al., 2002), and carboxymethylated (CM) SRs (Vereschagin et al., 1998) have been considered to be major glucan binding sites on macrophages and other immunocompetent cells. Receptor-bound β-glucan may mediate the production of inflammatory cytokines (interleukins, interferons, lymphokines, and tumor necrosis factor) and chemokines (CC, CXC, C, and CX_3C). These signaling proteins are believed to aggravate phagocytic activity and microbial killing efficiency of immune cells through oxidative burst and natural cytotoxic liquidation (Brown et al., 2003; Misra et al., 2006). Presently, instead of dectin-1 and CR3, attention has been given to toll-like receptor 2 (TLR-2) inducing important roles in innate immunity. Glucan instigates different cell types (macrophages, neutrophils, and T lymphocytes) to mediate its stimulatory signals by forming bondage to TLR-2 associating other cell membrane receptors (Aizawa et al., 2018). The process may first induce membrane receptors and then form subsequent complexation with TLR homologues. TLR homologues have been identified in Atlantic salmon and puffer fish. The homologues have also been described in zebrafish, flounder, and goldfish (Rodrigues et al., 2020).

CR3 (type ι membrane protein, an integrin dimer) consists of $\alpha_M\beta_2$ CD11b/CD18 (Cluster of differentiation 18) and is manifested exclusively by myeloid cells (evolved from progenitor cells and target oriented stem cells) comprising monocytes/macrophages, neutrophils, and NCCs (Blystone and Brown, 1999). They are frequently characterized by dendritic cells (DCs) and antigen-presenting cells having a central role in adaptive immune response (Banchereau and Steinman, 1998), present on the cell surfaces of skin, nose, lungs, stomach, and intestines. CR3 has multiple types of ligands (a molecule that binds to another molecule) inclined to bind with different fungal, parasitic, and microbial cells, and also encrypted with some complementary sites for inducing glycoprotein complex (fibrinogen), coagulation factors, and intercellular adhesion molecule-1 (ICAM-1). CR3, in association with complement component iC3b, phagocytizes complement-opsonized foreign particles (Blystone and Brown, 1999). Some studies suggest that CR3 may persuade the assemblage of low-affinity receptors and their binding to the interlinking protein filaments (cytoskeleton) of various microorganisms. Particular propensity to co-receptors proves that CR3 has distinguished binding capacity (5×10^{-8} M) to β-glucan and the domain is distinct from the iC3b binding site. Lectin domain is also involved in CR3 assemblage with urokinase plasminogen activator receptor (uPAR), an autogenous surface receptor, for binding to certain microbial carbohydrates that do not contain β-glucan (Xia and Ross, 1999). A recent study reveals that antibodies responsive to CR3 or lactosylceramide hamper β-glucan particle fixing to human neutrophils. Thus, CR3 generated signaling requires β-glucan-mediated interaction of CR3 with lactosylceramide-linked lyn-kinase. The finding suggests that CR3 may act as a co-receptor with lactosylceramide in binding β-glucan (Nakayama et al., 2008). A study on zebrafish genome revealed that two genes related to CR3 proved the indirect existence of the receptor in fish (Petit et al., 2019).

Lactosylceramide (a glycosphingolipid), which consists of an aquaphobic ceramide (waxy lipid) and an aquaphile sugar moiety

TABLE 1 | Some research findings on the uses of **β**-glucan in aquaculture.

Name of the species	β-glucan/ immunostimulants	Doses	Duration (days)	Effects	References
Atlantic salmon (*Salmo salar*)	β-1,3/1,6 glucan	1 g/kg diet	119	Increased resistance to *Moritella viscosa* and infectious salmon anemia virus. Improved the protective effect (relative percentage survival at 60% mortality) of vaccine against the pathogens.	Filho et al., 2019
Nile Tilapia (*Orechromis niloticus*)	β-1,3/1,6 glucan	0.5 g/kg diet	35	Significantly improved the fish immune status. Improved the level of protection and decreased mortality rate while challenged with *Aeromonas hydrophila*.	Sherif and Mahfouz, 2019
Nile Tilapia (*Orechromis niloticus*)	Algamune™	0, 100, 200, 400, 800 mg/kg diet	21	No significant effect on weight gain and disease resistance of fish; increased macrophage extracellular superoxide anion at 200 mg/kg diet	Yamamoto et al., 2018a
Nile Tilapia (*Orechromis niloticus*)	β-glucan	500 mg/kg diet	60	Improved growth, intestinal morphology, stress resistance, and immunity on fish crowding stress	Dawood et al., 2020
Tropical Gar (*Atractosteus tropicus*)	β-1,3/1,6 glucan	0.5%, 1.0%, 1.5%, and 2.0%	62	Chymotrypsin activity increased at dose 1.0% and 1.5% No adverse effects on other digestive enzymes.	Rodriguez et al., 2018
Silver catfish (*Rhamdia quelen*)	*β*-glucan	0.01% and 0.1%	42	Higher dose showed significantly higher complement activity. Specific resistance against *Aeromonas hydrophila* raised.	Domenico et al., 2017
Giant freshwater prawn (*Macrobrachium rosenbergii*)	β-glucan	1, 2, and 3 g/kg diet	60	Lowest dose increased microvilli length and number of goblet cells. Higher doses showed negative effects on gut morphology.	Meshram et al., 2014
Orange-spotted grouper (*Epihephelus coioides*)	Mushroom β-glucan	1 and 2 g/kg diet	30	Both doses showed significantly higher lysozyme activity. Higher phagocytic and oxidative burst activity recorded.	Chang et al., 2013
Mirror carp (*Cyprinus carpio*)	β-1,3/1,6 glucan (MacroGard)	1% w/w	28	No effects on heterotrophic aerobic intestinal bacteria No effects on allochthonus lactic acid bacteria Increased intestinal microvilli length and desity	Kuhlwein et al., 2013
European common carp (*Cyprinus carpio carpio*)	β-glucan (curdlan and MacroGard)	1 mg/fish with oral gavage	14	Immune modulation by activating C-type lectin receptor (CLR), proteins similar to Dectin-1, production of short-chain fatty acids (SCFAs), fermentation of β-glucan by naïve bacteria in intestine	Petit et al., 2019; 2022
Freshwater prawn, *Macrobrachium rosenbergii*	Brewer's yeast	0.5%, 1%, and 2%	75	Medium doses showed highest phenoloxidase activity, highest survival (70%), and hemocyte count (7.8×10^6 cells/ml). Highest dose produced highest superoxide anion.	Parmar et al., 2012
Nile tilapia	β-glucan laminaran yeast cell (*Saccharomyces cerevisiae cell*)	(0.1%); (0.1%); 10 g/kg	21	All three doses showed increased cellular and humoral immunity. β-glucan–mercuric chloride group showed enhanced phagocytic and lymphocytic activity.	El-boshy et al., 2010

(Continued)

TABLE 1 | Continued

Name of the species	β-glucan/ immunostimulants	Doses	Duration (days)	Effects	References
Atlantic salmon (*Salmo salar*)	β-1,3/1,6 glucan and Mannan oligosaccharide	0.5 and 1 g/kg 1 and 2 g/kg	70	Mannan oligosaccharide, 2 g/kg feed slightly enhanced feed efficiency ratio (10%) and growth rate (8%). β-glucan, 1 g/kg feed reduced significantly the frequency of salmon lice infestation by 28%.	Refstie et al., 2010
Hybrid striped bass (*Morone chrysops x M. saxatilis*)	β-1,3/1,6 glucan paramylon (PML) and Vitamin C (vit. C)	100 mg/kg PML 500 mg/kg vit. C	56	Synergistic positive effects of PML and vit C by enhancement of immunologcal responses	Yamamoto et al., 2020
Red drum (*Sciaenops ocellatus*)	Algamune™	0, 100, 200, 400, 800 mg/kg diet	21	No significant effect on production performance of fish; increased hemolytic activity in fish fed 100 and 200 mg/kg diet	Yamamoto et al., 2018b
Marron (*Cherax tenuimenus*)	β (1/3) glucan	0.08, 0.1, 0.2, 0.4, 0.8%	84	β-glucan (0.1%–0.2%) increased hemocyte and granular cell counts Survival and yield improved at 0.1% β-glucan supplemented diet.	Sang and Fotedar, 2010
Koi/Climbing perch (*Anabas testudineus*)	Barley β-glucan	0, 5, 10, and 15 mg/L^{-1}	7	Increased lysozyme and bactericidal activity. Raised superoxide anion activity The highest dose showed least mortality against ***Aeromonas hydrophila*** for 5 days.	Das et al., 2009
Rainbow trout (*Oncorhynchus mykiss*)	Mushroom β-glucan (lentinan)	0.2% and 0.4%	54	Showed less sensitivity of genes involved in acute inflammatory reactions	Djordjevic et al., 2009
Grass carp ***(****Ctenopharyngodon idella****)***	β-1,3 glucan	10 mg/kg fish weight	15	Superoxide dismutase activity and catalase activities of erythrocyte were higher	Kim et al., 2009
Golden mahseer (*Tor putitora*)	Yeast *β*-glucan	0%, 0.5%, 1.0%, and 1.5%	56	Dietary β-glucan (0.5%–1.0%) improved thermal tolerance, immune responses, and disease resistance in fish	Akhtar et al., 2021
Zebra fish (*Danio rerio*)	Yeast β-glucan	10 µl/fish at a dose of 0.5, 2, and 5 mg ml^{-1}	7	All three concentrations reduced *Aeromonas hydrophila*-induced mortality. The highest dose reduced the mortality and increased the percentage of myelomonocytic cells.	Rodriguez et al., 2009
White shrimp (*Litopenaeus vannamei*)	Inactive yeast cell wall	1 and 2 g/kg diet	28	The highest dose showed better SGR%/ day, FCR, and PER. Diets supplemented with 1 and 2 g kg^{-1} inactive yeast cell increased hemocyte and granular cell count	Chotikachinda et al., 2008
Mussel (*Mytillus galloprovincialis*) Carpet Shell clam (*Ruditapes decussatus*)	β-glucan	100 µl to each individual at a dose of 0.05, 0.1, 0.5, and 1 mg ml^{-1}	23	The higher doses (except 0.05 mg ml^{-1}) increased oxidative burst activity. β-glucan treated clam's hemolymph hindered the growth of bacteria.	Costa et al., 2008
Rainbow trout (*Oncorhynchus mykiss*)	β-glucan (MacroGard) Barley glucan	2 g/kg diet	63	No significant dietary effect on growth and feed conversion ratio. No significant effect on lysozyme and TNF mRNA expression. Showed higher antibody titre against infectious hematopoietic necrosis virsus following vaccination.	Sealey et al., 2008
Rainbow trout (*Oncorhynchus mykiss*)	Gas1-β-glucan	0.2% or 0.5%	36	No effects on growth performance; increased lysozyme activity, Ig proportion, immunity, and disease resistance	Cornet et al., 2021
Sea bass	β-1,3/1,6 glucan (Macrogard),	250, 500, and 1,000 mg kg^{-1} diet	25	The lowest dose increased respiratory burst activity of head kidney macrophages	Bonaldo et al., 2007

(Continued)

TABLE 1 | Continued

Name of the species	β-glucan/ immunostimulants	Doses	Duration (days)	Effects	References
Seabream *Sparus aurata*	Whole yeast cells	1 ml at a dose of 10^7 cells ml^{-1}	18	Increased macrophage mononcyte numbers after 48 h. Raised acidophilic granulocyte to higher level after 4 h post injection. Increased respiratory burst activity	Cuesta et al., 2007
Rohu *Labeo rohita*	Yeast cell wall preparation	(5 g/kg feed)	56	Oxidative radical and nitrite production raised to peak. Raised lymphocytes count on day 10.	Pal et al., 2007
Flounder (*Paralichthys adspersus*)	β-glucan–mannan oligosaccharide compound	5, 10, and 15 mg/L^{-1} water	10	The lowest dose showed higher survival and growth. Promoted monocytes expression in intestinal epithelium.	Piaget et al., 2007
Channel catfish (*Ictalurus punctatus*)	MacroGard BioMos aqua grade Betagard Levucell	1g/kg feed 2 g/kg feed 0.1 g/kg feed 0.1 g/kg feed	42	No effect on growth performance or immune function against infection by *Edwardsiella ictaluri*. Survival rate increased to 17.5% more.	Welker et al., 2007
Catla (*Catla catla*)	β-glucan lactoferrin	0.1 and 0.2 ml for each fish at 100 µg ml^{-1}	30	Proliferation of leukocytes and antibody production was higher in glucan adjuvanted vaccine. Lactoferin adjuvanted fish did not show any significant change.	Kamilya et al., 2006
Asian catfish (*Clarias batrachus*)	Lactoferrin β-1/3 glucan levamisole vitamin C	100 mg/kg 0.1% 50 mg/kg 500 mg/kg feed	30	Raised antibody titre against *Aeromonas hydrophila* β-glucan was the most effective followed by levamisole, lactoferrin and vitamin C.	Kumari and Sahoo, 2006
Rohu (*Labeo rohita*)	β-–glucan	100, 250, and 500 mg/kg diet	56	The medium dose raised leukocyte count, phagocytic ratio, lysozyme activity, complement activity, and serum bactericidal activity. The medium and highest dose also resulted in better feed conversion ratio and specific growth rate.	Misra et al., 2006
Fathead minnows (*Pimephales promelas*)	Yeast β-glucan (MacroGard)	10 g kg^{-1} diet	24	Increased neutrophil degranulation in non-stressed fish. Prevented a decrease in acute stressed fish. Returned to non-stressed level in chronically stressed fish.	Palic et al., 2006
Atlantic Cod (*Gadus morhua*)	Chrysolaminaran β-glucan (MacroGard) M-alginate	0.5 g/L for 500,000 rotifers/L	30	Chrysolaminaran and M-alginate showed higher survival MacroGard had no significant effect on larval survival.	Skjermo et al., 2006
European sea bass (*Dicentrarchus labrax*)	Macrogard Ergosan (alginic acid)	(0.1%) (0.5%)	60	Had significant effect on serum complement activity on day 15. Lysozyme activity and heat shock protein concentration in gill and liver raised on day 30.	Bagni et al., 2005
Common carp (*Cyprinus carpio*)	Yeast ***β***-glucan	100, 500, and 1000 µg/fish (peritoneal injection)	29	Leukocyte, macrophage, and monocyte counts were higher. Raised interleukin 1 mRNA and antibody titer against *Aeromonas hydrophila*	Selvaraj et al., 2005
Nile tilapia (*Oreochromis niloticus*)	β-glucan	50, 100, and 200 mg/kg diet	98	Had no boost up response against *Streptococcus iniae.* Had no effect on percent mortality and relative percent survival.	Whittington et al., 2005

(Continued)

TABLE 1 | Continued

Name of the species	β-glucan/ immunostimulants	Doses	Duration (days)	Effects	References
Trout	β-1,3/1,6 glucan (MacroGard)	0.5%	40	Increased the number of specific antibody secreting cells and immunoglobulin titer against *Yersinia ruckeri*.	Siwicki et al., 2004
Black tiger shrimp	β-1,3 glucan (laminaran)	1, 2, 10, and 20 g/ kg diet	44	The dose 10 g kg^{-1} had significantly higher survival. The highest three doses raised hemocyte count, superoxide anion, and phenoloxidase activity.	Chang et al., 2003
Snapper (*Pagrus auratus*)	β-glucan (EcoActiva)	0.1%	84	Increased respiratory burst activity on day 28 at temperature 12°CC and continued up to day 84.	Cook et al., 2003
Gilthead seabream (*Sparus aurata*)	Whole wild yeast fks-1	10 g/kg feed	42	Lysozyme activity increased after 4 weeks for fks-1. Raised respiratory burst activity and natural cytotoxicity	Rodriguez et al., 2003
Black tiger shrimp (*Penaeus monodon*)	Brewer's yeast whole cell (BWC) Brewer's yeast β-glucan (BYG) Yeast *β*-glucan (YGT)	0.2%	10	Showed elevated phenoloxidase activity during *in vitro* trial. The phenoloxidase activity increased with the purity of β-glucan, yeast YGT > BYG> BWC.	Suphantharika et al., 2003
Gilthead seabream (*Sparus aurata*)	Lyophilized whole yeast (*Saccharomyces cerevisae*)	1, 2, and 5 g yeast/ kg diet	28	Had no effect on serum complement titer. Phagocytic, oxidative burst, myeloperoxidase, and natural cytotoxic activities were enhanced	Ortuno et al., 2002
Atlantic Salmon	Yeast *β*-glucan Lipopolysaccharide	1–250 μg/ml 10–250 μg/ml	6	Showed dose-dependent lysozyme activity of head–kidney macrophages. Yeast β-glucan had five times more increment. Bacterial lipopolysaccharide had six times more increment.	Paulsen et al., 2001
Sea Bass (*Dicentrarchus labrax*)	***β***-1,3 glucan (MacroGard)	2%	280	Enhanced lysozyme and alternative complement activity. Serum protein content or albumin/ globulin ratio did not vary significantly.	Bagni et al., 2000
Black tiger shrimp *(Penaeus monodon)*	*β*-1,3 glucan extracted from *Schizophyllum commune*	2 g/kg diet	40	Showed higher survival. Enhanced phagocytic acitivity and superoxide anion production.	Chang et al., 2000

(a portion of a complex molecule), is found in microdomains (lipid rafts) on the cytomembranes of immune cells. Lactosylceramide (LacCer)'s ability to interact with (1/3)-β-D-glucans is first recognized through biochemical reaction of distinct human leukocyte membrane constituents (Zimmerman et al., 1998). The reaction between glycosphingolipid and β-glucans promotes cellular responses, the release of cytokines, and the release of MIP-2 (macrophage inflammatory protein-2) and TNF (tumor necrosis factor) (Evans et al., 2005). These signaling proteins initiate the respiratory oxidative burst and anti-microbial activities of leukocytes (Wakshull et al., 1999).

Dectin-1 (type ɯ membrane receptor) predominantly binds protein ligands (Brown et al., 2003) and is considered to be the prominent β-glucan binding receptor in mammals (Dennehy and Brown, 2007; Petit et al., 2019). Dectin-1 is assessed as a β-glucan receptor (βGR) for its particular (opsonin-independent) β-glucan binding affinity (Brown et al., 2003). It consists of a recognition domain (lectin-like carbohydrate) with a short stalk [C-terminal C-type lectin or C-type lectin receptor (CLR)] and a cytoplasmic tail (consists of 40 amino acids, with an amino terminal intracellular tail), and therefore has the ability to recognize carbohydrate having β (1/3) and/or β (1/6) glycosidic bondages (Dennehy and Brown, 2007). Dendritic cells and a subpopulation of T lymphocytes are reported to lower the expression level of C-type lectin (Taylor et al., 2002). This membrane protein can be detected by flow cytometry on a subcomponent of B and T lymphocytes, though its function on these cells is not well-defined (Willment et al., 2001). However, other lines of evidence reveal that Dectin-1, in association with toll-like receptor 2 (TLR-2), results in pro-inflammatory reaction to mycobacterial contamination (Yadav and Schorey, 2006). For structural resemblance, C-type lectin has higher affinity to β-glucans having (1/3)-β-D-glucopyranosyl backbones (Adam et al., 2008). Dectin-1, as a response to antigens, recruits a variety of macrophages, DCs, and neutrophils responsible for the production of inflammatory mediators and signaling proteins (cytokines, chemokines, etc.). These antigen-presenting substances are believed to accelerate phagocytosis, oxidative burst, and neutrophil degranulation into

the phagosomes of immune cells. An investigation on *Candida albicans*, *P. carinii*, and *Aspergillus fumigatus* has proven that βGR is capable of recognizing β-glucan resembling compounds (e.g., zymosan and curdlan) and defying fungal infections in mouse (Taylor et al., 2007; Werner et al., 2009). In fish genomes, there is no clear homologue of Dectin-1 identified so far (Petit et al., 2019). However, Dectin-1 homologues have yet been identified as β-glucan pattern recognition receptors on salmon macrophages and catfish neutrophils (Magnadottir, 2006). A receptor associated with dectin-1 activation through the CLR signaling pathway is reported to mediate the immunostimulatory functions of β-glucan in common carp macrophages (Petit et al., 2019). In a recent study, Petit et al. (2022) reported that β-glucans such as curdlan and MacroGard can be fermented and produced three dominant SCFAs such as acetate, butyrate, and propionate through the production of nitric oxide and expressions of several cytokines (interleukin-1b, -6, -10, and TNF-α) *in vitro* using head kidney leukocytes of common carp. Moreover, the researchers confirmed the fermentation of MacroGard (β-glucan) by specific bacteria and immunomodulation by β-glucan through the production of SCFAs in the GIT of common carp *in vivo*.

Scavenger receptors are a superfamily of cell surface receptors having the properties of recognizing and binding to diverse ligands (Patten and Shetty, 2018). These ligands usually include low-density lipoproteins (LDL), apoptotic cells (pyknotic cells due to apoptosis), phospholipids (lecithin and phosphatidylcholine), proteoglycans (testican and versican), and carbohydrates (mainly polysaccharides) (Murphy and Weaver, 2017). This wide recognition range allows scavenger receptors to play a vital role in homeostasis (equilibrium among biological functions), apoptosis, inflammatory disorders, and pathogen removal (PrabhuDas et al., 2017).

USES OF β-GLUCAN IN AQUACULTURE

Purified β (1/3, 1/6) glucan has been increasingly used as an immunostimulant (biologically active compounds) and/or an adjuvant (substances enhancing immune response) to improve the fish immune system (Petit and Wiegertjes, 2016; Filho et al., 2019). The most useful bioactive ingredient used in aquaculture is β-glucan extracted from yeasts, mushrooms, seaweeds, and cereal plants (Bagni et al., 2005). Researchers have proven that β-glucans increase fish resistance to infectious diseases primarily by boosting their non-specific defense mechanisms. Some studies in fish have demonstrated evidence of developing the specific defense as well (Siwicki et al., 2004). The biological extracts (Lentinan, Laminaran, and Schizophyllan) and/or therapeutic chemicals (Levamisole, MacroGard, EcoActiva, Ergosan, and VitaStim) trigger the immune cells or non-specific cytotoxic cells for microbial killing through phagocytosis and/or oxidative burst (Sakai, 1999). Among the frequently trialed immunostimulants, only a few are considered suitable for preventive measure in aquaculture (Siwicki et al., 1998). Major reports that have been made on the immunomodulatory effects of β-glucans in aquaculture are related to yeast, fungi, and macro algal extracts having molecular weights ranging from 5 kDa (kelp, *Laminaria digitata*) to 200 kDa (yeast, *Saccharomyces cerevisae*) (Bagni et al., 2005). Many experiments have been conducted on different fish species to determine the effects of purified β (1/3, 1/6) glucan on their immune responses (**Table 1**). Researchers have found that β-glucan from different sources, despite their similarity in structures, molecular weights, and solution strength, can differ significantly. Even the same β-glucan administered through different routes responds in varying magnitude (Chen and Seviour, 2007).

LYSOZYME ACTIVITY, OXIDATIVE BURST, AND PHAGOCYTOSIS

Misra et al. (2006) found that rohu fish (*Labeo rohita*) fed a diet containing 250 mg of β-glucan/kg showed maximum level of immune activities (serum bactericidal activity, lysozyme activity, complement activity, and phagocytic activity) after 42 days of feeding while the indices started increasing from the 28th day of the experiment. Superoxide anion and lymphokine production made a remarkable change in their volume. The magnitude of the activities started to decrease after 42 days, maintaining a peak at a dose of 250 mg of β-glucan kg^{-1} diet. In another investigation, Pal et al. (2007) reported similar results in rohu (*L. rohita*) fish. They evidenced that the ingestion of pelleted feed containing the yeast (*S. cerevisiae*) cell wall preparation (5 g/kg feed) increased the volume of reactive oxidative radicals and nitrogen intermediates (nitrite). Lymphocyte proliferation and phagocytic activity were also enhanced during the experimental feeding. These four parameters reached a peak in treated fish on day 10 and continued until day 20 with a significant difference on day 15 compared to the control group. Paulsen et al. (2001) found that head kidney macrophages of Atlantic salmon (*Salmo salar*), supplemented with yeast β-glucan, increased extracellular lysozyme production five times higher than control after 6 days of cell incubation. The optimum concentration for linear dose-dependent curve was between 1 and 250 μg/ml. The stimulated cells showed enhanced lysozyme gene (lysozyme mRNA) transcription responsible for escalated lysozyme secretion. Chang et al. (2013) observed that the orange-spotted grouper (*Epinephelus coioides*) fed mushroom β-glucan at 1 g and 2 g per kg diet had significantly higher lysozyme and complement activities against *Vibrio alginolyticus*. The activities started to increase from day 6 and continued up to day 30. On the contrary, phagocytic activity started to increase from day 9 and respiratory burst activity started to increase from day 12. In comparison to the control fish, the minimum level of incorporation (0.5 g/kg diet) of β-glucan also brought a significant change in these parameters after feeding for 15 days. El-boshy et al. (2010) observed similar responses in Nile tilapia when fed a diet supplemented with whole yeast (*S. cerevisiae*, 10 g/kg diet), β-glucan (0.1%), and laminaran (0.1%). β-glucan-treated Nile tilapia showed elevated phagocytic activity, phagocytic index, bactericidal activity, and total lymphocyte count when challenged with *Aeromonas hydrophila* for 21 days.

Bagni et al. (2000) conducted a similar research to assess the effect of an immunostimulant on sea bass (*D. labrax*) fed a diet supplemented with β-glucan (2%), ascorbic acid (500 ppm),

and α-tocopherol (500 ppm). After 40 weeks of feeding (diets fed at 2% of body weight for 14 days with 3 months interval), the treated fish showed increased lysozyme activity (672 mg/ml) compared to the control group (455 mg/ml). Plasma complement activity was also found to be significantly high (868 ± 157 units/ml) in the treated group. Bagni et al. (2005) also conducted another research on sea bass where the fish were fed with alginic acid (0.5%) and MacroGard (0.1%) for 30 days. The experiment showed elevated serum lysozyme and complement activity, which returned to the control level after 45 days from the start of the trial. Some immune parameters (lysozyme activities and bactericidal activities) of the treated fish were found to be significantly higher at week 4 compared to week 6. In a separate study, Cuesta et al. (2007) injected whole yeast (*S. cerevisiae*, a single peritoneal injection of 10^7 cells ml^{-1}) preparation to gilthead seabream (*S. aurata*) to assess the effect. The study showed increased respiratory burst and cytotoxic activity in peritoneal exudate leukocytes. The competent cells (monocytes and macrophages) started to increase their number after 24-h post-injection and reached the significant level after 48 to 72 h post-injection. A similar experiment was conducted by Cook et al. (2003) on snapper fish (*Pagrus auratus*) fed a diet containing EcoActiva (β-glucan-based immunostimulant) and showed increased macrophage respiratory burst activity through *in vitro* superoxide anion production on day 28 at 12°CC. Up to day 84, there was a higher burst activity but with no significant difference compared to the control. Castro et al. (1999) conducted a relevant experiment to assess the *in vitro* effect of MacroGard and Fibosel on respiratory burst activity of turbot (*Psetta maxima*) and gilthead seabream (*S. aurata*) where head kidney phagocytes were pre-incubated with β-glucan for 1, 3, and 6 h. MacroGard and Fibosel induced maximum responses when the cells were pre-incubated for 2 h with β-glucan (50–100 μg ml^{-1}). However, the activity started to decrease with higher β-glucan concentration becoming significantly low at 500 μg ml^{-1}. In contrast, Sealey et al. (2008) found no significant dietary effect on lysozyme and TNF-α mRNA expression when rainbow trout was fed a high amount barley β-glucan and commercial β-glucan (MacroGard, 2 g/kg diet).

ADJUVANTED EFFECT AND ANTIBODY TITER

Researchers have proven that β-glucan can be used as a vaccine adjuvant. Siwicki et al. (2004) conducted an investigation on rainbow trout (*Oncorhynchus mykiss*) fed diets containing β (1/3, 1/6) glucan (MacroGard) at a dose of 0.5% before immunization with anti-*Yersinia ruckeri* vaccine. The fish produced higher specific antibody secreting cells (ASC) and specific immunoglobulin levels in blood serum compared to control (provided vaccine only). The highest levels of specific antibody titers and highest number of ASCs were observed between 21 and 28 days after immunization (**Table 1**). Similar research was conducted by Sahoo and Mukherjee (2001) feeding 0.1% β (1/3) glucan to rohu fish (*L. rohita*). The fish raised some non-specific and specific immunity indices and resistance against *Aeromonas hydrophila* challenge compared to control fish. A remarkable rise (4.25 times higher than control group) in bacterial agglutination titer was shown by the glucan-fed fish group while the Aflatoxin B1-injected fish group showed a 10 times lower titer than the control group. The titer of the Aflatoxin-treated fish group was restored to the control level when they were supplemented with β (1/3) glucan in their diet. The serum bactericidal activity, the phagocytic ratio, and the leukocyte numbers were also significantly higher in glucan-fed fish. Selvaraj et al. (2005) obtained similar results in common carp (*Cyprinus carpio*) injected with β-glucan (100, 500, and 1,000 μg/fish). The treated fish showed a significant increase in total leukocyte counts and enhanced the proportion of neutrophils and monocytes. The fish pre-injected with β-glucan showed adjuvanted effect and ensured higher amount of antibody titers against vaccination with *Aeromonas hydrophila*. On the other hand, Parmar et al. (2012) conducted an experiment to assess the effect of brewer's yeast on immune response and resistance of giant freshwater prawn, *Macrobrachium rosenbergii*, to white muscle disease. The prawns supplemented with 1% brewer's yeast in their diet showed significantly higher ($p < 0.05$) phenoloxidase activity compared to the control. Significantly higher total hemocyte count (7.81×10^6 cells/ml) was also observed by this group. However, superoxide anion production differed significantly ($p < 0.05$) among the treatment groups, highest in prawns provided with 2% brewer's yeast. Dawood et al. (2015) found a significant combined effect on immunity (lysozyme activity, serum bactericidal activity, and complement activity) when red sea bream (*Pagrus major*) was treated with heat-killed *Lactobacillus plantarum* (0.025%) and β-glucan (0.1%). Bonaldo et al. (2007) vaccinated sea bass (*D. labrax*) with *Vibrio anguillarum* and the medium dose (500 ppm β-glucan) group showed the highest antibody titers at the second week. Sealey et al. (2008) found that rainbow trout fed with a higher amount of barley β-glucan displayed higher antibody titers following vaccination with infectious hematopoietic necrosis virus (IHNV) than the fish fed commercial β-glucan, MacroGard. In contrast, some researchers found no adjuvanted effect of β-glucan parallel to fish vaccination. Welker et al. (2007) did not observe any rise in antibody titers in channel catfish when challenged with *Edwardsiella ictaluri* for 21 days.

GROWTH AND SURVIVAL

Skjermo et al. (2006) conducted an experiment on Atlantic cod (*Gadus morhua*) fed algal (*Chaetoceros mulleri*) glucan chrysolaminaran (0.5 g/L rotifer culture), commercial β-glucan (MacroGard), and M-alginate to test the larval response. The stimulants were fed through rotifers and weaning from the supplemented diet was started on day 17 or 18 after hatching. The larvae fed *C. mulleri* glucan had higher survival and higher body weight at day 30. A similar experiment was also conducted by Misra et al. (2006) on rohu (*L. rohita*) fish to evaluate the effect of different doses of glucan on immunity, growth, and survival against *Aeromonas hydrophila* and *Edwardsiella tarda*. Doses between 250-500 mg β-glucan kg^{-1} diets showed better specific growth rate and food conversion ratio. Another study

was conducted by Piaget et al. (2007) for six days post-hatch larvae of flounder (*Paralicthys adspersus*) to assess the effect of β-glucan and mannan oligosaccharide (applied first five days of the experiment at 5, 10, & 15 mg L^{-1} culture water) on larval growth and survival. A histological study of intestinal epithelium suggested that combined effect of β-glucan and mannan oligosaccharide enhanced monocytes expression. The immunostimulants had positive impact on the growth and survival of the larvae when provided with 5 mg L^{-1} culture water. Dawood et al. (2015) tested red sea bream (*Pagrus major*) treated with heat-killed *Lactobacillus plantarum* (0.025%) and β-glucan (0.1%). The stimulants had significant combined effects on growth (feed intake, digestibility, growth rate, protein efficiency ratio, and plasma protein level). Kumari and Sahoo (2006) carried out a 30 day long investigation to assess the effect of lactoferrin (100 mg/kg feed), β (1/3) glucan (0.1%), levamisole (50 mg/kg feed) and vitamin C (500 mg/kg feed) on the immune function of Asian catfish (*Clarias batrachus*) vaccinated with formalin killed *A. hydrophila*. The study proved the stimulants efficient for inducing immunity against the bacteria and the immunosuppressant (the cyclophosphamide) injected peritoneally at a dose of 200 mg kg^{-1} body weight. These four substances significantly increased survival rates in both immunosuppressed and healthy vaccinated and non-vaccinated fish compared to their corresponding controls. On the contrary, channel catfish (*Ictalurus punctatus*) fed yeast subcomponents did not show a significant increase in survival percentage, though 5%–17.5% elevated survival was ensured in treated fish compared to the control ones (Welker et al., 2007). Sealey et al. (2008) conducted an investigation for 9 weeks on rainbow trout (*O. mykiss*) against three barley genotypes (with different amounts of β-glucan) regarding its growth and disease resistance. The high β-glucan barley diets (52 g/kg, 82 g/kg diet) had no significant effect on growth and feed conversion ratio compared to the lower amount of barley diet (38 g/kg diet) and the control diet (wheat supplemented).

CONCLUSION AND FUTURE OUTLOOK

β-glucan alone and/or coupled with other bioactive compounds (alginic acid, lactoferrin, mannan oligosaccharide, etc.) can be an effective immunostimulant. It may also be effective in improving specific immunity in fish for pursuing adjuvanticity. More than 3,000 papers have reported the effectiveness of β-glucan in improving fish immunity, but the detailed knowledge of the receptors involved in recognizing β-glucans and their downstream signaling mechanism is yet to be clarified in teleosts (Rodrigues et al., 2020). The β-glucans often differ in their activities. From thousands of published papers, it has been evidenced that β-glucans from different sources having similarities in structures, molecular weights, and solution strength can differ remarkably. Even the same β-glucan administering through immersion, dietary inclusion, or supplementary injection can respond in different ways (Chen and Seviour, 2007). For this, it is challenging to make a consensus on β-glucan sources, doses, and duration for an individual fish species.

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Conceptualization, ZH and MH. Supervision, ZH. Writing—reviewing and editing, MH, MM, MS, SB, TM, and ZH. All authors have read and agreed to the published version of the manuscript.

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Berberine in fish nutrition: Impact on hepatoenteric health, antioxidative and immune status

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Berberine, an isoquinoline alkaloid found in some traditional medicinal plants, such as *Berberis vulgaris* and *Coptis Chinensis*, has been considered as an effective drug in treating obesity, hypertension, type-2 diabetes, hyperlipidemia, and gout in humans and animals. It currently has certain applications in fish nutrition and health, mainly because it has strong biological and pharmacological properties, such as antioxidative, anti-inflammation, antidiarrheal, analgesic, antimicrobial, anticancer, hepatoprotective, and lipid- and glucose-lowering properties. Recent studies revealed that berberine supplementation in different fish diets could alleviate liver pathological changes, intestinal histological and microbiota alterations caused by high lipid and carbohydrate diets, as well as improve growth performance, antioxidative and immune status, and stress resistance ability of fish. However, the beneficial effects of berberine vary with fish species, basal diet, feeding modes, supplementation level, and etc. This review highlights the bioavailability and toxicity of berberine, and its mechanisms in lipid and glucose metabolism, antioxidation, anti-inflammation, and protection of intestinal health, as well as the other findings on supplementing berberine in the fish diet. Moreover, this review provides future perspectives on berberine application in fish nutrition and health.

KEYWORDS

Berberine, fish health, lipid metabolism, antioxidant, immune

Introduction

Aquaculture contributes to more than 50% of seafood production in the world and is responsible for increasing human protein consumption (FAO, 2018). Fish is a better source of protein, micronutrients (e.g., minerals and vitamins), and essential fatty acids compared with that of farm animals (Kiczorowska et al., 2017). However, the rapid

expansion of aquaculture is confronted by limited land source, and most aquaculture species are under stress in intensive farming conditions, leading to the outbreak of diseases. To reduce financial losses, chemicals and antibiotics are generally used to maintain good health condition and promote the growth of fish. Nevertheless, the overuse of antibiotics could increase the emergence of antibiotic-resistant bacteria, weaken the natural immunity of farmed fish, increase host susceptibility, and cause residue accumulation in fish tissues and water, thus threatening the environment and human health (Gangwar et al., 2013; Nm et al., 2018; Abdel-Latif et al., 2020). For the sake of reducing unnecessary use of antibiotics in aquaculture, herbal components are supplemented in aquatic feed due to their low toxicity, high safety, and environment-friendly advantage, in addition to their effects in enhancing disease resistance, immune and antioxidative status, promoting growth, and treating metabolic disorders (Zhu, 2020). Recently, the application of natural compounds has been widely explored in feed to replace antibiotics in monogastric animals, fish, and poultry (Alagawany et al., 2021; Tadese et al., 2022).

Berberine is a yellow odorless crystalline powder with an extremely bitter taste. Its molecular formula is $C_{20}H_{18}NO_4^+$ (16,17-dimethoxy-5,7-dioxa-13-azoniapentacyclo [11.8.0.02,10.0^{4},^{8}.015,20]henicosa-1(13),2,4(8),9,14,16,18,20-octaene) with a molecular weight of 336.37g/mol (Figure 1). It can easily dissolve in hot ethanol, but slowly dissolves in water, and is almost insoluble in low-polar organic solvents (Xu et al., 2021). It is an isoquinoline alkaloid that widely exists in the rhizome, roots, and stems of some traditional medicinal plants, such as *Berberis aristata* DC, *Coptis Chinensis* Franch, and *Berberis vulgaris* L. The berberine contents in these plants range from 0.05 mg/g to 96.10 mg/g, while *Berberis* is the richest natural source of berberine (Xu et al., 2021). Presently, chemical synthesis is a more productive way to obtain berberine, and berberine sulfate salt and chloride are commonly used in the clinic because they are relatively more soluble than berberine (Kumar et al., 2015).

There is a long history of using berberine in Chinese and Ayurvedic medicinal systems (Kumar et al., 2015). In traditional Chinese medicine, berberine is widely used for treating diarrhea, dysentery, fatty liver, hypertension, obesity, and type-2 diabetes (Liu et al., 2016; Wang et al., 2020a), owing to its strong antioxidant, anti-inflammation, antidiarrheal, analgesic, anticancer, and antimicrobial activities, as well as lipid- and glucose-lowering properties (Kumar et al., 2015; Pirillo and Catapano, 2015; Liu et al., 2016; Xu et al., 2021).

In fish feed, carbohydrate and lipid are widely used because of their "protein-sparing" effect, as well as they are important nutrients for fish. Suitable levels of dietary lipid and carbohydrate can promote growth performance, improve feed efficiency, reduce dietary protein requirement, reduce disease susceptibility, and maintain the health condition of fish (Ai et al., 2004; Wu et al., 2016; Kamalam et al., 2017; Wang et al., 2019). However, the lipid and carbohydrate levels in fish feed sometimes exceed the requirements because they are cheaper energy sources than protein. Excessive lipid and carbohydrate in the fish diet causes lipid accumulation in the liver, impairs liver functions, reduces the antioxidative and immune status of fish, and eventually leads to low yields and economic losses (Wu et al., 2016; Lin et al., 2018; Cao et al., 2019; Yang et al., 2019). The liver is a metabolic organ in charge of lipid and glucose homeostasis, plasma protein production, degradation of toxins, and bile synthesis (Zhou et al., 2021). Thus, maintenance of liver normal function is vital for fish health and growth. Previously, some herbal compounds were proven to attenuate liver dysfunction caused by high lipid (HL) diet and high carbohydrate (HC) diet (e.g., xylooligosaccharides (Abasubong et al., 2018), resveratrol (Shi et al., 2018), and curcumin (Bao et al., 2022)). Berberine has

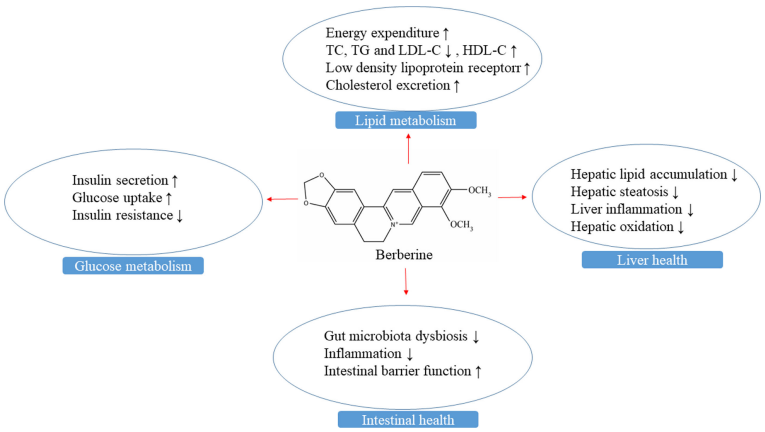

FIGURE 1
The mechanisms of berberine in maintaining liver and intestine health, and glucose and lipid metabolism.

immense potential to be a novel feed additive in treating liver dysfunction caused by HL and HC diets in fish, due to its strong effects in treating metabolic disorders, such as type-2 diabetes, fatty liver, hyperlipidemia, obesity, and non-alcoholic steatohepatitis (NASH) in clinical and animal studies, suggesting its important role in glucose and lipid metabolism (Xu et al., 2021). Besides, berberine supplementation also promoted growth performance, intestinal health, antioxidative capacity, and immune status of fish in several studies (Doan et al., 2020; Yu et al., 2020).

This review paper aims to summarize the bioavailability and toxicity of berberine, and its mechanisms in lipid and glucose modulation, antioxidant and anti-inflammatory effects, and regulation of intestinal microbiota demonstrated in clinical and animal studies. We also review the current berberine-related studies in fish health to increase its potential application in aquaculture.

Bioavailability

Feng et al. (2015) proposed that the gut microbiota converts insoluble berberine into dihydroberbreine (dhBBR), which showed a five times faster absorption rate than that of berberine in the intestine of rats. DhBBR is subsequently oxidized to berberine and enters blood circulation. Although intestinal microbiota contributes to the absorption of berberine, the bioavailability of berberine is still lower than 1% after oral administration (Imenshahidi and Hosseinzadeh, 2016). Several factors affect the intestinal absorption of berberine: 1) Intestinal first-pass elimination. Liu et al. (2010) reported that almost half of the intragastric administrated berberine disposed in the small intestine of rats due to the intestinal first-pass elimination, which caused very low oral bioavailability (0.36%); 2) Berberine is in the ion form and easily occurs self-aggregates in the stomach and upper intestine, with acidic environment (Spinozzi et al., 2014); 3) Berberine exhibits low permeability across the intestinal mucous membrane of rats, and is identified as a low permeable drug (Liu et al., 2010); 4) The re-excretion of berberine from the hepatoenteral circulation process inhibits its absorption (Imenshahidi and Hosseinzadeh, 2016; Liu et al., 2016); 5) Berberine is a substrate of P-glycoprotein (P-gp), which is a ATP-binding cassette transporter that located in the apical membrane of the epithelial layer of the gut wall, and is in charge of maintaining the integrity of intestinal barrier to keep body away from many drugs and exogenous toxins, thus P-gp is a crucial factor that influences bioavailability of orally administered drugs in the intestine (Chen et al., 2014). P-glycoprotein reducing berberine absorption by transporting berberine out of cells has been widely reported *in vivo* and *in vitro* (Maeng et al., 2002; Shitan et al., 2007; Pirillo and Catapano, 2015). Besides, P-gp expression in the intestine increased with increasing berberine levels and berberine exposure time, indicating the high berberine level or long-term administration of berberine may result in poor berberine absorption (Benet et al., 1999; Maeng et al., 2002). Similarly, Kheir et al. (2010) observed that a low concentration of berberine is rapidly absorbed by the intestine, whereas a high level of berberine caused absorption limitation in rats. Some studies have reported that P-gp antagonists, such as silymarin (Di Pierro et al., 2013), tetrandrine (Shan et al., 2013a), and cationized chitosan (Fratter and Servi, 2015) treatments significantly improved intestinal berberine absorption. Therefore, oral administration of berberine normally shows low bioavailability.

Toxicity and side effects

Generally, berberine has very low side effects and toxicity, and the toxicity is related to administration methods (Pang et al., 2015). A study in mice found that the LD_{50} of intraperitoneal (IP) and intravenous (IV) injections were 57.6 g/kg and 9.0 g/kg, respectively, whereas intragastric administration did not obtain LD_{50}, which might be due to the low oral bioavailability of berberine (Kheir et al., 2010). Rad et al. (2017) concluded that the toxicity of berberine varies with dosage, animal species, and routes of administration, and the oral route is less toxic than IP and IV injections. Similarly, mice fed 156 mg berberine/kg/day for 30 days did not cause any death (Yi et al., 2013). In a clinical study, the gastrointestinal adverse effects were only presented in the first four weeks in 34.5% of patients when treated with 0.5g berberine (twice a day) for 13 weeks (Yin et al., 2008). Thus, both clinical and animal studies have demonstrated that berberine exhibits low side effects and toxicity, hence potentially a safe drug.

Liver protection, antioxidative and immunomodulatory properties

The liver is the main accumulation organ of berberine after oral administration, followed by the kidney, muscle, heart, and pancreas in mice (Pirillo and Catapano, 2015). The high hepatic accumulation of berberine may account for its lipid-lowering effect on the liver (Liu et al., 2016). Berberine displays a potent hepatoprotective effect that has been extensively studied, which may be ascribed to its regulation of glucose and lipid metabolism, antioxidative and immune systems (Figure 1).

Regulation of glucose metabolism

Numerous studies demonstrated that berberine has anti-diabetic activity, and the main mechanisms are: 1) Promotion of insulin secretion. Glucagon-like peptide-1 (GLP-1) is produced

by enteroendocrine L cells in the gut which is sensitive to nutrient ingestion and in charge of the overall insulin response to glucose ingestion (Chen et al., 2014). Besides, berberine can improve insulin production and secretion through the insulin/insulin-like growth factor-1 signaling cascade in 3T3-L1 adipocytes (Ko et al., 2005). 2) Alleviating insulin resistance (IR). Berberine reduces IR by the protein kinase C (PKC)-dependent upregulation of insulin receptor substrate-2 (IRS-2) in rats (Xing et al., 2011; Yang et al., 2011; Yan et al., 2015; Zhao et al., 2017). In addition, berberine lessened IR by improving the activity of AMP-activated protein kinase (AMPK) in 3T3-L1 adipocytes (Lee et al., 2006). 3) Inhibiting gluconeogenesis. Glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) are two vital enzymes in the gluconeogenesis pathway. Berberine inhibited the expressions of PEPCK and G6Pase by upregulating the expressions of liver kinase B1 (LKB1) and AMPK, thus reducing the translocation of cAMP response element-binding protein (CREB)-regulated transcription coactivator 2 (TORC2) into the cell nucleus in STZ-induced diabetic rats (Jiang et al., 2015). 4) Promotion of glucose uptake. A study in 3T3-L1 adipocytes proved that berberine promoted glucose uptake through activating adenosine monophosphate-activated protein kinase, and increasing glucose transporter 1 (GLUT 1) activity (Zhou et al., 2007). Besides, berberine can increase glucose transporter 4 (GLUT4) translocation into the plasma membrane to improve glucose uptake through the insulin receptor substrate-1-phosphoinositide 3 Kinase-Akt (IRS1-PI3-Kinase-Akt) and insulin signaling pathways in 3T3-L1 adipocytes (Ko et al., 2005). 5) Promoting glycolysis. Berberine can inhibit the mitochondrial respiratory chain complex I to reduce ATP production, then elevate AMP/ATP ratio and activate AMPK, thus inhibiting gluconeogenesis and improving glycolysis in HepG2 hepatocytes and C2C12 myotubes (Xu et al., 2014). 6) Berberine reduces intestinal glucose digestion and absorption by inhibiting α-glucosidase activity, which is in charge of carbohydrates digestion and monosaccharides production (Pang et al., 2015) (Figure 2).

Regulation of lipid metabolism

Berberine exhibits an anti-hyperlipidemia effect by reducing triglyceride (TG), total cholesterol (TC), and low-density lipoprotein cholesterol (LDL-C) contents in the plasma of rats (Tang et al., 2006), as well as a hepatoprotective effect by reducing hepatic TG and TC contents (Zhou et al., 2021). The mechanisms of berberine regulating lipid metabolism are mainly: 1) Inhibition of lipid uptake. Sun et al. (2017) reported that berberine inhibited intestinal bile salt hydrolase secretion, increased taurocholic acid discharge, activated intestinal farnesoid X receptor (FXR) pathway, and reduced hepatic fatty-acid translocase Cd36 expression, which together inhibited long-chain fatty acid uptake of the mice liver. 2) Promoting lipid oxidation and inhibiting lipogenesis. Berberine can activate the AMPK signaling pathway, which is a target for metabolic diseases and is the upstream gene of many lipid-producing genes. Berberine promotes phosphorylation of AMPK and increases expression of fibroblast growth factor 21 (FGF21) to increase hepatic energy metabolism in primary

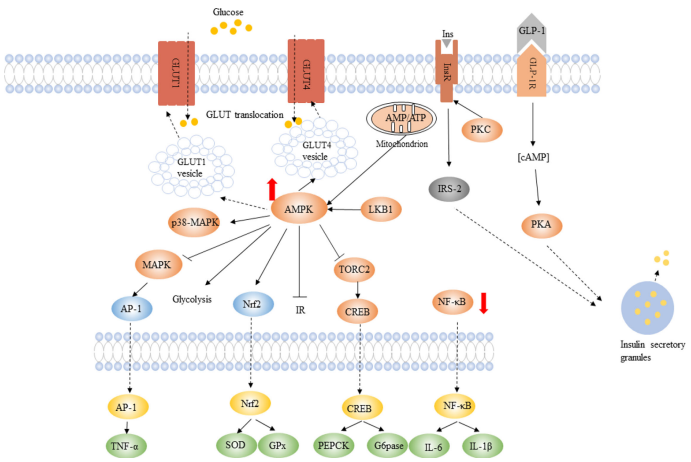

FIGURE 2
The potential mechanisms of berberine in regulating glucose metabolism, improving antioxidative and immune status. Berberine can enhance insulin sensitivity and improve insulin secretion. BBR induces glycolysis *via* activating AMPK pathway and increasing GLUT1 and GLUT4 translocation. BBR reduces the expression of PEPCK and G6Pase genes to suppress gluconeogenesis. BBR decreases the production of pro-inflammatory cytokines, for example, TNF-α. Up arrows indicate an increase or activation, and down arrows indicate a decrease or suppression. Modified from Xu et al. (2021) and Zhou et al. (2021).

mouse hepatocytes (Zhou et al., 2018). Besides, it also promotes phosphorylation of sterol-regulatory element binding protein-1c (SREBP-1c) to suppress the fatty acid synthase (FAS) and stearoyl-CoA desaturase (SCD) expressions, thus improving hepatic steatosis and reducing hepatic TG synthesis in mice (Zhu et al., 2019). In addition, Sirtuin 3 (SIRT3) is also activated by phosphorylation of AMPK, which affects carnitine palmitoyltransferase-1A (CPT-1A) and phosphorylation-acetyl-CoA carboxylase (ACC) expressions to increase liver steatosis of rats (Zhang et al., 2019). 3) Increasing lipid transportation. Excessive cholesterol intake generally causes massive cholesterol accumulation in the liver, which activates nonparenchymal cells and leads to liver metabolic abnormalities. Low-density lipoprotein (LDL) transports cholesterol from the liver to peripheral tissue cells to alleviate liver damage (Zhou et al., 2021). Berberine can increase low-density lipoprotein receptor (LDLR) expression in the liver, and it also works on the 5′ proximal section of LDLR mRNA 3′ UTR to improve the stability of LDLR mRNA, which is achieved by activating the signaling cascade of AMPK/Raf-1/MEK/ERK in human hepatoma cells (Kong et al., 2004). 4) Maintenance of the normal function of mitochondria. Gomes et al. (2012) reported that berberine alleviated mitochondrial dysfunction caused by hyperglycemia and the high fat (HF) diet partly *via* increasing mitochondrial biogenesis modulated by sirtuins 1 gene through the AMPK pathway in the skeletal muscle of rats. 5) The alleviation of IR by berberine also contributes to lipid metabolism (Zhou et al., 2021) (Figure 3).

Antioxidative and anti-inflammatory effects

Berberine and its derivatives have strong antioxidant activity, and both *in vivo* and *in vitro* studies revealed that berberine is able to inhibit reactive oxygen species (ROS) production (Shan et al., 2011; Siow et al., 2011). Firstly, berberine can quench nitric oxide (NO), superoxide anion (O_2^-) and the precariously reactive molecule, peroxynitrite ($OONO^-$) directly (Siow et al., 2011). A study in rats found that oral administration of berberine alleviated the decline of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) activities in serum by scavenging $ONOO^-$, NO, and O_2^- stress (Yokozawa et al., 2010). Besides, berberine can inhibit ROS-producing oxidase enzymes. Sarna et al. (2010) observed that berberine effectively inhibited the production of intracellular superoxide in LPS-stimulated macrophages by selectively inhibiting $gp91^{phox}$ expression. Similarly, berberine activated the AMP-activated protein kinase sensitive pathway and reduced iNOS expression to inhibit NO production in LPS treated murine macrophages (Jeong et al., 2009). Hur et al. (2009) also reported that berberine inhibited the production of ROS through the p38 mitogen-activated protein kinase (MAPK) pathway, and activated caspase-3 in the human hematoma HepG2 cells. Furthermore, berberine can modulate the activities of several endogenous antioxidant enzymes. For example, a study in macrophages revealed that pretreatment with berberine significantly increased SOD activity (Sarna et al., 2010).

Berberine can directly downregulate the expressions of pro-inflammatory cytokines, such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β), as well as indirectly affect the expressions of activator protein 1 (AP-1), nuclear factor-kappa B (NF-κB), AMPK, Rho GTPase signaling pathways, and MAPK- mediated pathways to decrease inflammation response in human macrophages (Chen et al., 2014). Berberine mitigated non-alcoholic steatohepatitis (NASH) by the C-X-C chemokine receptor type 4 (CXCR4)

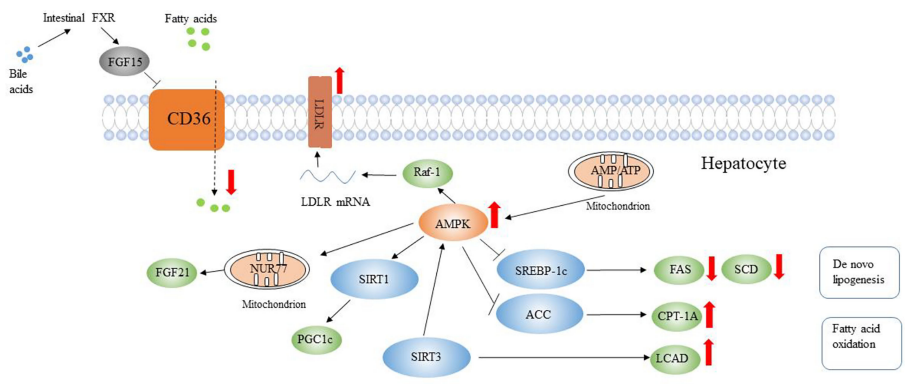

FIGURE 3
The potential mechanisms of berberine in reducing liver lipid accumulation. Berberine inhibits hepatic lipid absorption by the intestinal microbiota-bile acid-FXR pathway. Berberine inhibits *de novo* lipogenesis but improves fatty acid oxidation *via* activation of AMPK. Berberine increases LDLR expression and LDLR mRNA stabilization. Up arrows indicate an increase or activation, and down arrows indicate a decrease or suppression. Modified from Xu et al. (2021) and Zhou et al. (2021).

signaling pathway, and significantly reduced the expressions of interleukin-8 (IL-8), NF-κB, and phosphoinositide 3-kinase in mice (Yang et al., 2017). Likewise, berberine treatment increased the survival rate, anti-inflammatory cytokines (IFN-γ and IL-10) expressions, and downregulated proinflammatory cytokines (IL-17A, IL-6, IL-17F, and IL-1β) expressions in the liver and spleen of ducks infected by *Riemerella anatipestifer* (Fernandez et al., 2017). Besides, berberine also enhanced macrophage function through increasing apoptosis by caspase-3 activation in the DDS-induced colitis mice (Yan et al., 2012).

Protection of the intestine and regulation of intestinal microbiota

Due to the low bioavailability of berberine, more than 90% of oral-administrated berberine remains in the intestine, causing the intestine to become the main working site of berberine (Wang et al., 2017; Pan et al., 2019). Gu et al. (2015) reported that a stronger lipid-lowering effect was found in intragastrically administered hamsters than that of intraperitoneally administered hamsters, even though the former showed much lower bioavailability than the latter, indicating the gastrointestinal tract was the main target for the hypolipidemic effect of berberine.

The intestine is regarded as the first line of defense against antigens. The intestinal physical barrier determines the absorption capacity of nutrients, and its integrity is vital for animal health (Lin et al., 2020; Wang et al., 2020b). Berberine was demonstrated to restore barrier function and maintain epithelial gut permeability in diseased intestines. Berberine reduced the TNF-α-NF-κB-MLCK pathway by increasing tumor necrosis factorα-induced protein 3 (TNFAIP3) expression to protect intestinal epithelial tight junction and repaired the intestinal epithelial barrier in diarrhea-predominant irritable bowel syndrome rats (Hou et al., 2019). Besides, Gu et al. (2011) reported that pretreatment of berberine prevented the disruption of tight junction in intestinal epithelium caused by lipopolysaccharide (LPS) in mice, which might be linked to the down-regulating of the NF-κB and myosin light chain kinase pathway.

It is well-known that berberine has antidiarrheal activity, mainly because it has a direct bactericidal effect. Berberine was demonstrated to reduce bacterial adherence to the epithelial or mucosal surface, and decrease intestinal secretion of electrolytes and water caused by microbial enterotoxins (Chen et al., 2014). Besides, a wide range of animal experiments revealed that berberine has noteworthy effects on intestinal microbiota composition. Berberine could kill or repress some harmful intestinal bacteria, such as gram-negative bacteria (e.g., *K. pneumonia*, *P. mirabilis*, and *E. coli*) (Čerňáková and Košťálová, 2002), and promote the growth and reproduction of some beneficial bacteria (e.g., *Lactobacillus acidophilus* and *Bifidobacterium adolescentis*) (Chen et al., 2014). The antimicrobial activity of berberine also contributes to curing metabolic disorders. For example, Firmicutes and Bacteroidetes are two dominant gut microbiota, and a higher Firmicutes/Bacteroidetes (F/B) ratio may be an indication of more intestinal energy harvest (Jumpertz et al., 2011; Cao et al., 2016). Xie et al. (2011) found berberine supplementation in a HF diet increased fecal Bacteroidetes and reduced Firmicutes richness, along with reduced blood glucose and lipid of rats. A similar observation of intestinal F/B ratio was reported in rats when berberine was co-administrated with a high-fat diet (Sun et al., 2016). Berberine also reduced insulin resistance, plasma lipid contents and endogenous glucose production of rats fed a HF diet by regulation of the microbiota-gut-brain axis (Sun et al., 2016). In mammals, berberine regulated bacterial bile salt hydrolase and 7α-dehydroxylases activities, thus changing the bile acid pool, FXR signaling, and alleviating metabolic disease (Gu et al., 2015a; Sun et al., 2017), which is an important mechanism of regulating energy and immune by berberine. Moreover, berberine can promote intestinal butyrate-producing bacteria (e.g., *Ruminococcus*, *Butyricimonas*, and *Coprococcus*) growth to produce short-chain fatty acids (SCFAs) (Xu et al., 2021), which is beneficial for alleviating inflammation, improving gut barrier functions, creating a nonpermissive environment for pathogens, and helpful for obesity and insulin resistance-related metabolic abnormalities (Pang et al., 2015).

The role of berberine in fish nutrition

The effects of supplementing berberine in different fish diets are shown in Table 1. It includes a total of 18 papers, which reported the roles of berberine in protecting liver and intestine functions, improving growth performance, antioxidative and immune status, as well as increasing disease and stress resistance ability. Many of the berberine functions observed in fish have been reported in rodent animals and *in vitro*, but there are still some discrepancies with the previous findings.

Optimal supplementation level and feeding mode of berberine

Qin (2014) studied the pharmacokinetics of berberine hydrochloride in Nile tilapia (*Oreochromis niloticus*). After oral administration of 30 mg/kg body weight of berberine hydrochloride, the berberine concentrations in different tissues were liver > kidney > muscle > plasma. After intraperitoneal injection of 10 mg/kg body weight berberine hydrochloride, the berberine levels were kidney > liver > muscle > plasma. Oral administration of berberine hydrochloride showed slower absorption and elimination rates but wider distribution than

that of intraperitoneal injection. These pharmacokinetic patterns were similar to the results in other animals (Li et al., 2005; Feng et al., 2015; Kumar et al., 2015; Pirillo and Catapano, 2015).

As shown in Table 1, the supplementation level of berberine in fish feed ranged from 30 to 9000 mg/kg in different fish species. In Chinese Fisheries Pharmacopoeia, 30 mg/kg berberine is the highest level for controlling bacterial diseases

TABLE 1 Effects of berberine on fish nutrition.

Fish	Initial weight (g)	Feeding period (days)	Basal diets	Supplementation levels (mg/kg)	Feeding mode	Main results	References
Black sea bream	1.47	56	11.1% and 20.25 fat	50	Continuous	WG→; whole body and muscle lipid ↑; liver lipid ↓; hepatic lipid β oxidation genes ↑; lipid synthesis genes ↓; serum TG, LDL-C, and ALT ↓	Wang et al. (2021)
	1.52	56	23% starch diet	50	Continuous	WG →; hepatic lipid ↓; serum ALT and AST ↓; serum glucose ↓; liver mitochondria density ↑; hepatic GPx activity ↑; MDA content after ammonia challenge ↓	Wang et al. (2020a)
Blunt snout bream	8.15	56	15% fat	50	Continuous	Hepatic CPT 1 and PPAR α expressions ↑; PPAR β and PPAR γ expressions →	Lu et al. (2016)
	8.15	56	15% fat	50 and 100	Continuous	WG↑; hepatic fat ↓; hepatic CPT1, AOX, ApoB, ApoE, PGC-1α, PPARα, FATP, LPL, and LDLR expressions↑	Zhou et al. (2019)
	80	56	5% and 10% fat	50	Continuous	WG↑ (low-fat group); hepatocyte apoptosis ↓; hepatic LPO, MDA, and PC ↓; plasma ACP, LYZ, C3, and C4 ↑; SR ↑ after ammonia challenge	Chen et al. (2016)
	8.15	56	15% fat	50 and 100	Continuous	mitochondrial respiratory chain and density ↑; hepatic oxidative stress ↓; hepatocyte apoptosis ↓	Lu et al. (2017)
	4.70	56	5% and 10% fat	50	Continuous, two-week, and four-week interval	WG ↑; plasma TC and TG ↓; serum LYZ, C3 and C4 ↑; hepatic MDA and LPO ↓, SOD and CAT ↑; mortality ↓ after challenged by *Aeromonas hydrophila*	Xu et al. (2017)
	44.83	56	5% fat, 10% fat and 43% nitrogen-free extract	50	Two-week interval	WG (low fat group) ↑; microvilli length; goblet cells number, intestinal integrity and intestinal permeability ↑; intestinal TNF-α and IL-6 expressions ↓	Yu et al. (2020)
	20.36	70	43% carbohydrate	50	Continuous	WG →; plasma glucose, TG and TC ↓; hepatic lipid and glycogen ↓; glucose uptake and decomposition in liver ↑; glycogen synthesis ↓; hepatic lipid oxidation ↑; insulin pathway ↑	He et al. (2021)
Common carp	150	21	Normal diet	780	Continuous	Respiratory burst, LZM, MPO, and phagocytic activities, C3 level ↑; TNF-α, IL-1β, lysozyme-c and C3 expressions ↑, IL-10 expression ↓ in the head kidney; SR ↑ after infected with *Aeromonas hydrophila*	Zhou et al. (2016)
Grass carp	34.0	56	Normal diet	30mg/kg body weight	Continuous	WG →; Serum glucose, TC and TG ↓; liver TC and TG ↑; intestinal microbiota diversity ↑, Firmicutes to Bacteroidetes ↓	Pan et al. (2019)
Largemouth bass	122	56	HCD	500, 1000, and 2000	Continuous	WG ↑, serum glucose, TG, TC, and LPL-c ↓, hepatic antioxidative status ↑, hepatic gluconeogenesis ↓	Xia et al. (2022b)
	67.15	77	HCD	100 and 400	Continuous	WG →; serum glucose and hepatic glycogen ↓; insulin pathway genes ↑, hepatic antioxidative status ↑; intestinal potential pathogenic bacteria genera *Plesiomonas* ↓	Chen et al. (2022)
	8.70	56	Normal diet and HCD	1000	Continuous	WG →, serum glucose and hepatic glycogen ↓; the intestinal-FXR signal pathways ↑; modulation of intestinal microbiota	Xia et al. (2022a)
Nile tilapia	11.61	56	Norma diet	1000, 3000, 6000, and 9000	Continuous	WG ↑; skin mucus LYZ and peroxidase ↑; survival rate after *Streptococcus agalactiae* infection ↑	Doan et al. (2020)
Yellow drum	5.57	56	soybean-oil-based	50	Continuous	WG ↑, VSI ↓, hepatic lipid ↓, hepatic fatty acid β-oxidation ↑, hepatic proinflammatory genes expression ↓	Tan et al. (2022)

(Continued)

TABLE 1 Continued

Fish	Initial weight (g)	Feeding period (days)	Basal diets	Supplementation levels (mg/kg)	Feeding mode	Main results	References
Zebrafish	Three-month-old	0, 2, 4, 8, 14, 20, 25, and 30	HCD	1000 and 2000	Continuous	Serum TC, TG, and LDL-C ↓, Hepatic fat content and HMGCR expression ↓, LDLR and CYP7A1a expressions ↑	Han et al. (2015)
	5 days post-fertilization	10	HCD	0, 1, 5, and 25 μM	Continuous	Liver lipid accumulation ↓, ROS level ↓; lipid metabolism ↑; hepatic GSH ↑, MDA↓, and iron homeostasis ↑	Chen et al. (2020)

Symbols indicate an increase (↑), decrease (↓) or no effect (→) on the parameters. Survival rate (SR);high-cholesterol diet (HCD); myeloperoxidase (MPO); lysozyme (LZM); complement C3 level (C3); total cholesterol (TC); triglyceride (TG); low density lipoprotein cholesterol (LDL-c); high-density lipoprotein cholesterol (HDL-C); acid phosphatase (ACP); malondialdehyde (MDA); protein carbonyl (PC); lipid peroxide (LPO); lipoprotein lipase (LPL); carnitine palmitoyltransferase I (CPT 1); peroxisome proliferator-activated receptors α (PPAR α); 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR); low-density lipoprotein receptor (LDLR); cytochrome P450, family 7, subfamily A, polypeptide 1 a (CYP7A1a); fatty acid transport protein (FATP); Acyl-CoA oxidase (AOX); Apolipoprotein B (E), (Apo B (E)); peroxisome proliferator-activated receptor gamma coactivator-1α, (PGC1α); tumor necrosis factor alpha (TNF-α); interleukins-6 (IL-6); viscerosomatic index (VSI).

in fish (Pan, et al., 2019). A study in blunt snout bream (*Megalobrama amblycephala*) reported that supplementation of 50 mg/kg berberine in a HF diet (15% fat) increased weight gain (WG) and specific growth rate (SGR) significantly, while 100 mg/kg berberine did not influence the growth of fish, which might be because 100 mg/kg berberine reduced the palatability of the diet since feed intake (FI) was significantly reduced, the authors observed some fish spat out the feed after eating the 100 mg/kg berberine supplemented HF diet due to its bitter taste. Thus 50 mg/kg berberine was recommended as a suitable level in fish feed (Zhou et al., 2019), and widely applied in the studies on blunt snout bream (Chen et al., 2016; Lu et al., 2016; Xu et al., 2017; Yu et al., 2020; He et al., 2021) and black sea bream (*Acanthopagrus schlegelii*) (Wang et al., 2020a; Wang et al., 2021). However, Doan et al. (2020) supplemented 1, 3, 6, and 9 g/kg berberine in the Nile tilapia diet, but the authors did not report the feed utilization data. And the reduction of FI in black sea bream was only observed in the 50 mg/kg berberine supplemented normal diet group, not in the berberine supplemented HL diet group (Wang et al., 2021). On the contrary, 400 mg/kg berberine supplementation in a HC diet significantly improved FI of largemouth bass (Chen et al., 2022). Besides, 50 mg/kg berberine supplemented in the HC diet did not affect the FI of blunt snout bream (He et al., 2021) and black sea bream (Wang et al., 2020a). Thus, the bitter taste of berberine may influence feed palatability in some cases, which varies with supplementation level, fish species, and basal diet composition.

Nile tilapia were fed 1, 3, 6, and 9 g/kg berberine supplemented diets for 8 weeks, and the lowest supplementation level (1g/kg) obtained the lowest feed conversion ratio (FCR), with the highest WG, SGR, and immune parameters (Doan et al., 2020). Xu et al. (2017) explored the effects of supplementing berberine in 5% and 10% fat diets with different feeding modes (continuous, 2-week interval, and 4-week interval modes) on the growth and immunity of blunt snout bream. They found that the low-fat diet with 50 mg/kg berberine at 2-week interval mode obtained the best growth performance of fish, while fish fed berberine supplemented high-fat diet at 2-week interval or 4-weeks interval feeding mode showed similar or even better growth performance than that of the continuous feeding mode. These results indicate that high dietary berberine level or long berberine exposure time may reduce the function of berberine. One hypothesis is that the intestinal Pg-p expression being upregulated by high dosage, or long-term administration of berberine, which inhibits the absorption of berberine by the intestine as reported in mammals (Maeng et al., 2002; Shan et al., 2013b). However, this mechanism in fish has not been reported and deserves further study. Thus, supplementing a relatively low level of berberine in the diet with a discontinuous feeding mode may be an effective and economical way in fish feed. Considering the high discrepancy of berberine supplementation levels in the previous studies, the effective and economical dietary berberine levels of different fish species should be investigated. Furthermore, even 9 g/kg berberine supplementation of berberine enhanced the growth performance of Nile tilapia (Doan et al., 2020), indicating that berberine is a safe feed additive in fish.

Effects on growth

Enhanced growth performance was reported in blunt snout bream fed a 50 mg/kg berberine-supplemented normal diet (Chen et al., 2016; Xu et al., 2017), a 50 mg/kg berberine-supplemented HC diet (Yu et al., 2020), a 50 mg/kg berberine-supplemented HL diet (Zhou et al., 2019), Nile tilapia fed the 1, 3, 6, and 9 g/kg berberine-supplemented normal diet (Doan et al., 2020), and yellow drum (*Nibea albiflora*) fed 50 mg/kg berberine-supplemented soybean oil-based diet (Tan et al., 2022). The growth promotion effect of berberine might be ascribed to its hepatoprotective effect, like some other Chinese herbs (Zhou et al., 2015; Zhou et al., 2019). Doan et al. (2020) proposed that the modulation of the gut microbiota (especially

SUFA producing bacteria) by berberine might be a contributing factor for the enhanced growth performance of fish. Tan et al. (2022) hypothesized that the hepatic ribosome biogenesis might be the main reason accounting for improved growth performance of yellow drum fed berberine supplemented soybean oil-based diet, since ribosome biogenesis in eukaryotes was the most significantly enriched-KEGG pathway based on RNA sequencing. Largemouth bass fed 1000 mg/kg and 2000 mg/kg berberine for eight weeks obtained significantly improved growth performance, while 500 mg/kg did not significantly influence growth of fish (Xia et al., 2022b). Nevertheless, a nonsignificant effect on growth performance was reported in different species: black sea bream fed 50 mg/kg berberine supplemented in normal, high starch, and HL diets (Wang et al., 2020a; Wang et al., 2021); blunt snout bream fed a 50 mg/kg berberine supplemented HF diet (Xu et al., 2017; Yu et al., 2020) and a 50 mg/kg berberine supplemented HC diet (He et al., 2021); largemouth bass fed the 100 mg/kg and 400 mg/kg berberine supplemented HC diet (Chen et al., 2022), and grass carp (*Ctenopharyngodon idella*) fed a 30 mg/kg berberine supplemented normal diet (Pan et al., 2019). While studies in rats and humans revealed that chronic administration of berberine caused growth inhibition due to increased energy expenditure, alleviated growth of adipose tissue, and prevented obesity (Lee et al., 2006; Kim et al., 2009; Hu et al., 2012; Ilyas et al., 2020). Thus, the growth promotion effect of berberine may vary with fish species, basal diet composition, feeding mode, and other factors yet to be determined.

Lipid metabolism

The liver is the main organ for nutrient metabolism, and it also plays an important role in plasma protein production, bile synthesis, degradation of toxins, as well as lipid and glucose homeostasis in the body (Zhou et al., 2021). Liver diseases normally occur in fish partially because of excessive lipid and/or carbohydrate in the diet. Berberine was demonstrated to alleviate excessive hepatic lipid accumulation and pathologic changes caused by HL and HC diets in black sea bream (Wang et al., 2020a; Wang et al., 2021), blunt snout bream (Lu et al., 2017; Zhou et al., 2019; He et al., 2021), and zebrafish (Chen et al., 2020). Zhou et al. (2019) reported that HF diet caused excessive glycogen droplets, stricture of the hepatic sinus, inordinate and obscure hepatic cords, irregular arrangement of hepatocytes, diffused lipid vacuolization, large and electron-dense fat droplets in the liver of blunt snout bream, while 50 and 100 mg/kg berberine supplementation alleviated these hepatic abnormalities. Moreover, increased mitochondria density and repaired mitochondrial respiratory chain in hepatocytes by berberine inclusion were reported in blunt snout bream (Lu et al., 2017) and black sea bream (Wang et al., 2020a), which might be related to the improved liver energy expenditure and/or maintenance of mitochondria function. Besides, increased serum TG and TC contents are signs of liver dysfunction and steatosis and are regarded as poor health condition symbols (Wang et al., 2021). Reduced serum TG and TC contents by berberine treatment were reported in grass carp (Pan et al., 2019), zebrafish (Han et al., 2015), and blunt snout bream (He et al., 2021). The mechanisms of berberine alleviating hepatic lipid accumulation in fish are similar to those found in other animals, mainly by increasing lipid oxidation and transportation, reducing lipogenesis and fatty acids uptake. Berberine was demonstrated to downregulate hepatic lipogenesis genes, such as acetyl-CoA carboxylase α (ACCα), SREBP-1, 6-phosphogluconate dehydrogenase (6PGD), glucose 6-phosphate dehydrogenase (G6PD), and peroxisome proliferator-activated receptors γ (PPARγ) expressions in black sea bream (Wang et al., 2021); and hepatic FAS and ACCα expressions in blunt snout bream (He et al., 2021). In addition, berberine upregulated the expressions of hepatic lipid β-oxidation genes, such as carnitine palmitoyltransferase 1a (CPT1a) and hormone-sensitive lipase (HSL) expressions in black sea bream (Wang et al., 2021); CPT 1 in blunt snout bream (He et al., 2021); CPT 1 and PPARα in blunt snout bream (Lu et al., 2016); as well as Acyl-CoA oxidase (AOX), apolipoprotein B (ApoB), Apolipoprotein E (ApoE), peroxisome proliferator-activated receptor-gamma coactivator-1α (PGC-1α) and peroxisome proliferator-activated receptor alpha (PPARα) in blunt snout bream (Zhou et al., 2019). Furthermore, the fatty acid uptake and transportation genes including fatty acid transport protein (FATP), lipoprotein lipase (LPL), and low-density lipoprotein receptor (LDLR) were all upregulated by berberine supplementation in blunt snout bream (Zhou et al., 2019). However, some hepatoprotective mechanisms have not been reported in fish studies, such as if berberine can stabilize LDLR mRNA and regulate intestinal microbiota thus affecting fatty acid uptake.

Glucose metabolism

It is well known that fish show prolonged hyperglycemia after ingestion of HC diet, which is due to limited insulin secretion, low amount of insulin receptors in muscle, poor ability to use glucose in white muscle, endogenous glucose production, and low ability of lipogenesis from glucose (Kamalam et al., 2017). Therefore, fish exposed to HC diet may cause metabolic disorders and eventually threaten health status (He et al., 2021). Berberine supplementation in the HC diets reduced serum glucose in grass carp (Pan et al., 2019), black sea bream (Wang et al., 2020a), largemouth bass (Chen et al., 2022; Xia et al., 2022a; Xia et al., 2022b), and blunt snout bream (He et al., 2021). He et al. (2021) investigated the mechanisms of berberine regulating glucose metabolism in the liver. Berberine

upregulated hepatic insulin receptor substrate (IRS), phosphatidylinositol 3-kinase (PI3K), and protein kinase B (AKT) expressions, but downregulated forkhead transcription factor 1 (Foxo1) expression. The IRS/PI3K/AKT pathway is a major insulin signaling pathway, indicating that berberine activates the insulin pathway to reduce plasma glucose. The similar mechanism was also reported in largemouth bass fed 100 mg/kg and 400 mg/kg berberine supplemented HC diet (Chen et al., 2022). Besides, berberine reduced the expressions of hepatic gluconeogenesis genes: PEPCK, G6Pase, and glycogen synthase (GS), but upregulated pyruvate kinase (PK), and glucose transporter 2 (GLUT2) expressions. In largemouth bass also reported that berberine improved hepatic glycolytic enzymes activities and gene expression (HK (hexokinase) and PK), whereas gluconeogenic enzymes (G6Pase and PEPCK) activities and gene expression were reduced (Xia et al., 2022b). Thus, berberine inhibited hepatic gluconeogenesis and glycogen synthesis, activated insulin signaling, and promoted glucose transport and glycolysis, which in all contributed to reduced plasma glucose, liver glycogen, and lipid contents of some fish species.

Antioxidative and immune status

Berberine also shows strong antioxidant activity in fish. Chen et al. (2016) observed that 50 mg/kg berberine supplementation in a HL diet reduced hepatic malondialdehyde (MDA), lipid peroxide (LPO), and protein carbonyl (PC) contents of blunt snout bream through elevating superoxide dismutase (SOD) activity and total sulfhydryl (T-SH) levels. Another study in blunt snout bream found that HF (15% fat) diet damaged mitochondria normal structure and induced more ROS production in the liver, while berberine supplementation reduced hepatic MDA content along with increased glutathione (GSH) activity *via* upregulating Sirtuin 3 expression and increasing the complex I and II activities (Lu et al., 2017). Xu et al. (2017) also reported that berberine (50 mg/kg) supplementation in the normal and HF diets of blunt snout bream improved hepatic CAT and SOD activities, with a decreased MDA content, a similar result was found in largemouth bass fed the berberine-supplemented high starch diets (Xia et al., 2022b). Besides, berberine supplementation in a high starch diet of black sea bream increased hepatic GPx activity, and reduced MDA content after the ammonia challenge (Wang et al., 2020a). Chen et al. (2020) demonstrated that berberine could eliminate hepatic ROS production and reduce DNA damage caused by the high cholesterol diet of zebrafish. Chen et al. (2022) reported that berberine carried out its antioxidative capacity in largemouth bass through the Nrf2/Keap1 pathway, which is the same as that found in mammals. Up to now, the specific mechanisms of the antioxidative effect of berberine remain to be better illustrated in fish, especially the pathways (e.g., NF-κB and Nrf2) found in mammals.

Zhou et al. (2016) reported that common carp (*Cyprinus carpio*) fed a 0.78 g/kg berberine supplemented diet for 21 days significantly increased respiratory burst, lysozyme (LYZ), myeloperoxidase, and phagocytic activities, as well as serum complement C_3 level. Besides, berberine inclusion downregulated IL-10 expression but upregulated the TNF-α, IL-1β, lysozyme-c, and C3 expressions in the head kidney. The survival rate of fish infected with *Aeromonas hydrophila* was also enhanced by berberine supplementation. These results indicated that short-term berberine ingestion could enhance non-specific immunity and disease resistance ability, which might account for its immunomodulatory property. Likewise, plasma LYZ, acid phosphatase (ACP) activities, and alternative complement C3 and C4 contents were all improved by 50 mg/kg berberine supplementation in a HF diet of blunt snout bream (Chen et al., 2016). Berberine supplementation also reduced soybean oil-induced hepatic proinflammatory response mainly by decreasing cytokine–cytokine receptor interaction pathway-related genes expression in yellow drum (Tan et al., 2022). However, Xu et al. (2017) found the immune parameters (ACP, LYZ, C3, and C4) of blunt snout bream fed the 50 mg/kg berberine supplemented normal and HF diets at 2-week and 4-week interval feeding modes were similar or even higher than that of the continuous feeding mode. In addition, after being challenged by *Aeromonas hydrophila*, the cumulative mortality of fish fed the normal diet with 50 mg/kg berberine inclusion at the 2-week interval feeding mode was the lowest, while the other treatments showed no significant difference. The authors proposed that feeding fish berberine supplemented diet for the long term might cause immunosuppression or immunity fatigue, just like some other immunostimulants (e.g., β-glucan (Bai et al., 2010) and fructooligosaccharide (Zhang et al., 2014)). Besides, Nile tilapia fed the 1, 3, 6, and 9 g/kg berberine supplemented normal diets for 8 weeks all showed enhanced skin mucus lysozyme and peroxidase activities, as well as improved serum lysozyme, peroxidase, alternative complement (ACH50), phagocytosis, and respiratory burst activities. While the 1g/kg berberine supplemented group obtained the best immune status, and the highest survival rate after *Streptococcus agalactiae* infection (Doan et al., 2020). These results indicate that low dietary berberine supplementation level and discontinuous feeding mode may be more beneficial for the immune status of fish. A study on black sea bream found that fish fed a 50 mg/kg berberine supplemented high starch diet continuously for 8 weeks did not influence serum C3, C4, and IgM contents, as well as LYZ activity (Wang et al., 2020a). Yu et al. (2020) also observed that plasma IgG and IgM contents of blunt snout bream were not affected by berberine supplementation in the HC or HF diet.

Berberine supplementation also benefits intestinal immune function. Yu et al. (2020) reported that berberine reduced

intestinal TNF-α and IL-6 expressions upregulated by HF or HC diet in blunt snout bream, and it also upregulated intestinal major histocompatibility complex class gene expression. In addition, several *in vitro* studies revealed that berberine shows a strong ability against pathogenic bacteria in fish. Berberine hydrochloride effectively inhibited *Escherichia coli*, *Aeromonas hydrophila*, *Vibrio vulnificus*, *Pseudomonas fluorescens*, *Edwardsiella ictaluri*, and *Streptococcus agalactiae*, and it showed a synergistic bactericidal effect with enrofloxacin against these fish pathogenic bacteria (Zhang et al., 2010). Ji et al. (2012) found that pretreatment with berberine hydrochloride activated the complement system and significantly enhanced serum bactericidal activity of grass carp against *Edwardsiella ictaluri*.

Regulation of intestinal integrity and microbiota

Even though most studies in fish concerned the hepatoprotective effect of berberine, several studies investigated the effects of berberine supplementation on intestinal health and microbiota composition. Modulating of intestinal microbiota may be the main mechanism of regulating energy mechanism and immune system by berberine since most ingested berberine remains in the intestine and may regulate intestinal microbiota composition and metabolism, as reported in mammals. Pan et al. (2019) reported that berberine supplementation in a normal diet increased intestinal microbiota diversity and decreased the Firmicutes to Bacteroidetes ratio of grass carp. Based on Spearman's rank correlation, serum glucose content was negatively related to the 32 berberine-operational taxonomic units, indicating the glucose-lowering effect of berberine might be correlated with the regulation of intestinal microbiota composition. Berberine supplementation in the HC and normal diets improved intestinal microbiota diversity of blunt snout bream, whereas a reduced diversity and richness of intestinal microbiota were found in the berberine supplemented HF diet group (Yu et al., 2020). Moreover, berberine supplementation induced a high abundance of phyla Proteobacteria, Cyanobacteria, Firmicutes, and Bacteroidetes. It reduced intestinal *Verrucomicrobia*, *Planctomycetes*, *Dependentiae*, and *Chloroflexi* abundance increased by the HF diet, as well as reduced *Verrucomicrobia*, *Planctomycetes*, and *Chloroflexi* abundance elevated by the HC diet in blunt snout bream (Yu et al., 2020). Tian et al. (2022) demonstrated that the supplementation of Gly-β-MCA (an intestine-specific FXR inhibitor) alleviated the lipid-lowering effect of berberine of grass carp, which demonstrated intestinal FXR is the main working site of berberine. Similarly, berberine supplementation (500 mg/kg, 1000 mg/kg, and 2000mg/kg) significantly improved the expression levels of intestinal FXR and FGF-19 of largemouth bass, which led to improved glycolysis and inhibited gluconeogenesis (Xia et al., 2022a; Xia et al., 2022b). Currently, the bile acids-FXR axis has been demonstrated to regulate lipid and glucose metabolism, immune and antioxidative systems in many fish species (Du et al., 2018; Du et al., 2020; Du et al., 2021; Tian et al., 2021; Wen et al., 2021; Xu et al., 2022; Zhang et al., 2022). Thus, the modulation of intestinal bile acids metabolizing bacteria may account for the multiple functions of berberine in fish. Besides, berberine supplementation alleviated intestinal villus length reduction, intestinal integrity impairment, expanded lamina propria, decreased goblet cell count and mucosal folds, and elevated plasma diamine oxidase (DAO) and D-lactate (D-LA) contents caused by HF and HC diets in blunt snout bream (Yu et al., 2020). Modulation of intestinal microbiota by berberine is also related to nutrient utilization. Xia et al. (2022b) proposed that the enhanced intestinal digestive enzymes by berberine supplementation might be linked to the modulation of intestinal microbiota. Chen et al. (2022) reported that elevated intestinal abundance of *Cetobacterium* induced by berberine was contributing to the improved glucose utilization of largemouth bass.

Therefore, berberine supplementation is beneficial for maintaining intestinal structure and health, and its regulation of intestinal microbiota may be the important mechanism that exert various functions. And the relationship between berberine functions and its regulation of the specific intestinal microbiota is not clear enough.

Other applications

Recent studies have shown that dietary berberine decreased the cumulative mortality of blunt snout bream (Chen et al., 2016), and elevated the glucose level of black sea bream (Wang et al., 2020a) after acute ammonia challenge. These results might be due to improved liver and intestine health, as well as enhanced antioxidative and immune status of fish by berberine supplementation. Besides, injection of crucian carp (*Carassius auratus gibelio*) with berberine inhibited cytochrome P4501A (cyp1a) and cyp3a expression, which belong to the superfamily of monooxygenases, and play a vital role in drug metabolism in animal species. The authors proposed that when berberine is co-administrated with other drugs, it can reduce the dosage and increase the efficiency of other drugs, thus diminishing the pollution (Zhou et al., 2011). Besides, berberine also showed a strong antibacterial effect in fish *via* attenuating viral gene expression and host inflammatory response simultaneously. Berberine could inhibit cyprinid herpesvirus 2 (CyHV-2) replication and viral gene transcription *in vitro*, and single oral administration of berberine could against CyHV-2 infection in a dose-depend manner in crucian carp (Su et al., 2021). Su et al. (2022) reported that berberine could attenuate NF-κB signaling induced by acute CyHV-2 infection in crucian carp in a dosage-

dependent manner, thus suppressing the expression of inflammatory cytokines.

Conclusion and perspectives

Berberine has potential lipid- and glucose-lowering, anti-inflammatory, antioxidant, antimicrobial, and anti-stress activities in animals. Based on human and animal studies of rodents, berberine may be a safe, effective, and relatively cheap feed additive in the fish diet to protect the liver and intestine, and maintain good health condition. However, berberine also has limitations such as low bioavailability, and negatively influences feed palatability. Indeed, a major risk of berberine supplementation could be its negative impact on feed intake and growth performance during prolonged periods. In addition, some human and rodents studies also revealed that berberine promotes energy expenditure thus reducing body weight eventually. This discrepancy result with the studies in fish also needs further investigation.

Further studies may focus on improving the bioavailability of berberine in the feed, which can learn from studies *in vivo* and other animals. For example, Shan et al. (2013b) found that pseudoberberine (IMB-Y53), a berberine analogue, has a much lower ability to increase intestinal P-gp expression than berberine, but it showed a strong glucose-lowering effect similar to berberine in rats. A study in rabbits revealed that administration of nanoparticles berberine led to higher berberine in blood circulation and higher pharmacokinetic parameters, as well as a better liver protection effect compared with regular berberine (Sahibzada et al., 2021). Besides, intestinal absorption promoters may be beneficial for intestinal permeability. For example, sodium caprate has been approved by the FDA as a food additive for humans (Liu et al., 2016). Moreover, P-gp inhibitors such as silymarin (Wei et al., 2020), and tetrandrine (Shan et al., 2013b) were demonstrated to improve intestinal berberine absorption significantly. Additionally, using lipid microparticles to transport berberine could increase its solubility and permeability in the gastrointestinal tract, and enhance transportation to the lymphatic system (Liu et al., 2016). On the other side, many studies indicate that the main mechanism of berberine exerts its multiple functions is by modulating intestinal microbiota since most ingested berberine is highly accumulated in the intestine, as reviewed by Habtemariam (2020). Thus, the hidden link between berberine and intestinal microbiota may be a hot topic in the future.

The hepatoprotective effect of berberine has been well demonstrated in some fish species. Further studies should concern the effective and economical dietary berberine level to cope with different dietary levels of lipid and carbohydrate in different fish species. Berberine has strong antioxidant and immune regulation properties, which are beneficial for fish species under intensive aquaculture systems. However, long-term berberine feeding or high dietary berberine level may cause immune fatigue of immunosuppression. Thus, optimal dietary berberine levels with suitable feeding modes for different fish species deserve further study. Besides, most fish have limited ability to use dietary carbohydrates, especially carnivorous fishes, which are considered to have poorer ability than herbivorous and omnivorous fishes because of their slower blood glucose clearance and poorer intestinal glucose uptake rate (Kamalam et al., 2017). Previous studies have proved that berberine could improve insulin secretion, accelerate blood glucose clearance, and enhance glucose utilization. However, the beneficial effect of berberine supplementation on improving dietary carbohydrate utilization was only reported in blunt snout bream (herbivorous fish). Further research is needed about berberine on carnivorous and omnivorous fish.

From the economical aspect, supplementation of purified berberine may induce more economic costs for feed companies. However, berberine-enriched plant organs, such as *Forsythia suspensa* extract (Zhang et al., 2013), barberry root (Ramezanzadeh et al., 2020; Ramezanzadeh et al., 2021), barberry fruit (Shekarabi et al., 2022), fibrous root of *Rhizoma Coptidis* (Zhou et al., 2016), also showed considerable beneficial effects for animals and can be an economical choice.

The current studies of berberine are mainly conducted in rodents and humans. Though it shows excellent features in treating some diseases, there is still a long way for it to be widely applied in fish feed due to the limited studies. The application of herbal components is drawing more and more attention, and berberine is a promising candidate for fish feed additive in the future.

Author contributions

All authors have read and agreed to the published version of the manuscript. LW wrote the paper under the supervision of QS and YY; CG, BW, and CW helped collect data. GS 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.

Publisher's note

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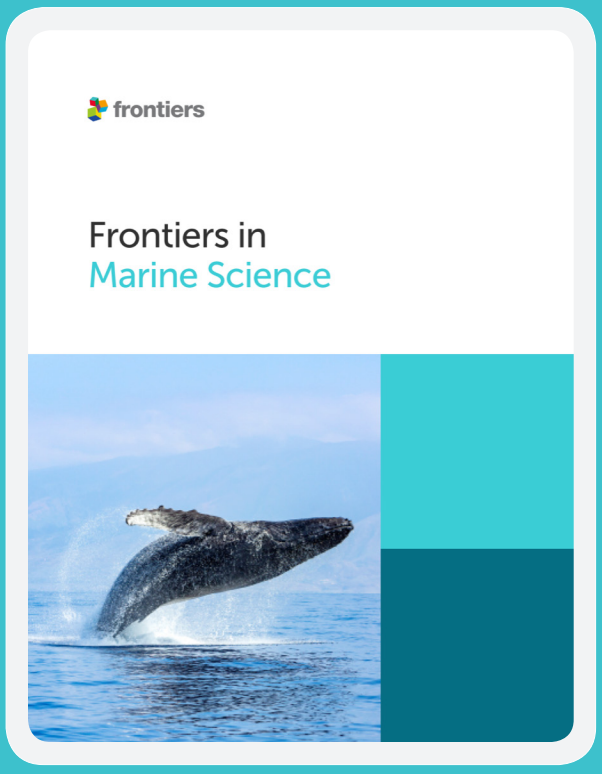

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