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Functional feed additives and intestinal health in aquatic animals

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Functional feed additives and intestinal health in aquatic animals

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Table of contents

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Editorial: Functional feed additives and intestinal health in aquatic animals

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feed additives, immunity, intestinal inflammation, antioxidant capacity, intestinal microbiota, metabolism

Editorial on the Research Topic
Functional feed additives and intestinal health in aquatic animals

The rapid expansion and intensification of aquaculture have been accompanied by various intestinal disease outbreaks and a drastic increase in mortality, especially when fish meal and oil in feed were replaced by plant source raw materials in large quantities (Glencross et al., 2020). Previous studies have identified dietary supplementation of functional feed additives as one of the alternative strategies and therapeutic interventions to prevent intestinal dysfunction that benefits the healthy growth of aquatic animals simultaneously (Dawood et al., 2018; Hossain et al., 2024). Except for digestion and absorption functions, the intestine also serves as the largest immunity organ and performs as the first barrier of defense between the organism and pathogens, and thus, it is crucial for fish growth and physiological functions. Therefore, it is utmost importance to investigate different functional additives to improve the intestinal health of aquatic animals.

The goal of this Research Topic is to focus on the effects of functional feed additives on intestinal health of aquatic animals by studying their benefits on the intestinal physiological state and underlying mechanisms. The areas covered include: 1) Feed additives and intestinal immunity; 2) Anti-inflammatory mechanism; 3) Antioxidant stress regulation; 4) The relationship between feed additives and nutrient metabolism; and 5) The modulating of the intestinal microbiome by feed additives.

In past decades, plant source raw materials have been widely used, and their proportion in aquatic feed have become higher and higher due to the shortage of fishery resources and their expensive price (Macusi et al., 2023). When basic feed resources become scarce, assessing alternative replacements the from natural environment becomes crucial to sustaining the fast growth of aquaculture, which supports billions of people by providing high-quality animal protein (Valenti et al., 2018). The studies included in this Research Topic are mainly focused on the effects of different diets derived from wheat, soybean oil, corn starch, and frass from black soldier on the growth, feed utilization, immune response, and welfare of aqua-farmed animals. Zhang et al. demonstrated the positive role of replacing of fish meal and soy protein with wheat gluten on growth performance, feed utilization, and nutrient digestibility and retention in Japanese seabass (*Lateolabrax japonicus*). Zuo et al. evaluated the effects of different carbohydrate-to-lipid

ratios (C/L) on growth and energy utilization and their mechanism in Chinese mitten crab (*Eriocheir sinensis*), and their results revealed that the optimal dietary C/L of 3.59 was beneficial for growth performance and carbohydrate and lipid metabolism. In this Research Topic, an article by Sankappa et al. highlighted that dietary inclusion of frass from black soldier fly larvae improved the immunity of the fish by activating the innate and adaptive immunity of channel catfish (*Ictalurus punctatus*).

Functional feed additives have been widely used in aquaculture due to their positive effects on aquatic animals (Vijayaram et al., 2022). An inclusion of selenium as a functional feed additive by Li et al. demonstrated its antioxidant effects in aquatic animals. The supplementation of 0.01%–0.02% fulvic acid in diet was identified to be beneficial for growth performance, digestive ability, and intestinal function of large yellow croaker (*Larimichthys crocea*) larvae through the inhibition of intestinal inflammation (Zhang et al.). Yang et al. highlighted that dietary supplementation of 1%–2% fermented *Astragalus membranaceus* could improve intestinal and hepatic morphology and regulate intestinal microbiota together with a significant enhancement in the growth performance of juvenile tiger grouper (*Epinephelus fuscoguttatus*). Pelusio et al. and Sun et al. reported that supplementation of yeast extract improved the growth, digestibility, intestinal histology, antioxidant capacity, and immune response and modulated the intestinal microbiota in fish positively. Moreover, Pelusio et al. pinpointed that nucleotides and nucleic acids extracted from yeast can be an excellent fish meal replacer. Li et al. investigated the application of fucoidan in juvenile common carp (*Cyprinus carpio*) and highlighted its positive effects on growth performance, immunity, antioxidant ability, digestive enzyme activity, and hepatic morphology. Bera et al. reported that the intestinal microbiome of *Oreochromis niloticus* could be optimized through nutritional interventions with Aloe vera extract polysaccharide, thereby improving the performance in commercial fish.

The underlying regulatory mechanism of functional feed additives improving the intestinal health of aquatic animal is far from being understood. The studies included in this Research Topic present the effects of wheat gluten, the variation in the ratios of dietary carbohydrates to lipids, and frass from black soldier fly larvae on organic physiology and highlighted the latest discoveries and advances of functional feed additives in aquatic animals. By compiling these 10 articles, we provide a base of knowledge for both researchers and professionals in the field of aquaculture for the better understanding of the application of functional feed additives in aquaculture farming. Hence, these articles will hopefully upgrade our understanding on the contribution of different types of diets and functional feed additives isolated from various natural sources on the wellbeing of aquatic animals.

Author contributions

GY: Writing–original draft, Writing–review and editing. ZZ: Writing–original draft. VK: Writing–review and editing.

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Effects of supplemental fulvic acid on survival, growth performance, digestive ability and immunity of large yellow croaker (*Larimichthys crocea*) larvae

Chenxiang Zhang[1], Yongtao Liu[1], Chuanwei Yao[1], Jianmin Zhang[1], Yuntao Wang[1], Jiahui Liu[1], Yucong Hong[1], Kangsen Mai[1,2] and Qinghui Ai[1,2]*

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A 30-day feeding trial was designed to evaluate the effect of supplemental fulvic acid (FA) on survival, growth performance, digestive ability and immunity of large yellow croaker (*Larimichthys crocea*) larvae (initial body weight 11.33 ± 0.57 mg). Four isonitrogenous and isolipids diets containing 0.00%, 0.01%, 0.02% and 0.04% FA were formulated, respectively. Results showed that the supplementation of 0.04% FA significantly improved survival rate of large yellow croaker larvae. Meanwhile, supplemental FA significantly increased final body weight and specific growth rate. Based on the specific growth rate, the optimal supplementation was 0.0135% FA. Larvae fed the diet with 0.01% FA had significantly higher villus height than the control. The supplementation of 0.01%–0.02% FA significantly increased the muscular thickness of intestine. Moreover, supplementation of FA significantly increased mRNA expression of intestinal epithelial proliferation and barrier genes (*pcna*, *zo-1* and *zo-2*). Diets supplemented with 0.02%–0.04% FA significantly increased the activity of trypsin in the intestinal segment, while 0.01%–0.02% FA significantly increased the activity of trypsin in the pancreatic segment. Compared with the control, supplementation of FA remarkably increased activities of alkaline phosphatase and leucine aminopeptidase in the brush border membrane of intestine. Larvae fed the diet with 0.01% FA significantly increased activities of lysozyme and total nitric oxide synthase. Furthermore, the supplementation of 0.01% to 0.02% FA significantly decreased the mRNA expression of pro-inflammatory cytokines (*tnf-α* and *il-6*). Concurrently, supplemental FA significantly increased anti-inflammatory cytokine (*il-10*) mRNA expression level. In conclusion, this study indicated that the supplementation of FA could improve the survival rate and growth performance of larvae by promoting intestinal development, digestive enzymes activities and innate immunity.

KEYWORDS

fulvic acid, large yellow croaker, larval nutrition, intestinal morphology, immune response

1 Introduction

Production of larvae is a critical bottleneck restricting the development of marine fish farming (Zhang et al., 2023). Inadequate digestive system and low immunity are the chief causes of high mortality of larvae (Comabella et al., 2013; Rojo-Cebreros et al., 2018). Therefore, finding ecofriendly feed additives to promote the maturation of digestive function and the development of immune system in larval stage is a powerful way for aquacultural flourish.

Fulvic acid (FA) is an organic substance found in humus with the lowest molecular size (around 2000 Da) but the highest activity (Isam et al., 2005; Plaza et al., 2005). Due to the various functional groups of FA, it has beneficial functions such as anti-inflammatory, antibacterial, stimulating the absorption of microelements, and enhancing immunostimulatory properties (Van Rensburg, 2015; Hullár et al., 2018; Goenadi, 2021). Studies in mammals have shown that FA has potential to prevent or treat diseases, such as diabetes and neurodegenerative disorders (Winkler and Ghosh, 2018; Dominguez-Meijide et al., 2020). In livestock and poultry, FA has been shown to improve lipid metabolism, intestinal morphology and meat quality (Bai et al., 2013; Chang et al., 2014; Taklimi et al., 2012). Researches of FA in aquatic animals have made progress in recent years. Studies proved that dietary FA can promote growth and digestive ability in *Carassius auratus* (Wu et al., 2016), regulate intestinal microbiota of *Paramisgurnus dabryanus* (Gao et al., 2017), improve antioxidant capacity of *Oreochromis niloticus* (Neamatallah et al., 2022), and enhance non-specific immunity of *Procambarus clarkia* (Zhang, 2018). However, the effects of supplemental FA on intestinal development and immune function of marine fish larvae have not been reported.

Large yellow croaker (*Larimichthys crocea*) is one of the main economic fish in China (He et al., 2022). Lots of studies in nutrition requirements, digestive physiology and feed manufacturing technology of larvae have been reported (Ai et al., 2008; Zhao et al., 2013; Cai et al., 2017; Liu et al., 2022). However, there is no study on effects of FA on digestive system maturation and immunity of large yellow croaker larvae. Therefore, this experiment was conducted to investigate the effects of

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

Ingredient% dry diet	Diets (FA%)			
	Control (0.00)	Diet 1 (0.01)	Diet 2 (0.02)	Diet 3 (0.04)
White fish meal[1]	36.86	36.86	36.86	36.86
Krill meal[1]	21.02	21.02	21.02	21.02
Squid meal[1]	14.27	14.27	14.27	14.27
Yeast extract[1]	2.00	2.00	2.00	2.00
Microcrystalline cellulose	0.04	0.03	0.02	0
Strong flour	4.00	4.00	4.00	4.00
α-starch	4.00	4.00	4.00	4.00
Sodium alginate	2.00	2.00	2.00	2.00
Vitamin premix[2]	1.50	1.50	1.50	1.50
Mineral premix[2]	1.00	1.00	1.00	1.00
Ascorbyl polyphosphate	0.20	0.20	0.20	0.20
Mold inhibitor	0.05	0.05	0.05	0.05
Antioxidant	0.05	0.05	0.05	0.05
Choline chloride	0.20	0.20	0.20	0.20
Fish oil	8.00	8.00	8.00	8.00
Soybean lecithin[3]	5.00	5.00	5.00	5.00
Fulvic acid[4]	0	0.01	0.02	0.04
Analyzed nutrients composition (dry matter basis)				
Crude protein (%)	50.05	51.18	49.97	50.35
Crude lipid (%)	16.84	17.60	17.55	16.91
Moisture (%)	6.21	6.38	6.65	6.56

[1]Ingredients for feed were purchased from Great Seven Biotechnology Co., ltd in shandong, China; The feed compositions were referred to Zhang et al. (2023).
[2]The composition of mineral and vitamin premix (g/kg) was referred to Zhang et al. (2023).
[3]Commercially available from Beijing Huaxia Houde Co., Ltd. (Beijing, China).
[4]Fulvic acid was purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). The purity was ≥85.0%.

supplemental FA on survival, growth performance, digestive capacity and immunity of large yellow croaker larvae.

2 Materials and methods

2.1 Diets formulation

Four isonitrogenous (crude protein 50.4%) and isolipidic (crude lipid 17.2%) experimental diets supplemented with 0.00%, 0.01%, 0.02% and 0.04% FA (purity 85%, Shanghai Macklin Biochemical Co., Ltd), respectively (Table 1). Each experimental feed ingredient was crushed to 150 mesh, then FA was mixed with powdered ingredients in the order of addition from less to more. The remaining ingredients were added to an electric blender and stirred for 30 min until a uniform, viscous pellet was formed. The pellet was made into 1 mm micro-diets, and then oven-baked for about 8 h at 50°C. The dried feed was broken and filtrated to obtain two micro-diets with particle sizes of 280–450 μm and 450–600 μm. The dried feed was stored at −20°C for use in subsequent experiments. Diets of 280–450 μm were fed to larvae 17–32 days after hatching (DAH), and diets of 450–600 μm were fed afterwards.

2.2 Experimental procedures

The experiment was carried out at the Ningbo Marine and Fishery Science and Technology Innovation Base in Zhejiang Province. The experimental objects were artificially hatched large yellow croaker larvae from the same batch. Larvae were fed with rotifers (*Brachionus plicatilis*) from three to eight DAH. From 6 to 11 DAH, the larvae were fed with brine shrimp (*Artemia nauplii*). From 10 to 17 DAH, larvae were feed copepod (*Calanus sinicus*) and micro-diet, with the proportion of diet gradually increasing until 17 DAH, when the experimental diet was fed exclusively. In the experiment, larvae (mean body weight 11.33 ± 0.57 mg) were randomly assigned to 12 cylindrical white barrels (water volume 220 L), with a density of 3,000 individuals per barrel. Larvae were fed the experimental diet, and four treatment groups were set up with three replicates per treatment. During the rearing period (17–47 DAH), the experimental feed was fed to larvae seven times a day (06:00, 09:00, 12:00, 15:00, 18:00, 21:00, 24:00) until satiation. The rearing conditions are strictly controlled and water temperature, pH, and salinity were ranging from 23°C to 26°C, 7.8 to 8.2, 21‰–25‰, respectively. Approximately 150%–200% of the water was replaced daily.

2.3 Sample collection

At the beginning, fifty larvae of 17 DAH were randomly selected from each holding bucket, and initial body weight (IBW) and initial body length (IBL) were measured using an analytical balance and vernier caliper, respectively. At the end of the feeding trial, larvae were fasted for 24 h to empty their digestive tracts. Larvae were counted and survival rates (SR) were calculated for each barrel. Fifty larvae from each tank were randomly collected to measure final body weight (FBW) and final body length (FBL). Fifty larvae were randomly selected from each barrel and dissected under a dissecting microscope at 0°C to get the pancreatic and intestinal segments (PS and IS) for the detection of digestive enzyme activity (Cahu and Infante, 1994). Forty larvae were randomly selected from each barrel and dissected at 0°C to get visceral masses (VM) for analysis immune enzyme activities. Ninety larvae were randomly selected from each barrel and dissected under a dissecting microscope at 0°C in a sterile environment. The whole intestine and liver were separated and stored in RNase-free centrifuge tubes and immediately frozen in liquid nitrogen for real-time quantitative PCR analysis. Fifteen larvae from each treatment group were randomly selected and fixed in 4% paraformaldehyde for 24 h, then transferred to 75% alcohol for HE staining. After sampling, the remaining larvae were divided into 10 mL RNase-free centrifuge tubes and snap-frozen in liquid nitrogen for analysis of body composition.

2.4 Analytical methods

2.4.1 Proximate composition analysis

Diets and larvae were oven baked at 105°C for 72 h to constant weight to determine water content. Crude protein content of diets and fish was determined by the Kjeltec nitrogen method (FOSS Kjeltec 8,400 Analyzer Uni, Sweden) and estimated by multiplying nitrogen by 6.25. Crude lipid content of diets and fish was examined by the Soxhlet extraction method (Soxhlet Extraction System B-801, Buchi 36,680, Switzerland).

2.4.2 Intestinal morphology analysis

Intestinal sections were prepared according to the methods described in the published paper (Mai et al., 2005). In brief, larvae intestinal segments were dehydrated in a gradient, embedded in paraffin, sectioned, and immediately stained with hematoxylin and eosin solutions. Sections were observed under a light microscope (Leica DM3000 LED, Germany), and professional image analysis software (Image Pro Plus 6.0, United States) was used to measure the villus height, enterocyte cell height and muscular thickness.

2.4.3 Digestive enzyme activities assay

The PS and IS samples were mixed and homogenized at a 1:9 ratio, centrifuged at 3,500 *g* for 15 min, and the supernatant was used for index determination. Purified brush border membranes (BBM) were obtained from homogenates of IS according to the method described by Crane et al. (1979). LAP activity was determined according to the method of Maroux et al. (1973). Trypsin, *α*-amylase, lipase and AKP activities were measured using commercial kits (Nanjing Jiancheng Bio-Engineering Institute, China). Total protein quantification (TP) was determined using the Beyotime BCA kit (P0011). All experimental procedures were performed in strict accordance with the instructions.

2.4.4 Immune enzymes activities assay

The VM sample were mixed and homogenized at a 1:9 ratio, centrifuged at 3,500 *g* for 15 min, and the supernatant was used for index determination. LZM, TNOS and iNOS activities were measured using commercial kits (Nanjing Jiancheng Bio-Engineering Institute, China).

TABLE 2 Primers used for quantitative PCR.

Gene	Forward (5′–3′)	Reverse (5′–3′)	References
occludin	AGGCTACGGCAACAGTTATG	GTGGGTCCACAAAGCAGTAA	Zhang et al. (2023)
zo-1	TGTCAAGTCCCGCAAAAATG	CAACTTGCCCTTTGACCTCT	Zhang et al. (2023)
zo-2	ACCCGACCTGTTTGTTATTG	ATGCCGTGCTTGCTGTC	Liu et al. (2020)
pcna	AGTTTGCCCGTATCTGCC	CTCTTTGTCTACATTGCTGGTCT	Liu et al. (2020)
odc	GAGCCAGGTCGCTTCTATG	CCGTGGTCCCTTCGTCT	Liu et al. (2020)
ifn-γ	TCAGACCTCCGCACCATCA	GCAACCATTGTAACGCCACTTA	Li et al. (2020)
tnf-α	ACACCTCTCAGCCACAGGAT	CCGTGTCCCACTCCATAGTT	Wang et al. (2022)
*il-1*β	CATAGGGATGGGGACAACGA	AGGGGACGGACACAAGGGTA	Wang et al. (2022)
il-6	CGACACACCCACTATTTACAAC	TCCCATTTTCTGAACTGCCTCT	Li et al. (2020)
il-10	AGTCGGTTACTTTCTGTGGTG	TGTATGACGCAATATGGTCTG	Li et al. (2020)
β-*actin*	GACCTGACAGACTACCTCATG	AGTTGAAGGTGGTCTCGTGGA	Liu et al. (2020)

Abbreviation: *zo-1*, zonula occludens-1; *zo-2*, zonula occludens-2; *pcna*, proliferating cell nuclear antigen; *odc*, ornithine decarboxylase; *ifn*-γ, interferon γ; *tnf*-α, tumor necrosis factor α; *il-1*β, interleukin-1β; *il-6*, interleukin-6; *il-10*, interleukin-10.

2.4.5 RNA extraction and real-time quantitative PCR

Total RNA was extracted from the intestine or liver of larvae using RNAiso Plus (Takara Biotech, Japan). cDNA was reverse transcribed from the RNA using the Prime Script-RT kit (Takara, Japan). The primer sequences were designed and synthesized based on the corresponding sequences in published papers (Table 2). Real-time quantitative PCR was performed by referring to Zhang et al. (2023).

2.5 Calculations and statistical analysis

The following calculations were performed:

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

$$\text{Specific growth rate (SGR, \%day}^{-1}\text{)} = (\text{Ln}W_t - \text{Ln}W_0) \times 100/d$$

Where N_t and N_0 were the final and initial number of larvae, respectively; W_t and W_0 were the final and initial body weights, respectively; d was the experimental period in days.

Statistical analysis was performed in SPSS 26.0 (SPSS Inc., United States). The data were analyzed by the one-way analysis of variance (ANOVA) followed by Tukey's multiple-range test. Statistically significant differences were determined using $p < 0.05$. Results were presented as mean ± S.E.M. (Standard error of means).

3 Results

3.1 Survival, growth performance and body composition

After 30 days, the supplementation of FA significantly improved the survival rate and growth performance of larvae (Table 3) ($p < 0.05$). When the FA supplementation reached 0.04%, the survival rate significantly increased from 20.64% to 23.48% ($p < 0.05$). Supplementation of 0.01%–0.04% FA increased FBL, FBW and SGR of larvae significantly ($p < 0.05$). The broken-line analysis for SGR indicated that the maximum growth of larvae appeared in the supplementation of 0.0135% FA (Figure 1). There were no significant differences in moisture, crude lipid and crude protein contents among supplementary treatments ($p > 0.05$) (Table 4).

3.2 Intestinal morphology

The supplementation of FA could improve the intestinal morphology of larvae (Figure 2). With an increase in FA supplementation, the VH, MT and EH increased firstly and then decreased. VH was significantly increased when FA supplementation was 0.01%. Compared with the control, the difference of MT was statistically significant when the supplemental level was 0.01%–0.02% ($p < 0.05$) (Table 5). However, no significant change of the EH was observed in the intestinal tract of larvae ($p > 0.05$) (Table 5).

3.3 Expression of proliferation-related and barrier-related genes in intestinal cell

Compared to the control, supplementation of 0.02%–0.04% FA had notably higher mRNA expression of *zo-1* ($p < 0.05$) (Figure 3). The *zo-2* mRNA expression of larvae with 0.01%–0.02% FA supplementation was significantly higher than the control ($p < 0.05$) (Figure 3). Meanwhile, supplementation of 0.01% FA significantly increased the *pcna* mRNA expression ($p < 0.05$) (Figure 3). However, supplemental FA had no significant effect on the mRNA expression of *occludin* and *odc* ($p > 0.05$) (Figure 3).

TABLE 3 Effects of supplemental fulvic acid on survival rate and growth performance of large yellow croaker larvae (Means ± S.E.M., n = 3)[1]

Parameters	Diets (FA%)			
	Control (0.00)	Diet 1 (0.01)	Diet 2 (0.02)	Diet 3 (0.04)
Initial body length (mm)	8.77 ± 0.22	8.77 ± 0.22	8.77 ± 0.22	8.77 ± 0.22
Final body length (mm)	24.03 ± 0.14^{b}	25.69 ± 0.12^{a}	25.93 ± 0.41^{a}	25.67 ± 0.30^{a}
Initial body weight(mg)	11.33 ± 0.57	11.33 ± 0.57	11.33 ± 0.57	11.33 ± 0.57
Final body weight(mg)	286.89 ± 8.59^{b}	323.00 ± 7.37^{a}	332.33 ± 9.90^{a}	319.22 ± 12.46^{a}
Specific growth rate(%/day)	10.77 ± 0.10^{b}	11.17 ± 0.08^{a}	11.26 ± 0.10^{a}	11.12 ± 0.13^{a}
Survival rate(%)	20.64 ± 0.85^{b}	20.36 ± 1.04^{b}	20.72 ± 0.77^{b}	23.48 ± 0.51^{a}

[1]Tukey's test showed significant differences in the data without the same superscript letter in the same row ($p < 0.05$).

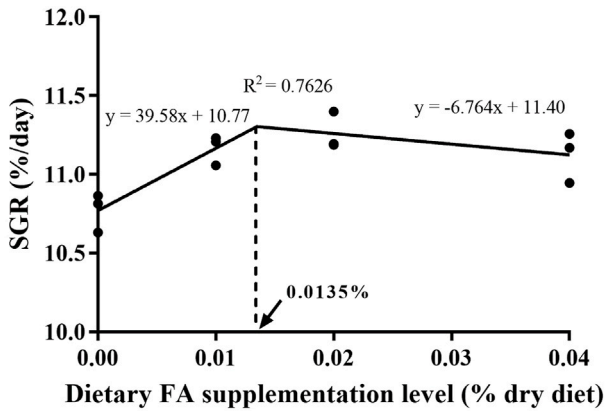

FIGURE 1
Relationship of specific growth rate (SGR, %/d) with supplemental FA levels of large yellow croaker larvae.

3.4 Digestive enzyme activity

The supplementation of FA increased the activity of digestive enzyme (Table 6). Supplemental FA increased activities of trypsin in IS and PS significantly ($p < 0.05$). However, supplemental FA did not affect try-IS/(PS + IS) ratio of larvae significantly ($p > 0.05$). Meanwhile, compared with the control, activities of amylase and lipase were increased but the differences among treatments were not significant ($p > 0.05$).

The results suggested that activities of AKP and LAP were significantly higher when supplemented with 0.01%–0.04% FA than in the control ($p < 0.05$) (Table 7). With an increase in FA supplemental level, AKP and LAP activities initially increased and then decreased, with the highest activity observed when the supplemental amount was 0.01%.

3.5 Non-specific immune-related enzyme activity

Supplementation of 0.01% FA significantly increased the enzyme activities of LZM and TNOS ($p < 0.05$) (Figure 4A, B). The supplemental FA increased iNOS activity, but the change was not statistically significant ($p > 0.05$) (Figure 4C).

3.6 Expression of inflammation cytokines genes

With an increase in FA supplementation, the mRNA expression of pro-inflammation cytokines *tnf-α*, and *il-6* decreased initially, then increased (Figure 5). When FA supplementation achieved 0.04%, no significant difference was found in the mRNA expression of *tnf-α* ($p < 0.05$). Supplementation of 0.02% FA significantly decreased the mRNA expression of *il-6* ($p < 0.05$). Simultaneously, the supplementation of FA significantly increased the mRNA expression of *il-10* ($p < 0.05$) (Figure 5). There was no significant difference in the mRNA expression of *ifn-γ* and *il-1β* among all groups ($p > 0.05$) (Figure 5).

TABLE 4 Effects of supplemental fulvic acid on body composition of large yellow croaker larvae (Means ± S.E.M., n = 3)[1].

Parameters	Diets (FA%)			
	Control (0.00)	Diet 1 (0.01)	Diet 2 (0.02)	Diet 3 (0.04)
Crude protein (%/w. w.[2])	8.35 ± 1.21	8.58 ± 0.11	8.48 ± 0.60	8.40 ± 0.75
Crude lipid (%/w. w.)	3.77 ± 0.09	3.82 ± 0.12	3.76 ± 0.13	3.79 ± 0.16
Moisture (%)	84.12 ± 0.42	84.00 ± 0.21	84.27 ± 0.49	84.10 ± 0.61

[1]Tukey's test showed significant differences in the data without the same superscript letter in the same row ($p < 0.05$).
[2]w.w., wet weight.

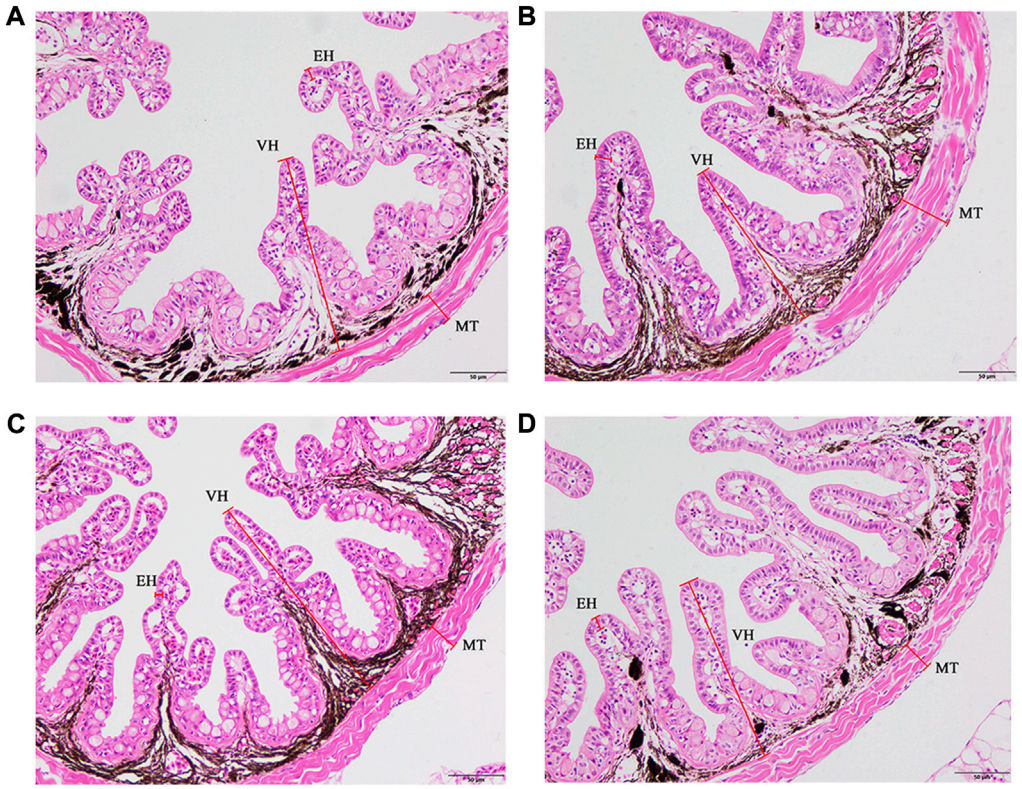

FIGURE 2
Effects of supplemental fulvic acid on intestinal morphology of large yellow croaker larvae. Control group (0.00%) **(A)**; 0.01% FA group **(B)**; 0.02% FA group **(C)**; 0.04% FA group **(D)**. MT, muscular thickness; VH, villus height; EH, enterocyte height (HE staining; Scale bar = 50 μm).

TABLE 5 Effects of supplemental fulvic acid on micromorphology of the intestine of large yellow croaker larvae (Means ± S.E.M., n = 3)[1].

Parameters	Diets (FA%)			
	Control (0.00)	Diet 1 (0.01)	Diet 2 (0.02)	Diet 3 (0.04)
Villus height (μm)	164.53 ± 23.14^{b}	224.27 ± 29.92^{a}	204.46 ± 21.85^{ab}	197.01 ± 21.59^{ab}
Enterocyte height (μm)	9.19 ± 1.15	10.59 ± 1.80	9.35 ± 2.21	9.65 ± 2.67
Muscular thickness (μm)	22.79 ± 3.36^{b}	32.11 ± 4.60^{a}	29.84 ± 3.61^{a}	28.37 ± 2.48^{ab}

[1]Tukey's test showed significant differences in the data without the same superscript letter in the same row ($p < 0.05$).

4 Discussion

FA is one of innocuous and eco-friendly organic substances, which has been proved to effectively reduce feed conversion rate and promote the growth of livestock and aquatic animals as a feed additive (Bai et al., 2013; Gao et al., 2018; Mao, 2019). In this study, the supplementation of FA could increase the survival and growth of larvae. The similar results have also been found in Nile tilapia (Jusadi et al., 2020) and Pacific white shrimp (Gao et al., 2018). These positive effects are related to the ability of FA to improve digestive capacity and immunity with its multiple active functional groups.

Intestinal development of marine fish larvae is important for improving digestive capacity (Pérez-Sirkin et al., 2020). In the present study, the intestinal VH and MT of larvae were significantly increased, indicating a more developed intestinal structure than that of the control group. Intestinal maturation is related to the proliferation and barrier function of epithelial cells (Liu et al., 2020). The mRNA expression of epithelial cell proliferation related gene (*pcna*) was significantly upregulated by supplementation of 0.01% FA. The permeability of tight junctions plays a crucial role in regulating the barrier function of the intestinal epithelium. This barrier allows for the absorption of nutrients and water while effectively blocking

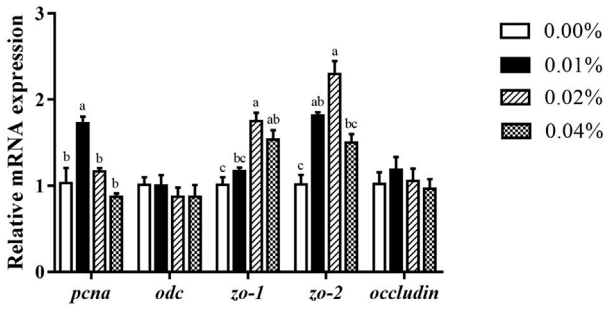

FIGURE 3
Effects of supplemental fulvic acid on intestinal cell proliferation-related and barrier-related genes mRNA expression in intestine of large yellow croaker larvae (mean ± S.E.M, n = 3). *Pcna*, proliferating cell nuclear antigen; *odc*, ornithine decarboxylase; *zo-1*, zonula occludens-1; *zo-2*, zonula occludens-2. Bars with different letters showed a significant difference ($p < 0.05$, Tukey's test).

the entry of pathogens (Turner, 2006; Elsabagh et al., 2018; Li et al., 2021). In the present study, we found that the mRNA expression of intestinal *zo-1* and *zo-2* was significantly increased in the treatment group. Gildea et al. (2017) found that 20% lignite extract (the main raw material for FA extraction) was able to alleviate damage to the tight junction protein ZO-1 caused by glyphosate. Consequently, supplemental FA could improve intestinal maturation of larvae by ameliorating intestinal morphology, stimulating intestinal epithelial cell proliferation and maintaining intestinal barrier function.

Digestive enzyme activities are widely used as the marker to assess the digestive capacity of larvae (Yao et al., 2020; Yin et al., 2022). The relatively low activity of protease enzymes is thought to be one of the causes of the poor growth of larvae fed on a micro-diets (Dabrowski, 1984; Segner et al., 1989; Kolkovski, 2001). The present study showed that supplemental FA significantly increased the trypsin activities of IS and PS, which was consistent with the results in *P. clarkia* (Zhang, 2018), *P. dabryanus* (Gao et al., 2017) and *C. auratus* (Wu et al., 2016). The increased and stable activity of intestinal BBM enzymes such as aminopeptidase and AKP indicates that the gut may fully mature and achieve full function at this stage of development (Kolkovski et al., 2009; Ma et al., 2005; Lallès, 2020). The present study found that supplemental FA could significantly improve AKP and LAP activities in the intestinal BBM, which means that FA promote the intestine maturation of larvae (Liu et al., 2020; Xu et al., 2022). Thus, larvae fed diets with FA improved digestive enzyme activity and promoted intestinal development, facilitating adequate digestion and absorption of nutrients in the feed sufficiently.

The innate immune system plays a vital role in the immune defense of fish. Effective prophylactic measures to enhance the innate immune system prior to infection are of special value in larviculture (Magnadottir, 2010; Rojo-Cebreros et al., 2018). Oral immunostimulants can increase stress resistance and stimulate potential killing activity of macrophages to defend themselves against pathogens (Geng et al., 2011). Our research found that

TABLE 6 Effects of supplemental fulvic acid on activities of digestive enzymes of large yellow croaker larvae (Means ± S.E.M, n = 3)[1].

Parameters		Diets (FA%)			
		Control (0.00)	Diet 1 (0.01)	Diet 2 (0.02)	Diet 3 (0.04)
Trypsin (U/mg protein)	IS[2]	254.91 ± 5.79^{b}	272.96 ± 6.18^{b}	343.90 ± 9.15^{a}	315.76 ± 14.32^{a}
	PS[2]	267.29 ± 20.02^{c}	329.35 ± 5.99^{ab}	382.65 ± 20.73^{a}	322.71 ± 22.59^{bc}
	Try-IS/(PS + IS)	0.49 ± 0.01	0.45 ± 0.01	0.47 ± 0.02	0.49 ± 0.02
Amylase (U/mg protein)	IS	0.050 ± 0.01	0.051 ± 0.01	0.063 ± 0.00	0.065 ± 0.00
	PS	0.034 ± 0.00	0.031 ± 0.00	0.038 ± 0.00	0.035 ± 0.00
Lipase (mU/mg protein)	IS	1.58 ± 0.09	1.84 ± 0.14	1.78 ± 0.14	1.50 ± 0.16
	PS	1.64 ± 0.18	1.87 ± 0.14	1.90 ± 0.16	1.68 ± 0.18

[1]Tukey's test showed significant differences in the data without the same superscript letter in the same row ($p < 0.05$).
[2]PS, pancreatic segments; IS, intestinal segments.

TABLE 7 Effects of supplemental fulvic acid on activities of AKP and LAP in BBM[2] of large yellow croaker larvae (Means ± S.E.M, n = 3)[1].

Parameters	Diets (FA%)			
	Control (0.00)	Diet 1 (0.01)	Diet 2 (0.02)	Diet 3 (0.04)
AKP[2] (mU/mg protein)	0.26 ± 0.04^{c}	0.62 ± 0.05^{a}	0.43 ± 0.03^{b}	0.47 ± 0.02^{b}
LAP[2] (mU/mg protein)	9.16 ± 0.62^{c}	21.52 ± 1.04^{a}	16.06 ± 1.23^{b}	15.08 ± 1.26^{b}

[1]Tukey's test showed significant differences in the data without the same superscript letter in the same row ($p < 0.05$).
[2]AKP, Alkaline-phosphatase; LAP, Leucine-aminopeptidase; BBM, brush border membrane.

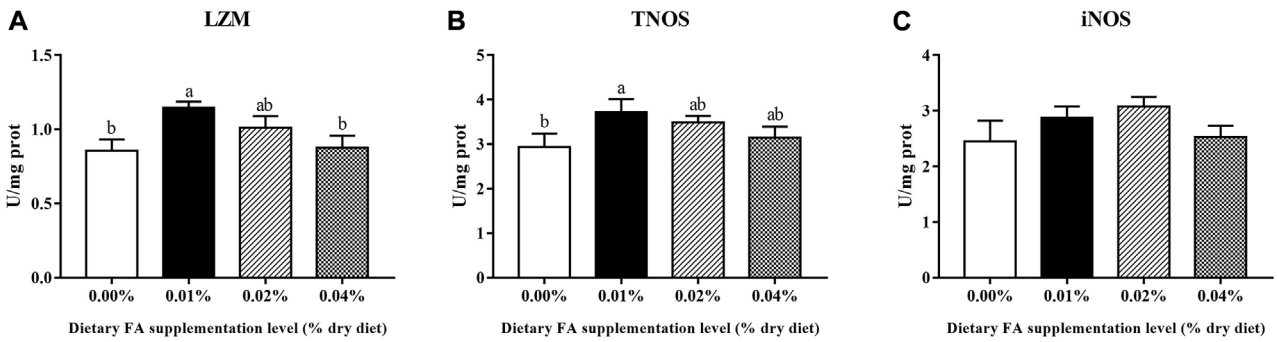

FIGURE 4
Effects of supplemental fulvic acid on LZM **(A)**, TNOS **(B)** and iNOS **(C)** activities in the visceral mass of large yellow croaker larvae (mean ± S.E.M, n = 3). LZM, lysozyme; TNOS, total nitric oxide synthase; iNOS, inducible nitric oxide synthase. Bars with different letters showed a significant difference ($p < 0.05$, Tukey's test).

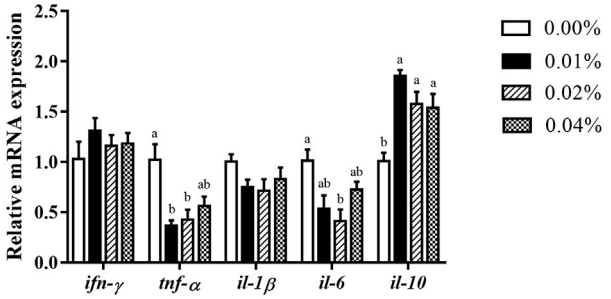

FIGURE 5
Effects of supplemental fulvic acid on genes related to inflammation mRNA expression in liver of large yellow croaker larvae (mean ± S.E.M, n = 3). *Ifn-γ*, interferon γ; *tnf-α*, tumor necrosis factor α; *il-1β*, interleukin-1β; *il-6*, interleukin-6; *il-10*, interleukin-10. Bars with different letters showed a significant difference ($p < 0.05$, Tukey's test).

supplemental FA effectively improved activities of non-specific immune enzymes. LZM is involved in an extensive range of defense mechanisms and can act on the peptidoglycan layer of bacterial cell walls leading to bacterial lysis (Saurabh and Sahoo, 2008). Therefore, the increased activity of LZM indicates an improved protection against bacterial invasion. In vertebrates, Nitric oxide (NO) produced by immune cells is synthesized and released for over time by iNOS, which has unique flexibility in responding to disease (Huang et al., 2020; Pederzoli and Mola, 2016). In RAW246.7 cells, FA regulate iNOS production and stimulate NO production by activating the NF-κB pathway (Jayasooriya et al., 2016). In our study, the increased activity of iNOS indicated that FA had an immunostimulatory effect on larvae. Moreover, FA acts as an immunomodulator and has anti-inflammation effect (Sherry et al., 2013; Van Rensburg et al., 2001). Cytokines, as signaling molecules for cellular communication, are key participants in the innate immune response (Magnadottir, 2010). In the present study, FA stimulation led to upregulation of anti-inflammatory cytokine *il-10* and downregulation of pro-inflammatory cytokines *tnf-α* and *il-6*. Therefore, our research suggested that supplemental FA can enhance innate immunity of larvae.

In conclusion, the present study showed that the supplementation of FA can improve the survival rate and growth performance of large yellow croaker larvae by improving intestinal morphology, promoting intestinal barrier function, increasing digestive enzymes activities, and enhancing non-specific immunity. Based on the specific growth rate, the supplementation of 0.0135% FA was optimal for the growth performance of large yellow croaker larvae under the current experimental conditions.

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 this study was executed in strict compliance with the Chinese Order NO. 676 of the State Council, revised 1 March 2017.

Author contributions

CZ designed the research. CZ, JZ, YW, and JL conducted the research. CZ, YL, and JZ analyzed the data. CZ wrote the manuscript. YL, CY, and YH reviewed manuscript. KM and QA

instructed the research. All authors reviewed and approved the submitted manuscript.

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Conflict of interest

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

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The effect of selenium on antioxidant system in aquaculture animals

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There will be generated some adverse conditions in the process of acquculture farming with the continuous improvement of the intensive degree of modern aquaculture, such as crowding stress, hypoxia, and malnutrition, which will easily lead to oxidative stress. Se is an effective antioxidant, participating and playing an important role in the antioxidant defense system of fish. This paper reviews the physiological functions of selenoproteins in resisting oxidative stress in aquatic animals, the mechanisms of different forms of Se in anti-oxidative stress in aquatic animals and the harmful effects of lower and higher levels of Se in aquaculture. To summarize the application and research progress of Se in oxidative stress in aquatic animals and provide scientific references for its application in anti-oxidative stress in aquaculture.

KEYWORDS

selenium, aquatic animals, antioxidant, oxidative stress, selenoprotein

1 Introduction

Selenium (Se) is an essential micronutrient that maintains growth, health and development in human and animals (Hatfield et al., 2012). Se exists in nature is available as inorganic form and organic form. In addition, there is also a new type of synthetic nano form. The biological functions of Se include promoting the growth and development of animals, improving the antioxidant capacity of the body, avoiding oxidative stress, enhancing immunity, improving the health of the body, and are widely used in the growth and development of aquaculture animals (Wischhusen et al., 2020; Khan et al., 2017; Jingyuan et al., 2020). However, Se cannot be directly digested and absorbed by organisms. Se appears functions in the form of selenoprotein and exerts multiple and complex effects (Wrobel et al., 2016).

There will be generated some adverse conditions in the process of aquaculture farming with the continuous improvement of the intensive degree of modern aquaculture, such as crowding stress, hypoxia, and malnutrition. Meanwhile, that will promote the production of excessive reactive oxygen species (ROS), change the balance of oxidation and reduction in cells and will lead to oxidative stress (Rider et al., 2009; Abdel Tawwab et al., 2019). Once oxidative stress occurs, it may lead to the dynamic imbalance of free radical stability, which ultimately the fish poor growth and decline the meat quality (Chadio et al., 2015). The damage of oxidative stress in production is increasingly prominent, so it is urgent to solve the problem of oxidative stress in aquaculture animals.

In order to prevent oxidative damage caused by adverse conditions, aquaculture animals must have an effective antioxidant system, such as endogenous free radical scavenging enzymes and exogenous antioxidants (Ighodaro and Akinloye, 2018). Se is just a extra-effective antioxidant and plays an important role in the antioxidant defense mechanism of fish. It protects the cell membrane from oxidative damage through regulate glutathione peroxidase (GPX) activity (Hamilton, 2004). Se can decompose and clear peroxides, thus preventing the accumulation of reactive oxygen species and free radicals such as hydrogen peroxide (H_2O_2) in the body tissues (Zuo et al., 2019), enhancing resistance to inflammation, maintaining immune function and stabilizing the internal environment (Wande et al., 2020). However, dietary Se deficiency will reduce the expression of selenoproteins, increase the peroxidation reaction of lipids, proteins, DNA and then pose a threat to the health of the body (Rao et al., 2020). Excessive Se will also produce toxic side effects on the body, leading to liver damage, physiological function decline, immune capacity reduction, and even death (Chadio et al., 2015).

At present, the research on Se antioxidant stress has involved inorganic Se (such as sodium selenite), organic Se (such as yeast Se, selenomethionines) and nano Se. In the study of the effects of sodium selenite and *lactobacillus* on the growth and immune response of rainbow trout, the results showed that the use of sodium selenite and *lactobacillus* as feed additives could improve the growth and metabolism. Also, the effect of *lactobacillus* on the natural immune response of rainbow trout was also enhanced under the effect of selenite (Kousha et al., 2020). Another study found that, the mortality rate and malondialdehyde (MDA) level of grass carp fed a high-fat diet supplemented with 0.3 and 0.6 mg/kg nano Se was significantly reduced after hypoxia stress, indicating that nano Se can alleviate lipid peroxidation, thus alleviating the oxidative damage of grass carp under hypoxia stress (Yu et al., 2020). Liu et al. (2017) showed that 0.4 mg/kg yeast Se significantly increased the activity of catalase (CAT) and GPX in the liver of blunt snout bream and significantly decreased the activity of glutathione reductase (GR) and the content of MDA, showing strong antioxidant capacity.

2 The role of selenoproteins in the antioxidant system of aquatic animals

In general, Se sources in animals will first be converted into selenides for play on organisms and then will be converted into proteins containing selenocysteine (Sec), that is, selenoprotein which then will be absorbed and used by organisms (Yoboue et al., 2018). During the process of fish growth, any pressure condition will directly or indirectly cause oxidative stress, leading to the level of intracellular ROS related to oxidative stress increasing, causing destructive effects on lipids, proteins, and DNA (Martínez Álvarez et al., 2005; Zenteno Savín et al., 2006). The lack of balance between the production of ROS and the antioxidant defense system of animals will lead to DNA hydroxylation, protein denaturation, lipid peroxidation, cell apoptosis, ultimately lead to cell damage (Yang et al., 2016). As an effective antioxidant, Se plays an important role in the antioxidant defense system of aquaculture animals. It can maintain balance in the body and antioxidant system by generating and removing ROS (Steinbrenner and Sies, 2009).

It has been found that the thioredoxin reductase system and glutathione peroxidase system play an important role in cellular oxidative stress defense (Lee et al., 2012; Benfeitas et al., 2014). The main function of GPX in mammals is to eliminate excessive free radicals in cells, including superoxide anions (O^{2-}), H_2O_2, and hydroxyl radicals (OH^-). Then through reduce peroxides to corresponding alcohols, prevent lipid peroxidation, and regulate the balance of redox in cells (Yoboue et al., 2018; Rocca et al., 2018). The Thioredoxin system is one of the main redox buffer systems in the body, which plays a key role in the transduction of redox signals (Canter et al., 2021; Shchedrina et al., 2010). In addition, the endoplasmic reticulum (ER) is one of the important places where redox reactions occur in cells, which can produce H_2O_2 and ROS (Varlamova, 2018). SELENOF, Recombinant Deiodinase, Iodothyronine, Type II (DIO2), SELENOS, SELENON, SELENOK, SELENOM, and SELENOT are considered to be seven endoplasmic reticulum resident proteins with oxidoreductase activity and can participate in a series of processes, such as the regulation of endoplasmic reticulum redox state, endoplasmic reticulum stress and intracellular Ca^{2+} homeostasis (Malandrakis et al., 2014; Pitts and Hoffmann, 2017; Drew et al., 2008; Addinsall et al., 2018; Rathore et al., 2021; Xu et al., 2022) Specific selenoprotein involved in redox reaction are shown in Table 1.

3 Application of different forms of Se on antioxidant system of aquatic animals

In many studies, Se has been found that supplement oxidative stress caused by hypoxia, crowding, heavy metal pollution, intake oxidized oil and other pressure conditions (Schieber and Chandel, 2014; Chen et al., 2013). Oxidative stress is considered to be a major problem in aquaculture because it is reported that this phenomenon will occur naturally in most aquatic environments, especially in high-density intensive fish farming which will induce metabolic changes and produce a large number of reactive oxygen species (Zenteno Savín et al., 2006). In order to avoid the imbalance of ROS production, aquatic animals can reduce the formation of ROS or reduce the high reactivity of such compounds through antioxidant components which are produced by cells (endogenous sources) or obtained through diet (exogenous sources). A Lack of balance between ROS formation and the ability of organisms to process reactive oxygen will lead to oxidative stress (Wang et al., 2013; Ji et al., 2019). At present, many aquatic animals have been studied on the antioxidant system, and there is evidence that they can prevent or repair oxidative damage (Biller and Takahashi, 2018) (Table 2).

4 Harmful effects of Se deficiency and excess on antioxidant function in aquatic animals

After these environmental pressures, Se may have protective effects on the pathology related to oxidative stress of aquatic animals. It is particularly important for intensive aquaculture animals, because environmental pressures may cause significant losses to fish growers. The demand for Se will increase after suffering environmental pressures, and supplementation is a

TABLE 1 Antioxidant functions of releated selenoproteins.

Type	Aquatic animal species	Antioxidant function	References
GPX1	*Danio rerio, Sparus aurata*	Neutralize reactive oxygen species and improve oxidative stress	Malandrakis et al. (2014), Rathore et al. (2021)
GPX2	*Oreochromis niloticus*	Reduction of H_2O_2	Xu et al. (2022)
GPX3	*Pelteobagrus fulvidraco*	Affects oxidative signaling	Guderley et al. (2003)
GPX4	*Gadus morhua, Thunnus maccoyi, Coho salmon*	Protection of vertebrate olfactory system from oxidative cell damage by lipid peroxidation	Solovyev (2015), Thompson et al. (2010), Wang et al. (2012)
TXNRD1	*Pelteobagrus fulvidraco*	Reduction of H_2O_2 and oxidative stress and regulation of redox-sensitive transcription factors that control cellular transcriptional machinery	Chernorudskiy et al. (2020)
TXNRD2	*Pelteobagrus fulvidraco*	Reduction of thioredoxin to protect cells from oxidation	Guderley et al. (2003)
SELENON	*Pelteobagrus fulvidraco*	Regulates calcium homeostasis and maintains redox activity	Saito (2021)
SELENOT	*Pelteobagrus fulvidraco*	Control protein processing in the ER and maintain ER homeostasis	Guderley et al. (2003)
SELENOP	*Zerbfish*	Reduction of phospholipid hydroperoxides	Zhang et al. (2022)
SELENOF	*Pelteobagrus fulvidraco*	Regulation of endoplasmic reticulum stress, regulation of lipid and glucose metabolism	Zhang et al. (2021)
SELENOM	*Pelteobagrus fulvidraco*	Regulation of ER stress and calcium homeostasis	Wang et al. (2018a)
SELENOK	*Oreochromis niloticus*	Regulation of Ca^{2+} metabolism in gills and mitigation of ER stress	Zhang et al. (2021)
SELENOL	*Oreochromis niloticus*	With thioredoxin structure, with redox function	Zhang et al. (2021)
SELENOS	*Oreochromis, niloticus, Pelteobagrus fulvidraco*	Regulation of lipid metabolism and endoplasmic reticulum stress	Zhang et al. (2021); Wang et al. (2018a)
SELENOW	*Oncorhynchus mykiss*	Protects skeletal muscle from inflammatory damage	Castellano et al. (2005)
SELENOJ	*Oreochromis niloticus*	Affects cellular resistance to stress	He et al. (2022)
SELENOU	*Ctenopharyngodon idella*	Regulation of lipid metabolism	Bityutskyy et al. (2020)
MSRB1	*Salmon fish*	Regulates the inflammatory response	Penglase et al. (2014)
SPS1	*Oreochromis niloticus*	Affects gill ion exchange and has oxidative stress defense	Rathore et al. (2021)

ER, endoplasmic reticulum; GPX1, glutathione peroxidase 1; GPX2, glutathione peroxidase 2; GPX3, glutathione peroxidase 3; GPX4, glutathione peroxidase 4; TXNRD1, thioredoxin reductase 1; TXNRD2, thioredoxin reductase 2; SELENON, selenoprotein N; SELENOT, selenoprotein T; SELENOP: selenoprotein P; SELENOF: selenoprotein F; SELENOM: selenoprotein M; SELENOK: selenoprotein K; SELENOL, selenoprotein L; SELENOS, selenoprotein S; SELENOW, selenoprotein W; SELENOJ, selenoprotein J; SELENOU, selenoprotein U; MSRB1, methionine sulfoxide reductase B1; SPS1, selenophosphate synthetase 1.

necessary condition to meet the needs of fish growth (Ashouri et al., 2015). Se can act as a dietary supplement to effectively improve the oxidation status of aquaculture animals. Although, dietary Se application in aquatic animal feed is considered to have a narrow intake range for high concentration may be toxic (Nasir et al., 2015) and lack may have adverse effects on fish health, leading to tissue damage and physiological function weakening (Khan and Bano, 2016). Therefore, their safety in aquaculture animals is controversial.

4.1 Harmful effects of selenium excess

Se is an essential micronutrient required for fish, which plays an important role in the antioxidant system and also affects the lipid metabolism, sugar metabolism and amino acid metabolism of aquatic animals (Yoboue et al., 2018; Fontagné Dicharry et al., 2015; Abdel Tawwab et al., 2007). Although Se supplementation in aquatic feed can alleviate the oxidation state of cultured animals, but excessive Se content will also have some toxic effects (Jobling, 2012). When the Se content in the aquatic feed is slightly higher than the demand, it will have other adverse effects including oxidative stress, cytotoxicity and genotoxicity (Gobi et al., 2018). If the fish is exposed to excessive Se, the degree of oxidative stress in the body can be significantly increased, then cause oxidative stress. Glutathione (GSH) is largely used to maintain the homeostasis of ROS in the body, can cause an imbalance in the antioxidant system (Atencio et al., 2009). In Atlantic salmon, higher Se levels (at least 15 mg/kg in diet) can lead to oxidative stress and change the lipid metabolism of organic and inorganic Se (Berntssen et al., 2017). In addition, in Acipenser sturgeon (*Acipenser transmontanus*), excessive Se in the diet leads to vacuolar degeneration of cells and necrosis of the liver (Tashjian et al., 2006). In a word, the mechanism of the negative effect of high Se intake on the antioxidant system of aquatic animals is still unclear.

4.2 Harmful effects of Se deficiency

The necessity and demand for dietary Se have been estimated in various fish (Khan et al., 2017). Although Se poisoning may cause multiple damages to fish, the effect of dietary Se deficiency in fish

TABLE 2 Effects of different forms of Se on the antioxidant system of aquatic animals.

Type	Aquatic animal species	Antioxidant mechanism	References
Inorganic Se	*Rainbow Trout*	Inhibition of increased lipid peroxidation, inhibition of antioxidant transcript levels, inhibition of glutathione levels in the blood	Fontagné Dicharry et al. (2020)
	Nile tilapia (*Oreochromis niloticus L.*)	Inhibits lipid peroxidation in gills, increases non-protein thiol levels, participates in fish excretion	Giuliani Durigon et al. (2018)
	Eriocheir sinensis	Inhibits hepatopancreas lipid peroxide production and enhances GPX activity	Qiang et al. (2020)
	Micropterus salmoide	Enhancement of hepatic GPX and CAT activity	Chen et al. (2013)
Organic Se	*Litopenaeus vannamei*	Catalyzing the dismutation of superoxide radicals into H_2O_2 and oxygen, and the conversion of H_2O_2 into water, protecting cell membranes from oxidative damage, participating in polyunsaturated fatty acid metabolism	Yu et al. (2022)
	Acanthopagrus schlegelii	Regulation of hepatic lipid metabolism, enhancement of hepatic GPX activity	Wang et al. (2019)
	Gilthead seabream (*Sparus aurata*)	Inhibition of hepatic lipid peroxide production	Mechlaoui et al. (2019)
	Rainbow Trout (*Oncorhynchus mykiss*)	Inhibits muscle lipid peroxide production, enhances muscle GPX activity, inhibits SOD and CAT activity	Wang et al. (2018a)
Nano-Se	*Nile tilapia* (*Oreochromis niloticus*)	Affects fish excretion and gill ion exchange	Rathore et al. (2021)
	Grass carp (*Ctenopharyngodon Idella*)	Regulation of Nrf_2 signaling and selective expression of antioxidant enzyme genes	Yu et al. (2020)
	Cyprinus carpio	Enhance hepatic GPX, SOD and CAT activities and inhibit hepatic peroxide production	Saffari et al. (2017)

SOD, superoxide dismutase.

cannot be ignored. It has been reported that Se deficiency is closely related to the occurrence of many diseases in aquatic animals and the impairment of physiological functions under various conditions (Zheng et al., 2018; Wang et al., 2018). Se deficiency can inhibit organs growth and reduce immune functions, thus leading to many inflammatory diseases (Zheng et al., 2018; Hofstee et al., 2017). Se has been proved to be a protective trace element in the liver of many animals. Se deficiency can lead to immune deficiency, inhibit fish growth and induce oxidative stress in the liver (Zheng et al., 2018; Byron and Santolo, 2018). Oxidative stress can stimulate liver inflammation, thereby aggravating liver injury (Mukhopadhyay et al., 2018). In a research on the mechanism of carp liver inflammation, dietary Se deficiency can lead to Se deficiency in blood and liver, leading to overexpression of heat shock protein HSP60 in the liver, aggravating and forming inflammatory damage to the liver (Gao et al., 2019). Se deficiency also impairs the structural integrity of the head kidney, spleen and skin of juvenile grass carp, leads to oxidative damage and aggravating cell apoptosis due to downregulate the activities of antioxidant enzymes (SOD, CAT, GPX, GSTR, and GR) and the mRNA levels related to the signal transduction part (Zheng et al., 2018).

5 Effects of Se-rich fish on humans

Food is the main source of Se intake by the human. As like fish, Se is mainly involved in regulating human various physiological activities in the form of selenoproteins. Se deficiency or Se excess can have adverse effects on the human (Rayman, 2008). Fish is a kind of food with the highest natural Se content and tender flesh, rich in mineral elements, proteins and vitamins, which is the preferred ingredient for people to supplement trace elements through diet. Fish reared in a Se-rich water environment and fed with Se-rich feed can be processed and prepared into bioactive peptide foods such as fish collagen peptides. Eating Se-rich bioactive peptide foods can improve the absorption rate of Se in the body and Se supplement is more efficient (Xia et al., 2022).

Se can reduce the incidence rate of heart disease in humans, but ROS formed during oxidative stress can initiate lipid peroxidation, damage normal cell function, and lead to myocardial ischemia reperfusion (I/R) injury. Se enzyme and the whole thioredoxin system are directly reduce the content of lipid peroxide, protect vascular endothelial cells from oxidative damage, and inhibit Low Density Lipoprotein (LDL) oxidation (Furman et al., 2004; Venardos and Kaye, 2007). SELENOM may be able to alleviate disorders of lipid metabolism and high-fat diet (HFD)-mediated mitochondrial damage in NAFLD. SELENOM regulates Parkin-mediated mitophagy *via* the $AMPK\alpha_1$–MFN_2 signaling pathway, blockade of the $AMPK\alpha_1$–MFN_2 pathway and inhibit mitophagy. Moreover, SELENOM deletion inhibits the expression of FAO-related genes (*Ppara*, *Cpt1α*, *Cdh15*, *Acox1* and *Acadm*), and increases the expression of lipogenic genes (*Gpam*, *Plin1*, *Scd1*, *Lipe*, *Fasn*, *Acly,* and *Pparg*) (Cai et al., 2022). It has been shown that during wound healing some selenoproteins (GPX, SELENOS and SELENOP) bind together with produce effects in the inflammatory phase, such as antioxidant, inhibition of inflammatory cytokines, scavenging of peroxynitrite and enhancing immune function by increasing the survival of phagocytes during phagocytosis (Lei et al., 2009). Se is play a key role in reducing and preventing cancer caused by several carcinogens and inducing apoptosis of cancer cells (Tinggi, 2008). Se level is negatively correlated with human cancer risk. Genomic studies and animal models have indicated that Se intake influences the expression

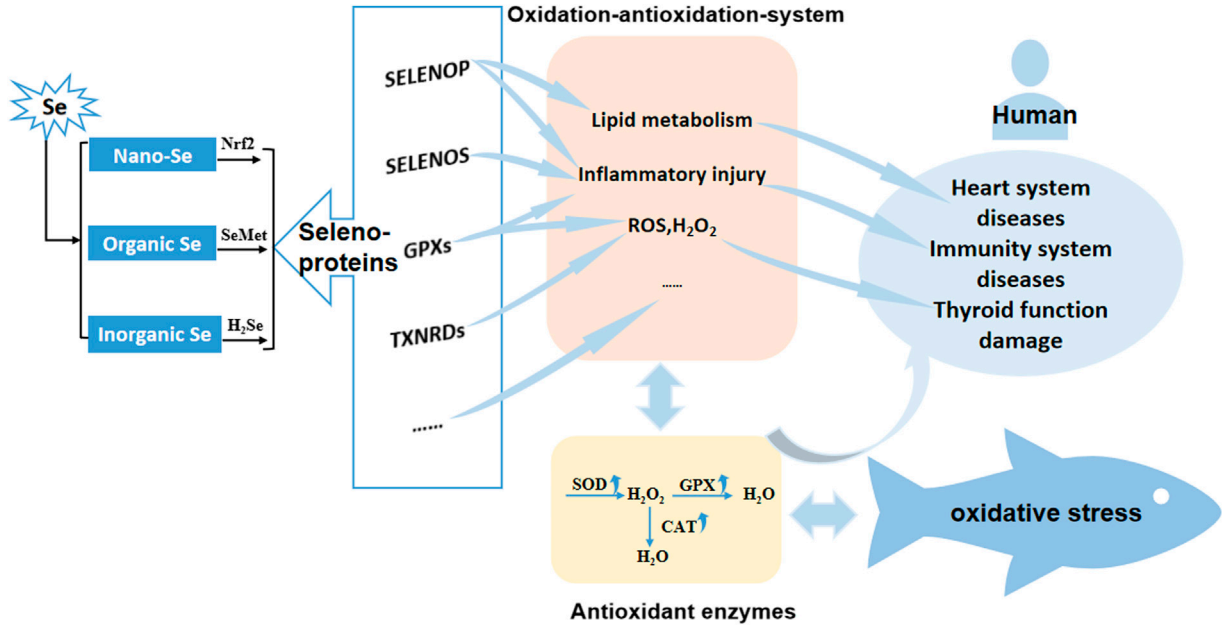

FIGURE 1
Antioxidant mechanism of different Se forms in fish. Nano-Se, Nano-Selenium; Organic Se, Organic Selenium; Inorganic Se, Inorganic Selenium; GPXs, glutathione peroxidase family; TXNRDs, thioredoxin reductase family.

of selenoprotein genes and pathways key to colorectal carcinogenesis such as the antioxidant response, immune and inflammatory pathways (including *NF-kB* and *Nrf*$_2$ signaling), the Wnt signaling pathway, protein synthesis pathway (Ye et al., 2021). Se also reduces the incidence of autoimmune thyroiditis and other related conditions, and the reduction of thyroid autoimmune effects is related to the role of GPX and thioredoxin reductase (TrxR) as antioxidant defense systems that scavenge ROS and excess H_2O_2 produced by thyroid cells during thyroid hormone synthesis, which can lead to thyroid cell necrosis and increased macrophage invasion in severe nutritional Se deficiency (Duntas, 2006).

6 Antioxidant mechanism of different Se forms in fish

In the intensive aquaculture system, cultured fish mainly obtain the selenium from the feed. There are three main forms of selenium additives: one is inorganic Se, which enters animals through passive diffusion in the form of Se^{4+}, and is quickly reduced to selenides (H_2Se) in the intestine; the second is organic Se, which is actively absorbed by fish in the form of amino acids, combines with plasma protein to form SeMet; the third is Nano-Se can activate Nrf$_2$ signal transduction pathway to express genes related to antioxidant enzymes. No matter inorganic Se, organic Se or Nano-Se, it will be converted into selenoproteins to play a role after being absorbed by fish. Selenoproteins related to fish antioxidant mechanism include GPXs, TXNRDs, SELENOP, SELENOS, and other selenoproteins, which regulate lipid metabolism, inflammatory damage, ROS and H_2O_2 formation oxidative stress through antioxidant enzymes (SOD, GPX, and CAT) in fish antioxidant system. In addition, the regulation of selenium deficiency in the human antioxidant system will cause diseases of the heart system, immune system and thyroid function damage (Yu et al., 2020; Wischhusen et al., 2019) (Figure 1).

7 Conclusion

In summary, Se is an essential mineral element for antioxidant stress of aquatic animals and plays an important role in the antioxidant system of aquaculture. We can further study the most effective form of Se in the antioxidant system of aquatic animals, selectively prevent the oxidative stress of aquaculture animals, clarify the mechanism of selenoproteins in the antioxidant system of aquatic animals, and provide direction for the study of Se toxicity. Due to the various functions of selenoproteins, the mechanism of specific selenoprotein function and expression can be considered to prevent the oxidation state of aquatic animals. In the future, we can also compare the effects of Se on other organisms and study the mechanism of Se on the antioxidant system of aquatic animals. This will provide important information for the extensive application of antioxidant systems in aquaculture. This will provide important information for the extensive application of antioxidant systems in aquaculture.

Author contributions

Conceptualization, Z-ML and L-SW; methodology, J-QH; software, Z-ML validation, Z-ML, X-MJ, and L-SW; formal

analysis, Z-ML; investigation, Z-ML; resources, J-QH; data curation, Z-ML; writing—original draft preparation, Z-ML; writing—review and editing, Z-ML, JQH, and L-SW; visualization, X-MJ; supervision, L-SW; project administration, L-SW; funding acquisition, L-SW. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

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Yeast-extracted nucleotides and nucleic acids as promising feed additives for European sea bass (*Dicentrarchus labrax*) juveniles

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Nowadays functional ingredients have a significant potential for improving current low fish meal (FM) aquafeed formulation in sustaining growth and enhancing animal robustness for Mediterranean aquaculture. Among them, nucleotides (NT) and nucleic acids (NA) drew attention for their application in the last two decades. NT are organic molecules involved in many life-supporting pathways, and are the building blocks of NA, which stand as genetic repositories. NT are naturally present in organic ingredients, and among them FM is known to be one of the highest NT sources. When this NT source is seriously limited, fish might be under the minimum NT requirements, especially in fast growing life stages of carnivorous species. Hence, a trial on European sea bass juveniles was carried out, testing two dietary FM levels (FM10, FM20 as 10% and 20% FM, respectively) supplemented with 500 mg kg^{-1} yeast-originate NT or NA dose over 80 days. Thereafter, fish were exposed to one week of sub-optimal thermal and dissolved oxygen condition (30°C and 4.0 mg/L O_2) to further explore the effect of NT and NA inclusion on immune response and gut microbiome alteration. At the end of the growth period NT increased feed intake at both FM dietary levels. FM20 combined with NA and NT further improved growth performance, enhancing lipid efficiency and increased anti-inflammatory TGF-β. After sub-optimal environmental conditions both NT and NA exerted prebiotic functions on gut microbiome by promoting beneficial lactic acid bacteria such as *Weissella* and *Leuconostoc*. At the same time NT in 10% FM diet increased the abundance of *Bacillus* taxon. In conclusion, the combination of NT/NA included at 500 mg kg^{-1} was able to promote growth when included in 20% FM level, assuming higher nutritional NT requirement when combined with 10% FM. On the other hand, NT/NA added in 10% FM upregulate proinflammatory IL-1β and favor beneficial gut bacterial taxa.

KEYWORDS

aquaculture, feed additives, nucleotides, fishmeal, growth, cytokines, gut microbiome

1 Introduction

To meet the continuously rising demand, Mediterranean marine aquaculture sector urges a continuous production improvement, especially for aquafeed, which covers approximately up to 70% of the total running expenses and is mainly bound to fish meal (FM) as the major but unsustainable protein source, especially for carnivorous fish species (Guillen and Carvalho, 2016; Dawood and Koshio, 2020). So far, the main solution has been the consistent FM replacement with alternative agricultural crop-derived ingredients though with some problems related to anti-nutritional and nutrient requirements issues (Hua and Bureau, 2012; Nasopoulou and Zabetakis, 2012; Bonvini et al., 2018a; Sutili et al., 2018; Parma et al., 2019; Pelusio et al., 2022). As a further step beyond, tailored functional aquafeeds able to improve growth, feed utilization, general health and stress resistance of animals, represent a new emerging paradigm of current fish nutrition to compensate the negative effects of alternative or low FM based diets and improve cost-effectiveness (Hossain and Koshio, 2017a; Dawood et al., 2018). Initially used as feed attractants, nucleotides (NT) and nucleic acids (NA) drew the attention of research and industrial communities as functional ingredients on account of their many features as alternative nitrogen sources and health promoters in the last two decades (Li and Gatlin, 2006; Hossain and Koshio, 2017a). NT are low molecular weight biological compounds involved in most of the biochemical pathways essential for life support. They stand as metabolic energy currency, cell signaling mediators and components of enzyme cofactors. Moreover, they are the constituents of the molecular repositories of genetic information: deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). The structure of every protein, biomolecule, and cellular component, is a product of information programmed into the NT sequence of cellular NA (Bowyer et al., 2019). In feedstuffs, NT are naturally present in all animal and vegetable origin ingredients mostly as nucleoproteins and in low quantities as free NT and among them, FM is one of the richest sources (Hossain et al., 2020). Since NT can be synthesized endogenously by the organism, they are not considered essential nutrients (Low et al., 2003). On the other hand, their synthesis is a metabolically costly process, and the dietary uptake of NT and NA isolated from yeasts may optimize cell proliferation to promote rapid growth, especially during early development stages with high metabolism or fast growth (Ringø et al., 2012; Dawood et al., 2018). Many studies show that NT or NA supplementation can improve growth and nutritional uptake in several aquaculture species such as European sea bass (Oliva-Teles et al., 2006; Rawling et al., 2019), gilthead sea bream (El-Nokrashy et al., 2021), amberjack (Hossain et al., 2017b), Nile tilapia (Barros et al., 2015; Kader et al., 2018), red sea bream (Hossain et al., 2016; Hossain and Koshio, 2017a; Hossain et al., 2017c), Atlantic salmon (Burrells et al., 2001), turbot (Peng et al., 2013) and rainbow trout (Hunt et al., 2014). Moreover, when fish undergo sub-optimal and stressful farming conditions such as handling, elevated temperatures and fluctuating levels of dissolved oxygen, additional NT pools might be required for further signal transduction or immune cell proliferation with a modulatory effect (Carver and Allan Walker, 1995; Cosgrove, 1998; Li and Gatlin, 2006). Many studies reported modulation to stress response (Kenari et al., 2013; Palermo et al., 2013), resistance to disease and pathogens (Burrells et al., 2001; Tahmasebi-Kohyani et al., 2011), lymphocytes activity, macrophage phagocytosis, immunoglobulin and cytokines responses (Gil, 2002; Low et al., 2003; Singhal et al., 2008; Reda et al., 2018). As a further gut health biomarker, gut microbiome composition and its eventual modulation may indicate the status of commensal bacteria related to stressful events or unbalanced diet formulation (Perry et al., 2020). So far, encouraging and consistent NT and NA applications on gut health have been reported in many aquatic species (Burrells et al., 2001; Guo et al., 2019; Rawling et al., 2019; El-Nokrashy et al., 2021). Nowadays, one of the main issues related to their addition in formulated feeds is to establish the right dose for each specific life stage and species. While terrestrial monogastric animals cannot tolerate high levels of dietary NT due to high serum uric acid from purine metabolism and associated toxicity, as well as negative effects on other nutrients pathways, fish such as salmonids and sea bass may tolerate high levels of NT/NA/yeasts thanks to active liver uricase (Hossain et al., 2020). So far, the most promising dose concentration for blend NT has been targeted at 0.5% dietary level, while for single purified nucleotide dosages the range has been between 0.1% and 0.6% of dry weight in different fish species (Hossain et al., 2020). European sea bass (*Dicentrarchus labrax*) is one of the major Mediterranean aquaculture species in the European Union and it is well known to be a model organism (Vandeputte et al., 2019). In recent years, though its carnivorous regime, sea bass diet formulation has been achieving even low FM contents with commercial potential application (Pelusio et al., 2022). For these reasons, the aim of the present study was to evaluate the effects of dietary NT and NA addition on growth and gut health of European sea bass juveniles fed different low FM level.

2 Materials and methods

2.1 Experimental diets and additive dosages

Ingredients and chemical composition of the experimental diets are presented in Table 1. Six isonitrogenous (45.16% protein) and isolipidic (17.43% lipids) diets were formulated to contain 2 low different FM levels (20 and 10%) as control treatments. Then, nucleotides (NT) and nucleic acid (NA) were added at the same dosage of 500 mg kg feed^{-1} in accordance with recent recommendations on same and other fish species (De Cruz et al., 2020; El-Nokrashy et al., 2021; Magouz et al., 2021). Experimental diets were produced via extrusion (pellet size = 2.0 mm) by the Danish Technological Institute (Taastrup, Denmark).

2.2 Fish and rearing conditions

The trial was performed at the Laboratory of Aquaculture, Department of Veterinary Medical Sciences of the University of Bologna, Cesenatico, Italy. European sea bass (*Dicentrarchus*

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TABLE 1 Feed formulation and chemical composition of experimental treatments at different fish meal (FM) level and nucleotide (NT) or nucleic acid (NA) inclusion.

Diet	FM10	FM10NT	FM10NA	FM20	FM20NT	FM20NA
Formulation (% of diet)						
Rapeseed lecithin	2.00	2.00	2.00	2.00	2.00	2.00
Wheat	8.96	8.96	8.96	14.49	14.49	14.49
Corn gluten	21.00	21.00	21.00	15.00	15.00	15.00
Hi-pro soya	14.00	14.00	14.00	10.00	10.00	10.00
Wheat gluten	13.77	13.77	13.77	13.77	13.77	13.77
Sunflower meal	14.00	14.00	14.00	10.00	10.00	10.00
Fish meal	10.00	10.00	10.00	20.00	20.00	20.00
Fish oil	12.87	12.87	12.87	12.20	12.20	12.20
L-Lysine	1.08	1.08	1.08	0.74	0.74	0.74
Monoamoniumphosphate	1.20	1.20	1.20	0.75	0.75	0.75
Vitamin premix	0.50	0.50	0.50	0.50	0.50	0.50
Mineral premix	0.20	0.20	0.20	0.20	0.20	0.20
Choline chloride	0.32	0.32	0.32	0.26	0.26	0.26
Yttrium premix	0.10	0.10	0.10	0.10	0.10	0.10
Feed additive inclusion (ppm)						
NT	–	500	–	–	500	–
NA	–	–	500	–	–	500
Proximate composition (% on a wet basis)						
Moisture	6.86	6.87	6.28	5.77	6.19	6.48
Crude protein	45.03	45.34	45.56	45.25	44.72	45.06
Crude lipid	17.68	17.63	17.53	17.56	17.47	16.72
Ash	4.99	5.09	5.09	5.85	5.84	5.84

NT, yeast-extract rich in nucleotides (22% nucleotides, 7.7% RNA).

NA, yeast-extract rich in nucleic acid (26.5% RNA, 8.0% nucleotides).

labrax) juveniles were transferred from Ittica Caldoli S.A.R.L (Poggio Imperiale, Foggia, Italy) to the laboratory facilities and adapted for one week before the beginning of the trial. Then, 60 fish tank^{-1} (initial weight: 14.33 ± 0.18 g, mean ± standard deviation, SD) were randomly allocated in eighteen 800 L tanks. Diets were randomly assigned in triplicate condition and animals were fed for 80 days. Tanks were provided with natural seawater using a closed recirculation system (overall water volume: 20 m^3) according to Pelusio et al. (2021). The recirculation system consisted of a mechanical sand filter (PTK 1200, Astralpool, Barcelona, Spain), ultraviolet lights (SH-88, BLUGEO S.r.l., Parma, Italy) and a biofilter (PTK 1200, Astralpool, Barcelona, Spain). The water exchange rate in each tank was 100% every hour, while the overall water renewal in the system was 5% per day. The temperature was kept at 22.0 ± 0.5°C while photoperiod was set at 12 h light and 12 h dark. The oxygen level (8.0 ± 1.0 mg L^{-1}) was provided through a liquid oxygen system regulated by a software programme (B&G Sinergia snc, Chioggia, Italy). Ammonia (total ammonia nitrogen ≤ 0.1 mg L^{-1}) and nitrite (≤ 0.2 mg L^{-1}) were daily measured (Spectroquant Nova 60, Merck, Lab business, Darmstadt, Germany), while salinity (30 g L^{-1}) was analysed using a salt refractometer (106 ATC, Giorgio Bormac S.r.l., Carpi, Italy). Sodium bicarbonate was provided regularly to keep pH at 7.8–8.2 (Pelusio et al., 2021). Fish were fed ad libitum twice a day (8:30 h and 16:30 h) for six days a week by means of automatic feeders using an overfeeding approach with a daily feeding ration 10% higher than the daily ingested ration of the previous days as reported by Bonvini et al. (2018b). Each meal lasted 1 h, after which the uneaten pellets of each tank were collected, dried overnight at 105°C, and weighed for further calculations of feed intake.

2.3 Suboptimal rearing conditions

After the end of the feeding trial, fish were exposed to high temperature (28.9 ± 1.8°C) and low oxygen (4.1 ± 0.8 mg L^{-1}, 55.3 ±

4.7% saturation level) for 8 days while keeping the same feeding conditions. The sub-optimal environmental conditions were designed in order to simulate unfavourable Mediterranean summer condition as reported by Busti et al. (2020). Specifically, temperature was increased at a rate of 2-degree day^{-1} while water oxygen level was decreased (concentration from 8.0 ± 1.0 mg L^{-1} to 4.6 ± 0.6 mg L^{-1} and saturation from 105.0 ± 1.0% to 67.6 ± 5.0%, respectively) within 24 h. Temperature and oxygen level were constantly measured through an automatic system regulated by a software programme (B&G Sinergia snc, Chioggia, Italy). The environmental parameters were chosen according to previous studies on oxygen and thermal tolerance of the species (Dülger et al., 2012; Makridis et al., 2018; Busti et al., 2020).

2.4 Sampling

At the beginning and at the end of the feeding trial (day 80) fish were anaesthetised and individually weighed. Specific growth rate (SGR), feed intake (FI) and feed conversion ratio (FCR) were calculated. Proximate carcass composition was determined at the beginning of the trial on a pooled sample of 15 fish and on pooled samples of 5 fish tank^{-1} at the end of the trial. Protein efficiency ratio (PER), gross protein efficiency (GPE), lipid efficiency ratio (LER), and gross lipid efficiency (GLE) were calculated. At the end of the trial, condition factor (CF), viscerosomatic index (VSI), hepatosomatic index (HSI) and mesenteric fat index (MFI) were also obtained from 3 fish per tank. Concerning immunological parameters, at the beginning (T0, 9 fish in total), at the end of the feeding trial (T1, 6 animals' diet^{-1}) and at the end of the suboptimal rearing conditions (T2, 6 animals' diet^{-1}) fish were sampled to assess inflammatory and immune response gene expression of distal intestinal mucosa (1 cm before the rectum) (Busti et al., 2020; Pelusio et al., 2020; Parma et al., 2023). At the end of the feeding and suboptimal rearing conditions periods, samples of feces from distal intestine in 6 fish diet^{-1} were also individually collected and placed at −80°C for gut bacterial community characterisation (Parma et al., 2019). The experimental design scheme of the feeding trial is reported in Supplementary Figure 1. All experimental procedures were carried in accordance with European directive 2010/63/UE on the protection of animals used for scientific purposes.

2.5 Cytokines gene expression analyses by real–time polymerase chain reaction

Activation of local (intestine) immune and inflammatory response associated with different diets (FM level and NA/NT supplementation) has been assessed through transcript quantification of target genes in distal intestine. In particular genes of two inflammatory cytokines such as interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α) and two anti-inflammatory cytokines interleukin 10 (IL-10) and transforming growth factor β (TGF-β) were investigated. Total RNA was isolated from 20-30 mg of intestine samples stored in RNA Later (Sigma) using the NucleoSpin RNA extraction kit (Machery-Nagel) following the manufacturer's instructions. RNA quality was assessed by 260/280 ratio. The RNA extraction protocol includes a treatment with DNAse I in order to remove genomic DNA. The first strand of complementary DNA (cDNA) was synthesised by reverse transcription using the GoScript Reverse Transcriptase (Promega). cDNA concentration was quantified using a Qubit Fluorometer (ThermoFisher). Realtime PCR was performed with an ABI PRISM 7300 instrument (Applied Biosystems) using BRYT Green GoTaq qPCR (Promega). 10 ng of each cDNA sample was added to a reaction mix containing 2 × GoTaq qPCR Master Mix (Promega), 300 nM of CXR and 200 nM of each primer. The primers used for IL-1β, IL-10, TNF-α, TGF-β and the housekeeping S18 rRNA gene, are shown in Table 2. Reaction mixtures were incubated for 2 min at 95°C, followed by 50 cycles of 10 s at 95°C, 30 s at 60°C, and finally 15 s at 95°C, 1 min 60°C and 15 s at 95°C (dissociation stage). Before the experiments, the specificity of each primer pair was studied using positive and negative samples. A melting curve analysis of the amplified products validated the primers for specificity. After these verifications, all cDNA samples were analyzed in triplicate. Negative controls with no template and an internal control were always included in the reactions. For each sample, gene expression was normalized against S18 rRNA and

TABLE 2 Primer sequences used for gene expression analyses of immune genes.

Gene	Abbreviation	GenBank ID	Primer sequence (5′- 3′)	Amplicon (bp)	References
S18 rRNA	*18S*	AM490061	AGGGTGTTGGCAGACGTTAC CTTCTGCCTGTTGAGGAACC	170	Sepulcre et al. (2007)
Interleukin 1 β	*il-1β*	AJ311925	ATCTGGAGGTGGTGGACAAA AGGGTGCTGATGTTCAAACC	106	Sepulcre et al. (2007)
Interleukin 10	*il-10*	DQ821114	CGACCAGCTCAAGAGTGATG AGAGGCTGCATGGTTTCTGT	199	Sepulcre et al. (2007)
Tumor necrosis factor α	*tnf-α*	DQ200910	AGCCACAGGATCTGGAGCTA GTCCGCTTCTGTAGCTGTCC	112	Sepulcre et al. (2007)
Transforming growth factor β	*tgf-β*	AM421619	GACCTGGGATGGAAGTGGAT CAGCTGCTCCACCTTGTGTTG	225	Faliex et al. (2008)

expressed as 2-ΔΔCt, where ΔCt is determined by subtracting the S18 rRNA Ct value from the target Ct. Gene expression of animals fed control and experimental diets collected at the end of the feeding trial (T1) and after suboptimal rearing conditions (T2) were expressed as "fold changes" relative to control animals sampled at T1 and T2, respectively (Pelusio et al., 2020).

2.6 Gut bacterial community DNA extraction and sequencing

The DNA was extracted as described by De Cesare et al. (2017). Distal intestine content aliquots of 0.30 g were initially suspended in 1 mL lysis buffer (500 mM NaCl, 50 mM Tris-Cl, pH 8.0, 50 mM EDTA, 4% SDS) with MagNA Lyser Green Beads (Roche, Milan, Italy) and homogenized on the MagNA Lyser (Roche) at 6500 rpm for 25 s. Afterwards, samples were heated at 70°C for 15 min, followed by centrifugation to split the DNA from the bacterial cellular wreckage. This process was repeated with a second 300 µL aliquot of lysis buffer. The samples were then subjected to 10 M v/v ammonium acetate (Sigma, Milan, Italy) precipitation, followed by isopropanol (Sigma) precipitation and a 70% ethanol (Carlo Erba, Milan, Italy) wash and resuspended in 100 µL 1X Tris-EDTA (Sigma). The samples were then treated with DNase-free RNase (Roche) and incubated overnight at 4°C, before being processed through the QIAmp DNA Stool Mini Kit (Qiagen, Milan, Italy) according to the manufacturer's directions, with some modifications. Samples were measured on a BioSpectrometer (Eppendorf, Milan, Italy) to assess DNA quantity and quality. Libraries were prepared following the 16S Metagenomic Sequencing Library Preparation protocol (Illumina, San Diego, CA, USA), amplifying the V3 and V4 hypervariable regions of the 16S rRNA gene in order to obtain a single amplicon of approximately 460 bp. Sequencing was performed in paired-end employing MiSeq System (Illumina) with MiSeq Reagent kit v2 500 cycles (Illumina), characterised by a maximum output of 8.5 Gb.

2.7 Analytical methods

Chemical analyses were conducted according to Pelusio et al. (2021). Shortly, diets and fish carcass were analyzed for proximate composition. Moisture was determined by weight loss after drying samples in a stove at 105°C overnight. Crude protein was analysed as total nitrogen (N*6.25) using Kjeldahl's method according to the AOAC International (AOAC, 2010). Total lipids were measured according to Bligh and Dyer (1959) extraction method. Ash content was determined by incineration in a muffle oven at 450°C overnight.

2.8 Statistical and bioinformatic analysis

For statistical analysis a tank was used as the experimental unit to analyse growth, while pools of five individuals per tank were considered to describe CF, VSI, HSI. Growth, nutritional, and somatometric indices were analyzed by one-way ANOVA, followed by Tukey's multi-comparison test. Gene expression values have been expressed as mean of fish subjected to each treatment ± standard deviation (SD). Diet effect on gene expression was assessed by two-way ANOVA using NA/NT supplementation (S) and FM level as variables; then Tukey's *post hoc* test was performed. The normality and/or homogeneity of variance assumptions were validated for all data preceding ANOVA. Growth, nutritional and somatometric indices and gene expression data were analyzed using GraphPad Prism 9.0 for Windows (Graph Pad Software, San Diego, CA, USA).

Gut microbiome samples before and after suboptimal rearing conditions (T1 and T2) were analysed adopting a bioinformatic pipeline based on QIIME2 (http://qiime.org/). Raw sequences were processed with the dada2 algorithm in order to denoise and to merge forward and reverse sequences per each pair. The taxonomic classification of cleaned data was performed by applying the VSEARCH-based classifier implemented in QIIME and using the Greengenes 13_8 97% OUT dataset as reference. The mean values for the relative frequency of abundance of each taxonomic level in each sample were compared using the the two-way anova followed by the Dunnet *post hoc* analysis (pakages aov::stats and dunn_test:: rstatix respectively). P-values ≤ 0.05 were considered statistically significant. β-diversity was calculated using "vegdist" (vegan "package) and the PCoA analysis using "cmdscale" ("stats" package). Statistical differences were calculated using the "beta_pcoa_stat" ("amplicon"package).

3 Results

3.1 Growth, biometric indices and nutritional uptake

Data on growth performance, somatometric indices, proximate composition and nutritional indices are represented in Table 3. At the end of the growth period the FBW was higher in FM20NT and FM20NA compared to FM20. On WG the lowest level was displayed by FM20 animals, while FM10 was lower than FM20NA. Concerning SGR, FM20 were lower than FM20NT and FM20NA; at the same time FM20NA was higher than FM10. Regarding FCR, FM20 was higher than FM20NA. VFI of FM10 and FM20 fish levels were lower than FM10NT and FM20NT. No significant differences were detected among treatments for survival. Concerning somatometric indices, no significant differences occurred among treatments for CF, VSI, HSI and MFI. Regarding proximate composition, no significant difference was shown for crude protein while the highest crude lipid was measured in fish fed FM20NT. At the same time crude lipid in FM10NT was higher than FM10NA. Moisture was higher in FM10NA than FM10NT, FM10NT, FM20NT, and FM20NA. No significant differences were detected in ash content. Regarding nutritional indices, PER was higher in FM20NA compared to FM20, FM10NA, FM10NT,

10.3389/fmars.2023.1145660

TABLE 3 Growth performance, somatometric indices, proximate composition and nutritional indices of European sea bass fed the experimental diets over 80 days.

	Experimental diets						
	FM10	FM10NT	FM10NA	FM20	FM20NT	FM20NA	p-value
Growth performance							
IBW (g)	14.3 ± 0.08	14.5± 0.05	14.4 ± 0.04	14.2 ± 0.34	14.4 ± 0.15	14.2 ± 0.10	0.2397
FBW (g)	69.9 ± 3.12^{a}	72.3± 1.48^{abc}	71.7 ± 2.46^{ab}	66.1 ± 1.37^{a}	75.9 ± 2.55^{bc}	78.7 ± 3.12^{c}	0.0006
WG (g)	55.6 ± 3.19^{ab}	57.8 ± 1.51^{abc}	57.3 ± 2.49^{ab}	51.9 ± 1.47^{a}	61.5 ± 2.60^{bc}	64.5 ± 3.18^{c}	0.0008
SGR (% day^{-1})	1.98 ± 0.08^{ab}	2.01 ± 0.03^{abc}	2.01 ± 0.07^{abc}	1.93 ± 0.06^{a}	2.08 ± 0.07^{bc}	2.14 ± 0.04^{c}	0.0022
FCR	1.24 ± 0.07^{ab}	1.29 ± 0.02^{ab}	1.30 ± 0.08^{ab}	1.33 ± 0.05^{b}	1.21 ± 0.04^{ab}	1.15 ± 0.05^{a}	0.019
VFI (g kg ABW^{-1} day^{-1})	67.1 ± 2.90^{a}	74.9 ± 1.14^{b}	73.7 ± 1.82^{ab}	67.0 ± 3.08^{a}	74.5 ± 2.90^{b}	72.1 ± 2.56^{ab}	0.0043
Survival (%)	94.4 ± 0.96	98.3 ± 2.89	98.3 ± 1.67	97.2 ± 1.92	99.4 ± 0.96	95.0 ± 5.00	0.2020
Somatometric indices							
CF	1.29 ± 0.06	1.37 ± 0.21	1.23 ± 0.09	1.20 ± 0.06	1.23 ± 0.06	1.30 ± 0.09	0.2336
VSI (%)	12.51 ± 0.85	13.36 ± 3.20	12.07 ± 0.48	12.37 ± 0.49	12.92 ± 0.95	12.73 ± 0.96	0.8964
HSI (%)	2.19 ± 0.12	2.37 ± 0.09	2.17 ± 0.12	2.40 ± 0.04	2.37 ± 0.18	2.40 ± 0.16	0.2284
MFI (%)	7.14 ± 0.47	7.26 ± 1.76	7.84 ± 0.70	6.81 ± 0.76	7.34 ± 1.12	7.26 ± 1.76	0.8586
Proximate composition (%)							
Crude protein	17.20 ± 0.14	17.10 ± 0.30	17.15 ± 0.49	17.23 ± 0.22	16.97 ± 0.14	17.33 ± 0.24	0.3322
Crude lipid	16.44 ± 0.64^{ab}	17.75 ± 0.34^{b}	16.24 ± 1.35^{a}	16.61 ± 0.23^{ab}	19.17 ± 0.46^{c}	17.51 ± 0.67^{ab}	<0.0001
Ash	3.76 ± 0.06	4.04 ± 0.41	3.77 ± 0.40	3.91 ± 0.23	3.68 ± 0.46	3.55 ± 0.30	0.2644
Moisture	62.04 ± 0.69^{bc}	60.81 ± 0.30^{ab}	63.33 ± 1.63^{c}	61.90 ± 0.55^{bc}	60.02 ± 0.98^{a}	61.44 ± 0.70^{ab}	<0.0001
Nutritional indices							
PER	1.84 ± 0.10^{ab}	1.70 ± 0.07^{a}	1.71 ± 0.08^{a}	1.71 ± 0.07^{a}	1.85 ± 0.10^{ab}	1.98 ± 0.06^{b}	0.0062
GPE (%)	31.61 ± 2.06^{ab}	29.07 ± 1.85^{a}	29.21 ± 2.26^{a}	29.53 ± 1.71^{ab}	31.24 ± 1.59^{ab}	34.40 ± 1.54^{b}	0.0318
LER	4.69 ± 0.26^{a}	4.38 ± 0.18^{a}	4.43 ± 0.20^{a}	4.42 ± 0.18^{a}	4.73 ± 0.24^{a}	5.35 ± 0.16^{b}	0.0008
GLE (%)	87.53 ± 3.65^{ab}	88.78 ± 4.78^{abc}	81.65 ± 10.5^{a}	84.06 ± 2.14^{a}	103.45 ± 7.36^{bc}	105.19 ± 6.29^{c}	0.0022

In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).
Growth performances, proximate composition and nutritional indices are given as the tanks mean (n =3) ± SD. Somatometric indices are given as the mean (n=15) ± SD. IBW Initial body weight, FBW Final body weight, WG Weight gain, SGR Specific growth rate = 100 * (ln FBW- ln IBW)/days, FCR Feed conversion rate = feed intake/weight gain, VFI Voluntary feed intake = g feed/fish, CF Condition factor = 100 * (FBW/length^{3}), VSI Viscerosomatic index = 100 * (viscera weight/FBW), HSI Hepatosomatic index = 100 * (liver weight/FBW), MFI Mesenteric fat index = 100* (mesenteric fat/FBW), PER Protein efficiency rate = (FBW – IBW)/total protein intake, GPE Gross protein efficiency = 100 * [(% final body protein content * FBW) – (% initial body protein * IBW)]/total protein intake fish, LER Lipid efficiency rate = (FBW – IBW)/total lipid intake, GLE Gross lipid efficiency = 100 * [(% final body lipid content * FBW) – (% initial body lipid * IBW)]/ total lipid intake fish, SD = standard deviation.

and FM10. GPE was higher in FM20NA compared to FM10NA and FM10NT. Concerning lipid efficiency, LER was higher in FM20NA compared to the other treatment while GLE was higher in FM20NA than FM20, FM10NA, and FM10.

3.2 Cytokines gene expression

The gene expression of four genes involved in the immune and inflammatory response are presented in Figure 1. Significant effect of FM level was found for IL-1 β and TGF-β at the end of the feeding trial (T1). Moreover, a significant interaction between FM level and NA/NT supplementation (FMxS) was observed for TGF-β at the end of the feeding trial (T1). In particular at this time point (T1) the IL-1β was upregulated in animals fed with FM10 diet supplemented with NA or NT compared to FM10 control diet with a significant increase in FM10NA group. Conversely, the TGF-β was downregulated in FM10NA and FM10NT compared to FM10 with a significant decrease in FM10NT group. In FM20 diets supplemented with NA/NT IL-1β was not upregulated and resulted less expressed compared to NA/NT supplemented FM10. Furthermore, a significant difference was observed for TGF-β between FM10NA/NT and FM20NA/NT groups: animals fed FM20NA/NT showed significantly higher expression than those

10.3389/fmars.2023.1145660

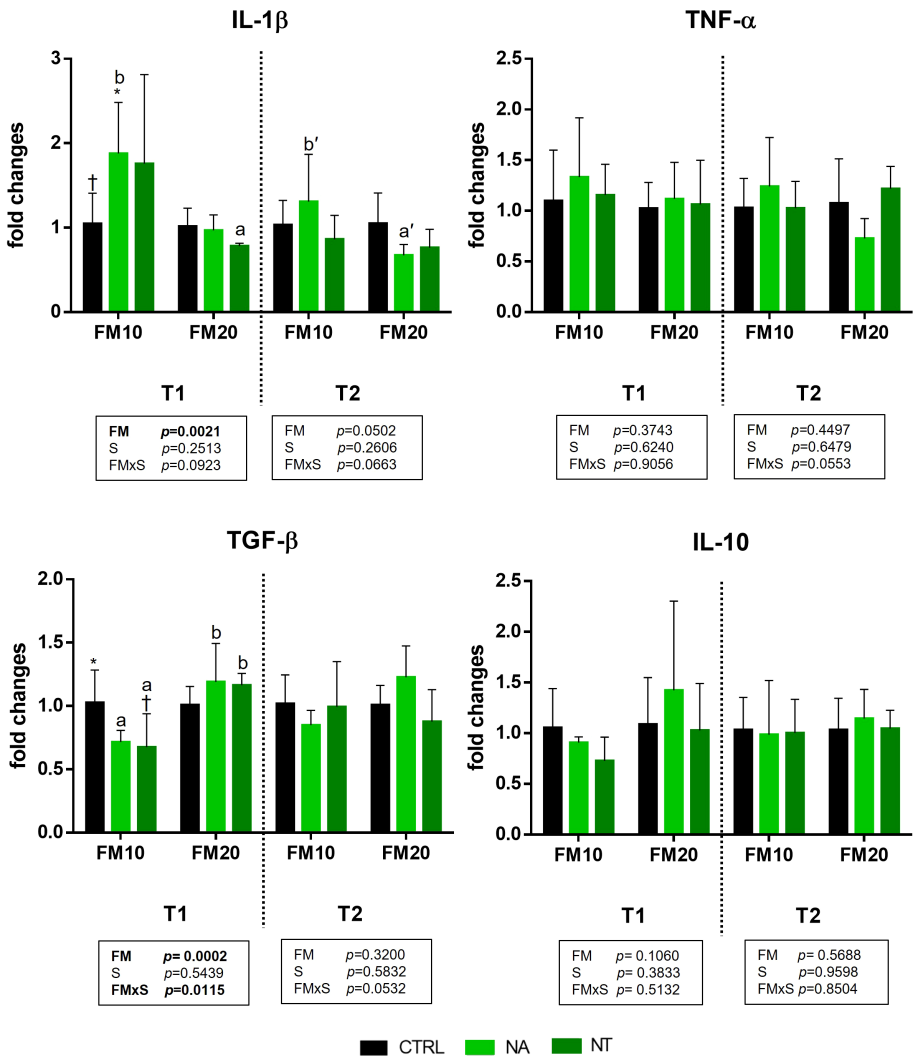

FIGURE 1
Immune and inflammatory cytokine gene expression in intestinal mucosa of European seabass fed with feed additives (nucleotides and nucleic acids) at two different fish meal levels over 80 days (T1) and after suboptimal rearing condition period (T2). Data are given as 6 individuals per diet. In each graph, significance is attributed to P ≤ 0.05. FM= fish meal level (10% and 20%) with inclusion of nucleotides (NT) or nucleic acids (NA) in diet at 500 ppm. IL-1β = Interleukin 1β; TNFα = Tumor necrosis factor α; TGFβ = Transforming growth factor β; IL-10 = Interleukin 10. Different symbols stand for significant differences among dietary treatments for the same FM level. Different lowercase letters (a/b, T1; a′/b′ T2) stand for significant differences between different FM levels.

fed FM10NA/NT. After suboptimal rearing condition (T2), no significant differences were observed among experimental groups by two-way ANOVA analysis. However, the *post hoc* test showed a significant upregulation of IL-1β in FM10 diet fed animals compared to the FM20 group.

3.3 Gut bacterial community

The 16S rRNA gene sequencing was performed on a total of 36 gut contents per each considered timepoint (T1 and T2) to assess how NT or NA dietary inclusion within 10% or 20% FM formulation levels could affect gut bacteria communities and consequently gut health of European sea bass before or after suboptimal rearing conditions. To this aim, samples were evaluated at different phylogenetic levels of phylum (Figure 2), family (Figure 3) and genus (Figure 4), respectively.

At phylum level, the most abundant taxa observed at both considered timepoints were Firmicutes (51.8% at T1, 52.5% at T2), Proteobacteria (20.5% at T1, 20.6% at T2), Cyanobacteria (17.5% at T1, 12.5% at T2), Actinobacteria (5.3% at T1, 7.2% at T2) and Bacteroidetes (3.3% at T1, 5.6% at T2) (Figure 2). The most dominating families were represented by *Leuconostocaceae* (22.2% at T1, 6.2% at T2), *Clostridiaceae* (4.6% at T1, 6.5% at T2), *Streptococcaceae* (5.5% at T1, 16.3% at T2), and *Lactobacillaceae* (3.8% at T1, 5.5% at T2) (Figure 3). At the end of the feeding trial the genus level abundance was mostly represented by *Weissella* (13.1% at T1, 4% at T2), followed by *Leuconostoc* (9.1% at T1), *Streptococcus*

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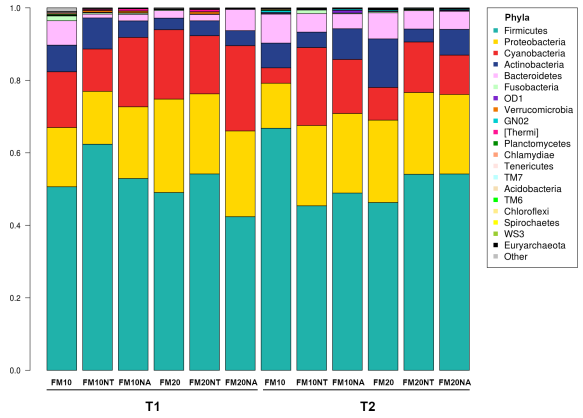

FIGURE 2
Microbiota composition of distal gut content of European seabass fed feed additives (nucleotides and nucleic acids) at two different fish meal levels over 80 days (T1) and after suboptimal rearing condition period (T2). Bar plot summarizing the microbiota composition at Phylum level of fish intestinal content. FM10 = fish meal 10% diet level; FM20 = fish meal 20% diet level; NT = 500 ppm nucleotide feed inclusion; NA = 500 ppm nucleic acid feed inclusion.

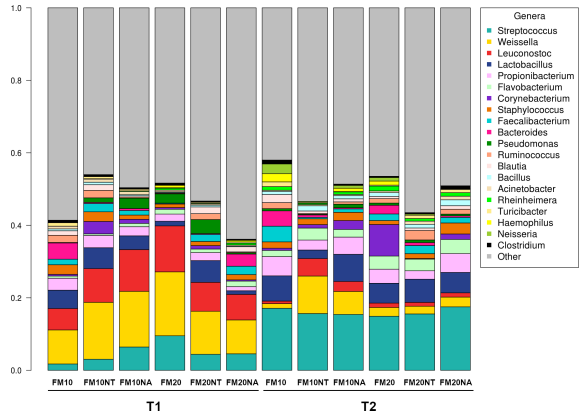

FIGURE 4
Microbiota composition of distal gut content of European seabass fed feed additives (nucleotides and nucleic acids) at two different fish meal levels over 80 days (T1) and after suboptimal rearing condition period (T2). Bar plot summarizing the microbiota composition at Genus level of fish intestinal content. FM10 = fish meal 10% diet level; FM20 = fish meal 20% diet level; NT = 500 ppm nucleotide feed inclusion; NA = 500 ppm nucleic acid feed inclusion.

(5% at T1, 16.1% at T2), *Lactobacillus* (3.8% at T1, 5.5% at T2), and *Propionibacterium* (2.3% at T1, 4% at T2). (Figure 4).

In Figures 5, 6 are reported the most represented taxa at family and genus level (mean rel. ab. > 1%) showing significant differences among treatments at T1 and T2. Supplementary Figures 2-4 reports differences among treatments of minor represented taxa (mean rel. ab. < 1%).

At T1, *Corynebacteriaceae* was significantly affected by S and FM level, with a significant interaction of SxFM level. In particular, *Corynebacteriaceae* abundance was higher in FM10NT compared to FM10, FM10NA and FM20NT. At T2, *Leuconostocaceae* abundance was significantly affected by S and FM level, with a significant interaction of SxFM level. In particular *Leuconostocaceae* abundance was higher in FM10NT than FM10 and higher in FM10NT than FM20NT. At the same time a significant interaction FMxS affected *Clostridiaceae* abundance which was lower in FM10NT than FM10. *Paenibacillaceae* abundance was affected by S, showing higher values in FM20NA compared to FM20 (Figure 5).

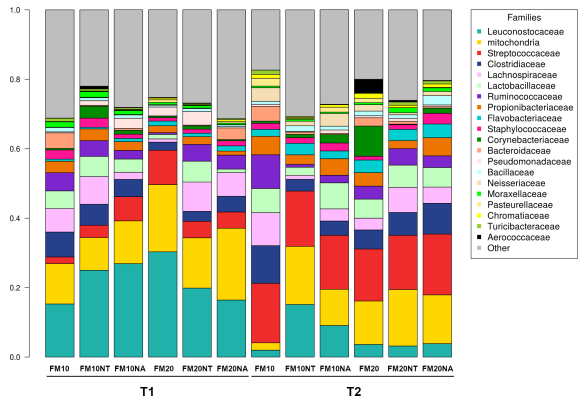

FIGURE 3
Microbiota composition of distal gut content of European seabass fed feed additives (nucleotides and nucleic acids) at two different fish meal levels over 80 days (T1) and after suboptimal rearing condition period (T2). Bar plot summarizing the microbiota composition at Family level of fish intestinal content. FM10 = fish meal 10% diet level; FM20 = fish meal 20% diet level; NT = 500 ppm nucleotide feed inclusion; NA = 500 ppm nucleic acid feed inclusion.

At T1 a significant interaction SxFM level affected *Weissella* abundance which was higher in FM10NT than FM10. At the same time, *Corynebacterium* was significantly affected by S and FM level, with a significant interaction of SxFM level. Specifically, *Corynebacterium* abundance was higher in FM10NT compared to FM10, FM10NA and FM20NT. At T2, *Weissella* and *Leuconostoc* abundances were significantly affected by S and FM level, with a significant interaction of SxFM level. Specifically, both taxa were higher in FM10NT than FM10 and FM20NT, while *Weissella* abundance was also higher in FM10NA than FM10. At the same time *Bacillus* abundance tended to increase in FM10NT compared to FM10, while *Clostridium* displayed an opposite trend with lower values in FM10NT than FM10 (Figure 6).

At each timepoint (T1 and T2), internal α-diversity gut microbiome was accessed by Shannon, Simpson and Fischer metrics at phylum, family and genus levels (Figure 7) while β-diversity is reported in Supplementary Figure 5. At T1 no significant differences at phylum level were detected while at T2 all the indexes showed a significant FMxS interaction with lower value in FM20NT than FM10NT (Figures 7A–C). At family level, alpha diversity metrics displayed at T1 a significant FM effect and FMxS interaction for Shannon and Simpson indexes, while a significant FM effect was detected in Fisher index. All the alpha diversity indexes at T1 showed at family level higher values in FM10 than FM20 without a significant effect of NT-NA supplementation (Figures 7D-F). At genus level, both Shannon and Simpson indexes displayed at T1 a significant FM and S effects, while a FM

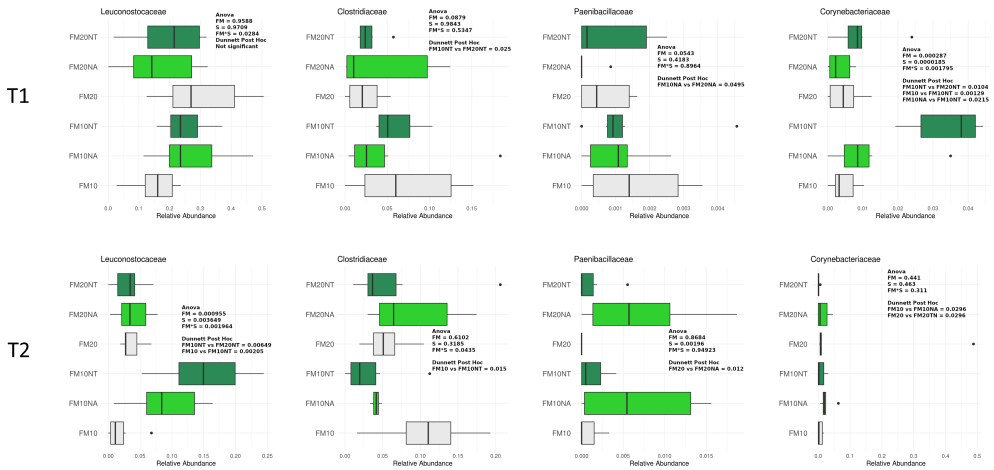

FIGURE 5
Taxonomic composition of bacterial communities of distal gut content of European seabass fed feed additives (nucleotides and nucleic acids) at two different fish meal levels over 80 days (T1) and after suboptimal rearing condition period (T2). Distributions of relative abundance of family that showed a significant variation between groups fed with different diets (two-way ANOVA, FM= fish meal level; S=feed supplemented with nucleotide, NT or nucleic acid, NA). Only the most abundant families with a mean relative abundance >1% in all groups were represented. FM10 = fish meal 10% diet level; FM20 = fish meal 20% diet level; NT = 500 ppm nucleotide feed inclusion; NA = 500 ppm nucleic acid feed inclusion.

effect was observed for Fisher index. Specifically, FM20NT displayed higher values compared to FM20 for all the alpha diversity metrics analysed at T1, while at T1 no differences were detected (Figures 7G-I). Significant separation in the principal coordinate analyses was mostly detected at T2 in FM10 *vs* FM10NT (Supplementary Figure 5).

4 Discussion

Since exogenous NT and NA supply have attracted attention in the last two decades, the present study is an attempt to fulfil some of the gaps in aquaculture research. Our results showed that 500 ppm of NT enhanced feed intake in 10 and 20% FM diet. Whether used as NA or NT, composition of nucleotides and their molecular form is crucial, since some compounds can stand as feed enhancer or feed intake inhibitors like free-adenine (Rumsey et al., 1992). The palatability mechanism is still not well known; however free 5'-NT are known as powerful gustatory stimulants on their own (Morais, 2017). Fish gustatory receptors (taste buds) have been found to have different positions on body surface among species and also attractiveness to different NT molecules is species-specific (Morais, 2017). For example, jack mackerel displayed appetite for IMP, GMP, UMP, UDP, UTP, while it was not attracted by nucleosides such as inosine, adenosine, guanosine and uridine, or other NT (AMP, ADP, ATP, IDP, GDP, xanthosine 5V-

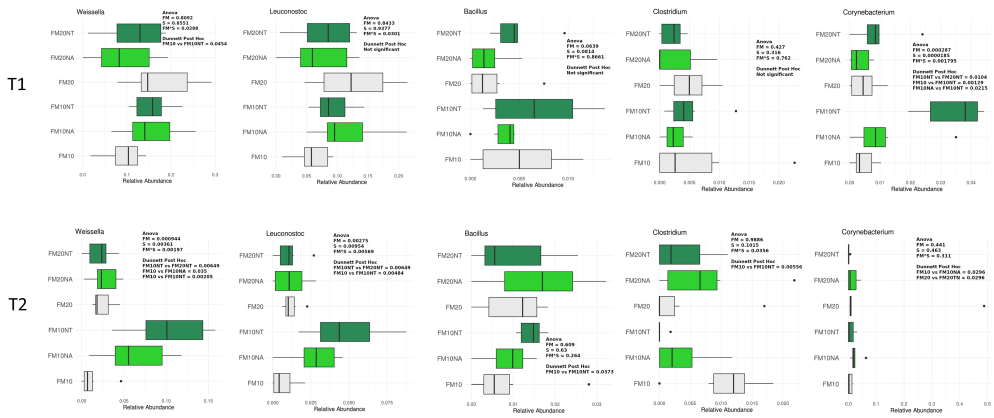

FIGURE 6
Taxonomic composition of bacterial communities of distal gut content of European seabass fed feed additives (nucleotides and nucleic acids) at two different fish meal levels over 80 days (T1) and after suboptimal rearing condition period (T2). Distributions of relative abundance of genus that showed a significant variation between groups fed with different diets (two-way ANOVA, FM= fish meal level; S=feed supplemented with nucleotide, NT or nucleic acid, NA), only most abundant genus with a mean relative abundance >1% in all groups were represented. FM10 = fish meal 10% diet level; FM20 = fish meal 20% diet level; NT = 500 ppm nucleotide feed inclusion; NA = 500 ppm nucleic acid feed inclusion.

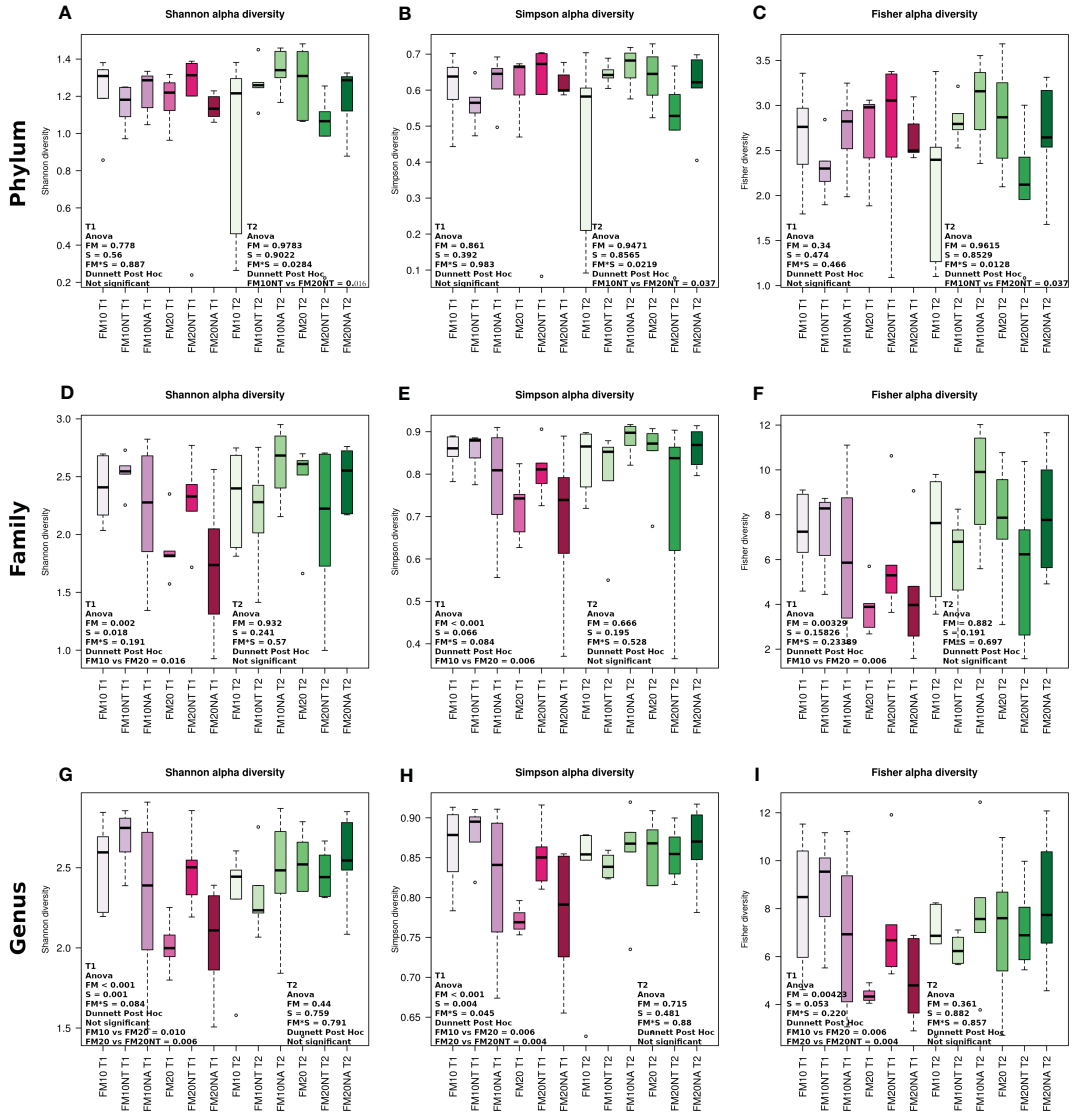

FIGURE 7
Alpha diversity of bacterial communities of distal gut content of European seabass fed feed additives (nucleotides and nucleic acids) at two different fish meal levels over 80 days (T1) and after suboptimal rearing condition period (T2). Boxplots show alpha diversity values measured by Shannon diversity, Simpson diversity and Fisher diversity at phylum **(A–C)**, family **(D–F)** and genus **(G–I)** level Simpson diversity and Fisher diversity. Differences were detected by two-way anova (FM= fish meal level; S=feed supplemented with nucleotide, NT or nucleic acid, NA). FM10 = fish meal 10% diet level; FM20 = fish meal 20% diet level; NT = 500 ppm nucleotide feed inclusion; NA = 500 ppm nucleic acid feed inclusion.

monophosphate, 3VIMP, 3V-UMP, 2-deoxy-IMP, allyltio-IMP), (Ikeda et al., 1991). In largemouth bass dietary IMP supply (2800 mg kg^{-1}) displayed an FI increase of 46% to soybean meal-based diet without additive, but FI of animals fed both IMP dose diets (2800 or 5600 mg kg^{-1}) was lower than fish fed 10% FM diet (Kubitza et al., 1997). The authors explained that probably FM is a relatively high source of IMP, therefore, the beneficial influence of IMP supplementation generally is not noticeable when fish meal is added to aquafeed formulations (Li and Gatlin, 2006). Concerning NA palatability effect, similar observations were reported by Peres and Oliva-Teles (2003), where low and high protein diets (39% and 50%, with 62.1% FM and 46.6% FM, respectively) supplied with yeast-RNA at 6.2% and 12.4% increased feed intake in European sea bass. Accordingly, the inclusion of 5.8% RNA extract combined with 44.4% FM led to a significantly higher FI and growth in gilthead sea bream (Oliva-Teles et al., 2006). On the contrary, RNA extract included at dietary levels of 3-6% in rainbow trout or 4-8% in turbot had no significant effect on FI (Fournier et al., 2002). In the present study, FCR was consistently reduced by 25.6% in FM20NA compared to FM20 control diet. Consequently, SGR of FM20 met a major enhancement by 11.0% in FM20NA diet. These findings are consistent with Magouz et al. (2021), where sea bass specimens fed increasing doses of NT blend (500, 1000, 1500 mg kg^{-1}) had improved SGR, especially at 500 mg kg^{-1}. On the same species another recent study testing diets containing 0%, 0.15% and 0.3% inclusion of a NT mixture found a positive effect on growth (Bowyer et al., 2019). However, rainbow trout fed 0, 8, 16 and 24 mg kg $diet^{-1}$ of same NT

blend supplied in diets with low FM levels of 0% and 5% were found to have a lesser growth and feed efficiency compared to a control group fed 25% FM without NT inclusion (Liu, 2016). Similar growth and feed efficiency improvements were also found in red drum *Sciaenops ocellatus* (Cheng et al., 2011), greater amberjack *Seriola dumerili* (Hossain and Koshio, 2017a), red sea bream *Pagrus major* (Hossain et al., 2016; Hossain and Koshio, 2017a), zebrafish *Danio rerio* (Guo et al., 2017), turbot *Scophtalmus maximus* (Peng et al., 2013), and gilthead sea bream *Sparus aurata* (Peres and Oliva-Teles, 2003; El-Nokrashy et al., 2021). In our study, the synergic growth and feed intake improvements of animals fed FM20NT may reveal a satisfied NT exogenous requirement for the considered life stage of this species. This hypothesis might confirm that exogenous supply of NT may promote growth of juvenile fish meeting their high rate of cell replication, especially when dietary FM is heavily limited (Li and Gatlin, 2006). In addition, the differences in feed and nutrient efficiency observed at the different FM levels may indicate that the requirements are met when 20% of FM is included, thus indicating that a possible higher NT/NA inclusion level should be considered for dietary FM level of 10%. Although both NT and NA at 20% FM promoted the overall growth, it seemed that NT had a greater role in enhancing feed palatability, while NA promoted feed efficiency. Though no significant differences occurred, HSI values were found to be in alignment with previous studies on same species and size (Peres and Oliva-Teles, 2003; Busti et al., 2020; Magouz et al., 2021). The liver size is an indicator of the nutritional status of fish (Hossain and Koshio, 2017a). A study on rainbow trout reported that dietary NT tended to change liver status of fish fed low FM diet by decreasing HSI and plasma alanine aminotransferase (ALT) activity (Ridwanudin et al., 2019). The increase of HSI was attributed as a biomarker of proper nutrient storage and health when red sea bream was fed diets with added UMP (Hossain and Koshio, 2017a). NA and NT diet showed the highest lipid efficiency when added to 20% FM. These outcomes are in contrast with findings described in European sea bass (Magouz et al., 2021 and Bowyer et al., 2019) and gilthead sea bream (Oliva-Teles et al., 2006). However, according to the present results, increased body lipid content was described in red drum juveniles fed a blend of NT supplemented diets (Li et al., 2004). The increase in carcass lipid content may indicate the involvement of NT in lipid metabolism or nutrient uptake at enterocytes level as stated by Li et al. (2015).

The fish intestinal surface represents the first line of defense against potentially harmful exogenous factors such as pathogens, antinutrients and environmental stressors; and eu- or dis-byosis assessment of gut microbiome (GM), and expression of innate immune genes changes are key actors of gut-health status (Busti et al., 2020). As immune biomarkers, cytokines are low molecular weight glycoproteins involved in the regulation of immune response, and can be elicited by immunostimulants (Zou and Secombes, 2016). They are mainly secreted by cells of both the innate and acquired immune system in response to microbial invasion and tissue injury, and act as signal cell messengers via specific-receptors on target cell surfaces, thus inducing physiological response (Sakai and Kono, 2021). When inflammatory response occurs, tumor necrosis factor alpha (TNF-α) secretion sparks the cytokine cascade with IL-1β followed by the release of a myriad of other molecules and chemoattractant to promote neutrophil and macrophage migration in loco, acting as pro-inflammatory markers (Pelusio et al., 2020). Transforming growth factor-β (TGF-β) is one of the anti-inflammatory cytokines that depicts a down-regulated effect on the expression of numerous cytokines and is associated with the deactivation of macrophages. Interleukin-10 (IL-10) is another anti-inflammatory cytokine that inhibits bacterial lipopolysaccharide (LPS) affecting pro-inflammatory cytokines (Reda et al., 2018). In the present study the gene expression analysis showed a significant effect of diets on the activation of local immune response on some intestinal cytokines. In fish fed FM10 groups an upregulation of the proinflammatory cytokine IL-1β and a concurrent downregulation of the anti-inflammatory TGF-β gene was observed prominently for fish fed the NA/NT supplemented diets signaling an activation of the immune response. Previous studies showed the upregulation of cytokines as an effect associated to nucleotide supplementation in turbot (*Scophthalmus maximus*) and Nile tilapia (*Oreochromis niloticus*) (Reda et al., 2018). NT enhanced the expression of immune genes from different organs involved in nonspecific defenses of fish like gills, kidney and intestine (Hossain et al., 2020). In particular, mRNA levels of intestinal cytokines including IL-1β, IL-10, TNF-α and TGF-β were significantly higher in Nile tilapia fed dietary NT (Reda et al., 2018). A later study on guanidinoacetic acid supplementation in the same species showed a different effect on liver proinflammatory and anti-inflammatory cytokines (Aziza and El-Wahab, 2019). In particular, IL-1β and TGF-β1 mRNA level were respectively downregulated and upregulated in Nile tilapia fed guanidinoacetic acid supplemented diet for 60 days. Similarly, anti-inflammatory effect was observed in zebrafish (*Danio rerio*) fed a diet supplemented with RNA yeast extract; in this case proinflammatory cytokines such as IL-1, IL-8 and TNF-α were downregulated, whereas the anti-inflammatory cytokine IL-10 was upregulated or not modulated depending on the growth stage of the investigated larvae (Falcinelli et al., 2018). In our study, we observed opposite expression trends of IL-1β and TGF-β in accordance to their pro- and anti-inflammatory roles respectively. As a matter of fact, in NA/NT supplemented FM10 diets an upregulation of the pro-inflammatory cytokine and a downregulation of the anti-inflammatory cytokine was observed. Moreover, European sea bass TGF- β modulation seems affected mainly by the FM and supplementation interaction. In effect, this anti-inflammatory cytokine was significantly more expressed in animals fed FM20NT and FM20NA than those fed with FM10NA and FM10NT at the end of the feeding trial. Actually, the combination of high FM diet and NA/NT supplementation showed the highest expression of TGF-β gene, depicting an increased ability to regulate inflammatory process. The upregulation of anti-inflammatory cytokines such as TGF-β could also contribute to protect the epithelial integrity decreasing intestinal epithelial permeability (Busti et al., 2020). On the other hand, IL-1β expression was affected only by FM level. Moreover, the NA/NT supplementation seems not to affect the gene expression after the suboptimal rearing conditions. Indeed, the two-way ANOVA

analysis showed no significant influence of diets. However, a FM-associated effect was observed for IL-1β expression levels limited to NA supplemented animals at this time point with the FM20NA group showing the lowest IL-1β expression.

Though many studies have turned their attention on NT and NA, to the best of our knowledge the present study is the first to investigate their effects on gut microbiota on this species. A different response in the microbial internal diversity was observed. At the end of the growing period, NT increased internal diversity at genus level when included in 20% FM diet. Increase in GM diversity may have positive implications for gut health due to increased competition against opportunistic pathogens (Parma et al., 2020; Moroni et al., 2021). In addition, a wider range of bacteria supported by a higher diversity may promote a more diverse number of host functions (Solé-Jiménez et al., 2021). In accordance, an increase in gut microbiome alpha diversity was also detected in gilthead sea bream fed 20% inclusion of bacterial single cell protein (Marchi et al., 2023).

At phylum level the gut bacterial community of sea bass was mainly represented by Firmicutes followed by Proteobacteria, Cyanobacteria and Actinobacteria, findings which are consistent with previous works on the same species (Parma et al., 2019; Busti et al., 2020). After the suboptimal environmental condition NT and NA added to 10% FM diet were able to promote beneficial taxa such as *Weissela* and *Leuconostoc*, when both taxa displayed a general decreasing trend. At this time a significant separation in the PCoA analyses also indicated an overall impact of NT and NA supplemented diet on the gut microbiome layout. These lactic acid bacteria genera are well known to have positive influence on host gut intestinal health in fish species. For instance, *Leuconostoc* has been described to be able to produce bacteriocins tolerant to high thermal stress and wide range of pH, which form pores in the target membrane of eventual bacteria pathogens (Ringo et al., 2018). Beneficial probiotic effects reported on *Weissella* in aquaculture have been associated with intestinal histology improvement by increasing height, width, number and mucosal productivity of villi and has been proposed for its application as a probiotic in aquaculture (Ringo et al., 2018; Pelusio et al., 2021). A similar overall decrease of LAB species after suboptimal rearing conditions (high temperature and low oxygen) was also described in previous studies (Busti et al., 2020) on European sea bass. In aquaculture species, LAB feed supplementation or their proliferation in GM has been attributed to positive outputs such as improved performance (LeBlanc et al., 2017; Xia et al., 2020), immune response and disease resistance, though their action mechanisms are still poorly understood (Ringo et al., 2018; Sun et al., 2022). After suboptimal condition, dietary NT was also able to increase *Bacillus* abundance in low fish meal diet. *Bacillus* is a well-known probiotics taxon in fish species and recently its abundance was found to significantly increase in gilthead sea bream fed bacterial single cell protein, raw material which are rich in nucleic acid (Marchi et al., 2023). In addition, after the suboptimal environmental condition, dietary nucleotides inclusion at 10% FM was also able to counteract *Clostridiaceae* abundance compared to its control group. The growth of this anaerobic bacteria family was observed under similarly high temperature and low oxygen; conditions that were able to negatively affect the overall GM structure of the same species (Busti et al., 2020).

5 Conclusions

NT and NA feed inclusion at 500 ppm enhanced voluntary feed intake when included in vegetable-based diet containing 10% and 20% dietary FM. A further improvement of feed efficiency was displayed in fish fed FM20 which overall led to the highest growth performance parameters. NT and NA combined with FM20 improved lipid efficiency. Furthermore, NT and NA combined with FM20 had also positive impact on intestinal mucosa by increasing anti-inflammatory TGF-β and decreasing pro-inflammatory IL-1 β, possibly translated in an improved gut-health functionality with a consequent improved nutritional uptake and growth performance. On gut microbiome, NT and NA when included in 10% FM diet were able to exert prebiotic properties stimulating the development of beneficial bacteria taxa such as *Weissella* and *Leuconostoc* when fish underwent suboptimal elevated temperature and low oxygen levels.

In conclusion, the combination of NT/NA included at 500 ppm was able to promote growth and lipid efficiency when included in 20% FM level, assuming higher nutritional NT requirement when combined with 10% FM. On the other hand, NT/NA added in 10% FM upregulate proinflammatory IL-1β and downregulate anti-inflammatory TGF- β intestinal level and favour beneficial gut bacterial taxa after the exposure to high temperature and low oxygen.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: BioProject, PRJNA933537.

Author contributions

AB, LP, PC, OM, PPG, conceived and designed the experiment. NFP, LP, AB, EV, SC, FE wrote the first draft of the manuscript. NFP, SN, conducted the fish rearing and sampling. SC, EV, FE performed cytokine gene expression analyses, ADC, VI carried out microbiota and bioinformatic analysis. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author PC is employed by Prosol S.p.A.

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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1145660/full#supplementary-material

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Microbiome analysis reveals potential for modulation of gut microbiota through polysaccharide-based prebiotic feeding in *Oreochromis niloticus* (Linnaeus, 1758)

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Characterization and functional profiling of the gut microbiota are essential for guiding nutritional interventions in fish and achieving favorable host-microbe interactions. Thus, we conducted a 30 days study to explore and document the gut microbial community of *O. niloticus*, as well as to evaluate the effects of a polysaccharide-based prebiotics with 0.5% and 0.75% Aloe vera extract on the gut microbiome through genomic analysis. The V3–V4 region of 16S rRNA was amplified and sequenced using Illumina HiSeq 2500, resulting in 1,000,199 reads for operational taxonomic unit (OTU) identification. Out of 8,894 OTUs, 1,181 were selected for further analysis. Our results revealed that Planctomycetes, Firmicutes, Proteobacteria, Verrucomicrobia, Actinobacteria, and Fusobacteria were the dominant phyla in both control and treatment samples. Higher doses of prebiotics were found to improve Planctomycetes and Firmicutes while decreasing Proteobacteria and Verrucomicrobia. We observed increasing trends in the abundance of Bacilli, Bacillaceae, and *Bacillus* bacteria at the class, family, and genus levels, respectively, in a dose-dependent manner. These findings were consistent with the conventional colony count data, which showed a higher prevalence of *Bacillus* in prebiotic-supplemented groups. Moreover, predicted functional analysis using PICRUSt indicated a dose-dependent upregulation in glycolysis V, superpathway of glycol metabolism and degradation, glucose and xylose degradation, glycolysis II, and sulfoglycolysis pathways. Most of the energy, protein, and amino acid synthesis pathways were upregulated only at lower doses of prebiotic treatment. Our findings suggest that the gut microbiome of *O. niloticus* can be optimized through nutritional interventions with plant-based polysaccharides for improved growth performance in commercial fish.

Abbreviations: OTU, Operational Taxonomic Unit; cfu, Colony forming unit; GIFT, Genetically Improved Farmed Tilapia; GALT, Gut associated lymphoid tissue; HSD, Honest significant difference; BHT, Butylated hydroxytoulene.

KEYWORDS

Fish, *Oreochromis niloticus*, gut, microbiome, prebiotics, plant polysaccharides

1 Introduction

The gut microbiota comprises a diverse group of microbes that inhabit the host's intestinal environment, thereby influencing host physiology (Feng et al., 2018). In terrestrial and aquatic vertebrates, the microbial ecosystem of the gastrointestinal tract involves host-microbe and microbe-microbe relationships that support digestion, nutrition, and health (Viney and Riley, 2014; Wang et al., 2017). The gut microbiota is crucial for gut epithelium development, enzymatic functioning, nutrient supply, and immune system stimulation (Nayak, 2010), and it is known as an extra organ due to its critical role in host growth and health (Feng et al., 2018). Dysbiosis in the gastrointestinal (GI) micro-ecosystem can lead to digestive enzyme dysfunction, gut epithelium damage, and penetration of pathogens and toxins from the lumen (Chassaing et al., 2015; Zhou et al., 2015). Researchers have become interested in the relationship between changes in the micro-ecosystem and various health problems (Qin et al., 2015). In recent years, researchers have explored the significant role of gut microbial communities in maintaining host homeostasis, growth, and disease resistance in fish. Nutritional technologies such as feed additives, probiotics, and prebiotics have been used to modulate gut flora (Hoseinifar et al., 2019; Peng et al., 2020). Probiotic supplemented diet was successfully used to achieve optimum growth performance through modulation of gut microbial community and improved digestive enzyme activity in *Labeo rohita* (Ghori et al., 2022). Use of better quality prebiotics alone or in combination with suitable probiotics have marked impact on microbiota in gut ecosystem that influence gut function and health of Atlantic salmon (Dhanasiri et al., 2023). Understanding the composition and structure of the gut microbiota and their effects on host growth and health is crucial for maintaining metabolic stability. Modulating the gut microbiota can improve host-microbe interaction and serve as a therapeutic strategy for metabolic disorders (Ojeda et al., 2016).

In this context, prebiotic substances are noteworthy as they do not directly contribute to any new flora, rather, they create a congenial environment for the favorable flora to flourish. Tilapias (*Oreochromis niloticus*) are excellent and cheap sources of animal protein worldwide. Their mode of reproduction, growth, and stress tolerance made them an ideal fish species to be intensively cultured both in enclosed and open waters (Tarnecki et al., 2017; Nicholson et al., 2019). Therefore, considering the economic importance of fish in the aquaculture industry, the functional profiling of the gut microbiota of tilapia is of utmost significance. This will help to modulate the supplements added to the feed with reduced mortality and higher yield which can promote the growth of beneficial bacteria and combat the harmful bacteria colonizing the fish gut (Ghanbari et al., 2015).

However, the exact mechanism of gut microbiota modulation by prebiotics is poorly understood in *O. niloticus* even though a few studies are available (Hasan and Banerjee, 2020; Souza et al., 2020). Of late, researchers have employed comparative microbiome analysis to evaluate the nutrition, growth, immunity, and health status of mammalian, avian, and piscine species. In our previous experiments, we observed positive growth effects of a plant-based polysaccharide prebiotic formulation (unpublished data). Therefore, to investigate the effects of the prebiotic on the gut microbiome and its prediction functionality for *O. niloticus*, we undertook the present study at two different dosage levels using a microbial community genomic approach.

2 Materials and methods

2.1 Fish rearing and feeding management

Tilapia (*Oreochromis niloticus*) fish, 200 nos, weighing 15–20 g were procured from institutional fish breeding facility, kept in fiberglass reinforced plastic (FRP) tanks, and acclimatized for 15 days. The feed was formulated and prepared in the institute's feed laboratory, and it was provided to the experimental fish reared in FRP tanks for 30 days. The Institute Animal Ethics Committee (protocol code IAEC/2021/07; DATE: 10 12 2021), ICAR-CIFRI duly approved the entire experiment.

2.2 Extraction of Aloe vera

The Aloe vera polysaccharide mostly made up of acetylated glucomannan, mannose, cellulose, pectin and xylose were extracted from freshly harvested leaves using hot water extraction, followed by ethanol precipitation, according to the method described by Sun et al. (2011). After homogenization, the leaf gel, free of yellow sap (aloin), was extracted in hot water and precipitated with ethanol. The resulting white precipitate was washed with ethanol, acetone,

TABLE 1 Diet composition (g/100g) for experimental fishes of different groups.

Ingredients	TCON	TPB1	TPB2
Soyabean meal	25.0	25.0	25.0
Rice bran	25.0	25.0	25.0
Mustard oil cake	20.0	20.0	20.0
Wheat flour	10.0	10.0	10.0
Fish meal	05.0	05.0	05.0
Groundnut oil cake	10.0	10.0	10.0
Vitamin C	01.0	01.0	01.0
BHT	0.2	0.2	0.2
Vegetable oil	1.0	1.0	1.0
Fish oil	2.0	2.0	2.0
Choline chloride	0.01	0.01	0.01
Phytase	0.01	0.01	0.01
Aloe vera extract	—	0.5	0.75

and ether to remove impurities and then dried in a hot air oven at 70°C for 8 h, yielding an off-white powder.

2.3 Feeding experiment

The fish were divided into three groups, with 45 fish in each group. Each group was kept in three replicates. Group Tilapia Pre-Biotic 1 (TPB1) and Tilapia Pre-Biotic 2 (TPB2) were provided with 0.5% and 0.75% Aloe vera extract, respectively, in the feed. On the other hand, fish in Group Tilapia Control (TCON) were kept under the control diet (Table 1). Some upper and lower doses were also tried in preliminary study. But these two doses were found to be effective without altering feed composition. Feed was prepared by adding the prebiotic on top. Since the dose was below 1%, replacement of any component was not considered. After 30 days of feeding trials, three representative fish were randomly selected from each replicate and sacrificed for gut collection and further analysis.

2.4 Sample collection and processing

The Fish were euthanized using clove oil at 50 ppm in water (Griffiths, 2000). After dissection, the whole intestine was aseptically removed and placed into sterile phosphate buffer saline (pH 7.2). The intestine samples were chopped and homogenized using a hand-held homogenizer. From each replicate, three fish were taken, and all nine samples were pooled for each group. The processed samples were used for bacterial colony counting and gut microbiome study.

2.5 Colony counting

The total bacterial count, *Bacillus*, and Bifidobacterium counts were determined using Nutrient Agar, *Bacillus* Medium, and Bifidobacterium Agar plates (Himedia, India), respectively. The method followed the standard protocol of tube dilution and plate counting described earlier (Wayne, 2011). Bacterial loads were expressed in cfu/gm of the whole gut sample and compared among the groups.

2.6 DNA isolation, 16S rRNA V3-V4-based Illumina library preparation, and sequencing

The DNA was isolated from gut samples using the DNeasyPowerSoil kit (Qiagen, United States) as per the manufacturer's guidelines. The DNA concentration was estimated using the QubitFluorimeter (V.3.0). The V3–V4 region of the 16S rRNA was amplified using the specific V3 Forward primer, CCTACGGGNBGCASCAG, and V4 Reverse primer, GACTACNVGGGTATCTAATCC. The amplified product was checked on a 2% agarose gel, and gel purification was performed to remove non-specific amplifications. Library preparation was done using 5 ng of the amplified product from the NEB Next Ultra DNA library preparation kit. Quantification and quality estimation of the prepared library were done using the Agilent 2200 TapeStation and sequenced on Illumina HiSeq 2500 using 2*250 cycles of chemistry.

2.7 Quality check

The raw reads obtained from Illumina sequencing were demultiplexed and quality checked using the Fast QC program version 0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) under default parameters. Before bioinformatics analysis, the base quality (Phred score; Q), base composition, GC content, ambiguous bases, and adapter dimers were checked. Chimeras were then removed using the *de novo* chimera removal method, UCHIME (version 11), implemented in the VSEARCH tool.

2.8 Operational taxonomic units (OTU) and classification

The OTU picking and taxonomic classification were performed using the consensus V3–V4 sequences. Based on the sequence similarity, the sample pre-processed reads were pooled and clustered into OTUs using the UCLUST program (similarity cutoff = 0.97), which is available in the QIIME software (Caporaso et al., 2010). A total of 8,894 OTUs were identified from 1,000,199 reads. Out of 8,894 total OTUs, 7,713 OTUs with fewer than five reads were removed, and 1,181 OTUs were selected for further analysis. To analyze the microbial diversity within samples, Shannon and Chao1 matrices were calculated.

2.9 Bioinformatics analysis

To speculate on the role of microbes in host metabolism, functional profiling predictions were performed using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Langille et al., 2013). The type of functional predictions was set to Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs. The obtained findings from PICRUSt were evaluated using versatile matrix visualization and analysis software (MORPHEUS) (https://software.broadinstitute.org/morpheus) (Starruß et al., 2014) for significant differentiation in microbial function among the fish groups due to the supplementation of prebiotics in feed. The software helps to execute the hierarchical clustering, sort and filter the discrete data based on numerous descriptive quantitative measures.

3 Results

The total bacterial count in the control fish (TCON) was 3.24×10^7 cfu/gm in the whole gut sample (Figure 1A). Treatment with plant-origin polysaccharides influenced the total bacterial count, increasing it upto 4.52×10^7 cfu/gm in the treated group TPB1, and up to 8.03×10^7 cfu/gm in the treated group TPB2. The *Bacillus* count was also higher in the treatment groups (Figure 1B) [1.92×10^6 CFU/gm and 2.04×10^6 CFU/gm in 0.5% (TPB1) and 0.75% (TPB2) prebiotic-supplemented groups, respectively] than in the

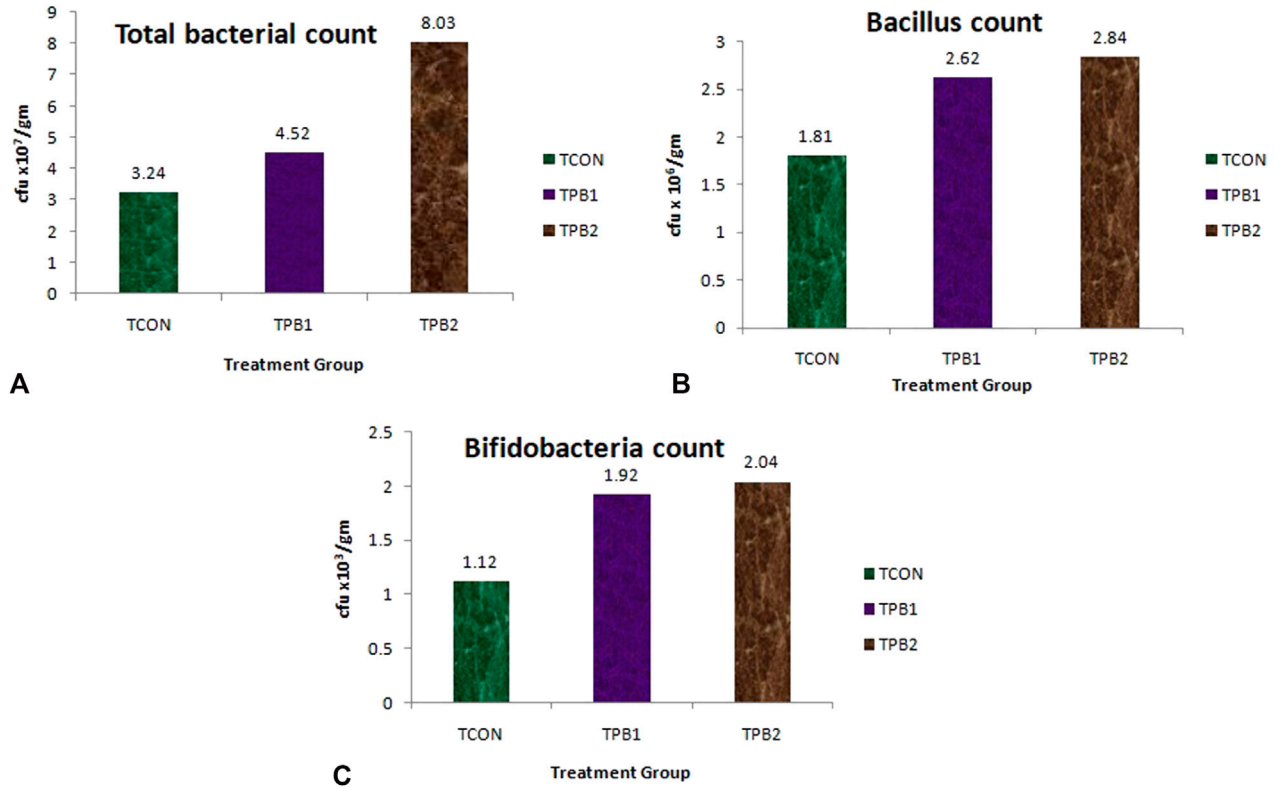

FIGURE 1
(A) Total bacterial load (cfu/mL), **(B)** *Bacillus* count and **(C)** Bifidobacteria count in whole gut of *O. niloticus* in different treatment group of fishes.

control (TCON) group (1.12×10^6 CFU/gm). In the control group of *O. niloticus*, the Bifidobacterium count was 1.81×10^3 cfu/gm of the whole gut (Figure 1C). Moreover, both treatment doses showed it's higher count (2.62×10^3 and 2.84×10^3 cfu/gm, respectively) (Supplementary Figure S1).

3.1 Operational taxonomic units (OTU) and classification

The process of OTU picking and taxonomy classification was conducted using pre-processed consensus V3–V4 sequences. The pre-processed reads from all samples were pooled and clustered into OTUs based on their sequence similarity using the UCLUST program (similarity cutoff = 0.97), which is available in the QIIME software. A total of 8,894 OTUs were identified from 1,000,199 reads. Out of these 8,894 OTUs, 7,713 OTUs with less than five reads were excluded, leaving 1,181 OTUs for further analysis.

3.1.1 Phylum

Based on the top 50 hit analyses, 13 phyla were recorded in the control group of tilapia fish. The highly dominant phylum was Proteobacteria (64.78%), followed by Planctomycetes (9.77%), Actinobacteria (6.59%), Fusobacteria (5.30%), Firmicutes (3.6%), Verrucomicrobia (3.06%), Chlamydiae (1.64%), and Bacteroidetes (1.17%). Other Phyla noted were Chloroflexi, Patescibacteria, Dependentiae, Epsilonbacteraeota, and Tenericutes. The fish supplemented with a lower dose of polysaccharides (0.50%) showed an equal number of presences of the same phylum as the control group, whereas in the 0.75% supplemented group, three phyla, namely, Chlamydiae, Epsilonbacteraeota, and Tenericutes could not be detected. The abundance of Planctomycetes and Firmicutes had increased, and that of Proteobacteria had reduced in the higher dose-supplemented group of fishes (TPB2 group) (Figure 2A).

3.1.2 Class

At class-level analysis, a total of 19 classes were noted in the control fishes and the lower dose-supplemented group (0.5%). Dominating classes were Alphaproteobacteria (49.35%), Gammaproteobacteria (12.72%), Planctomycetacia (9.77%), Actinobacteria (5.65%), Fusobacteria (5.30%), Verrucomicrobiae (3.06%), Deltaproteobacteria (2.70%), Bacilli (2.35%), Chlamydiae (1.64%), and Bacteroidia (1.17%). When compared to other prebiotic doses and the control group, Chlamydiae, Anaerolineae, Campylobacteria, and Mollicutes were absent in the higher dose group (TPB2). When fishes were supplemented with a higher dose of polysaccharide (0.75%), Planctomycetacia and Bacilli increased, but Gammaproteobacteria were reduced (Figure 2B).

3.1.3 Order

Analyzing the order level in the top 50 hits, a total of 47 orders were recorded in control *O. niloticus* fish. The dominating orders

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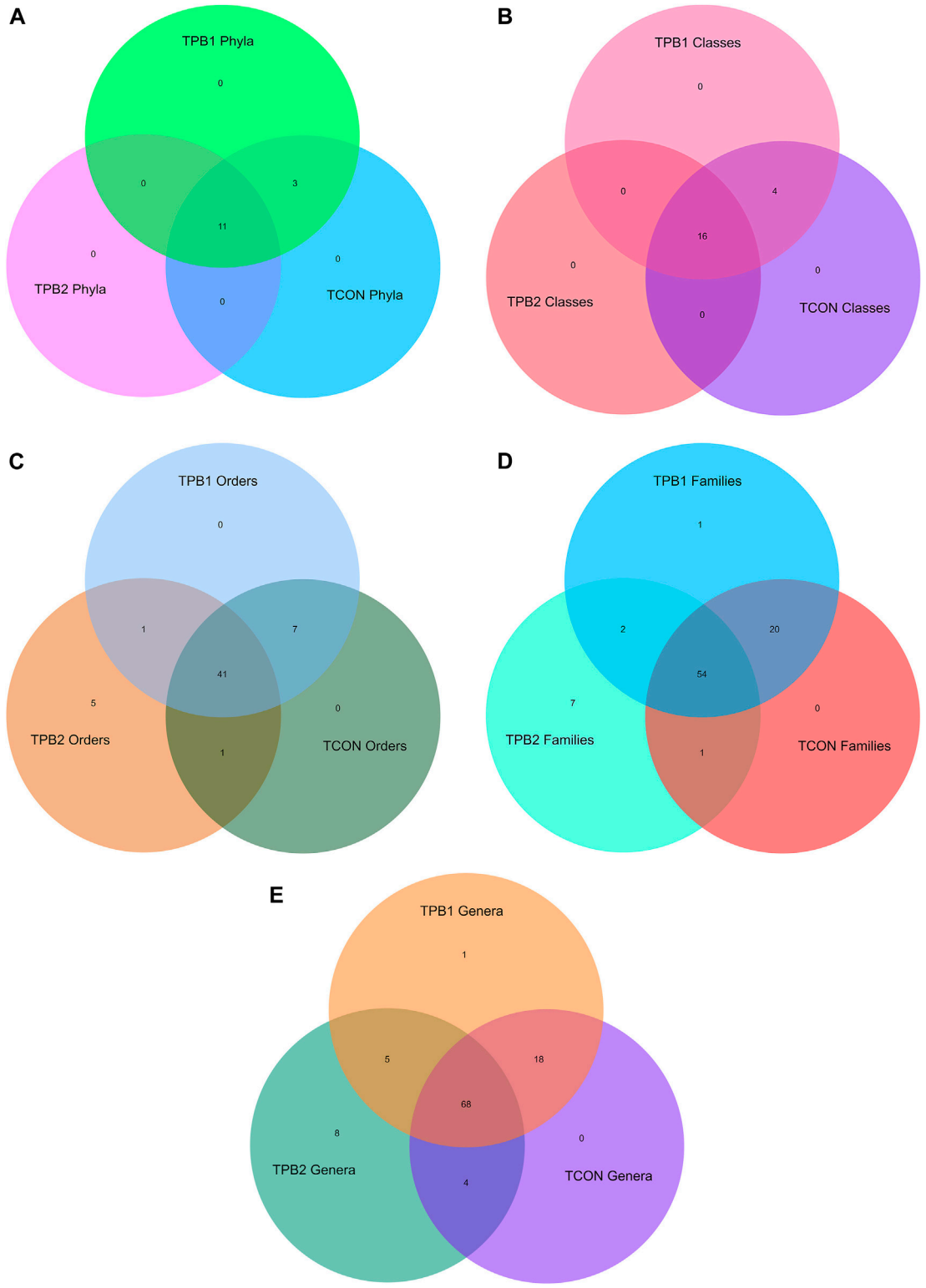

FIGURE 2
(A) Venn diagram of shared OTUs of bacterial phylum in gut amongst different treatment and control group fishes. **(B)** Venn diagram of shared OTUs of bacterial classes in gut amongst different treatment and control group fishes. **(C)** Venn diagram of shared OTUs of bacterial classes in gut amongst different treatment and control group fishes. **(D)** Venn diagram of shared OTUs of bacterial families in gut amongst different treatment and control group fishes. **(E)** Venn diagram of shared OTUs of bacterial genus in gut amongst different treatment and control group fishes.

were Rhizobiales (36.74%), Rhodobacterales (7.06%), Fusobacteriales (5.30%), Legionellales (4.0%), Pirellulales (4.0%), Micrococcales (3.53%), Verrucomicrobiales (2.82%), Gemmatales (2.23%), Reyranellales (2.23%), Pseudomonadales (2.00%), Isosphaerales (1.88%), Chlamydiales (1.64%), Planctomycetales (1.64%), Bdellovibronales (1.53%), Oligiflexales (1.17%), and Enterobacteriales (1.17%). Supplementation with prebiotics in a lower dose (0.50%) did not alter the microbial community, whereas, in a higher dose (0.75%), seven orders could not be detected. Besides, the abundance of Rhizobiales and Fusobacteriales were slightly reduced, whereas Rhodobacteriales were increased in both the supplemented groups. Supplementation with a higher dose of polysaccharide to the fish caused significant enhancement of Bacillales, Gemmatales, and Isosphaerales counts and a reduction in Reyranellales, Verrucomicrobiales, Betaproteobacteriales, and Corynebacteriales counts in comparison to the lower dosage (0.50%) and control group. Chlamydiales could not be detected in the higher-dose treated group (TPB2) fish, but the same was recorded in lower-dose-supplemented (TPB1) and control fish (TCON) (Figure 2C).

3.1.4 Family

A total of 43 families were recorded in healthy (control) tilapia fishes. The most dominating family was Rhizobiales incertae sedis (17.78%), followed by Rhodobacteriaceae (7.06%), Beijerinckiaceae (5.53%), Fusobacteriaceae (5.06%), Legionellaceae (4.00%), and Pirellulaceae (4.00%). The comparison revealed that an equal number of families were seen in the TPB1 and TCON, whereas TPB2 showed only 38 families of bacteria. Dose-dependent enhancement of bacteria was noted in Rhodobacteraceae and Bacillaceae, whereas dose-dependent decreased abundance was recorded in Reyranellaceae, Bdellovibrionaceae, and Pseudomonadaceae. Furthermore, it was observed that Fusobacteraceae and Hypomicrobiaceae were increased in both the treated groups. The higher dose group (TPB2) showed a significantly higher abundance of Gemmataceae and Isosphaeraceae, whereas a lower abundance was recorded in Rhizobiaceae and Rubritaleaceae (Figure 2D).

3.1.5 Genus

The genus-level composition analysis of the top 50 hits revealed that healthy control tilapia fish harbored 44 genera of bacteria in their gut system, with the highest dominance of Cetobacterium (4.94%), followed by *Legionella* (4.0%), Alpha cluster (3.41%), Pirellula (3.14%), Reyranella (2.23%), Rhodobacter (1.76%), *Bdellovibrio* (1.53%), *Pseudomonas* (1.41%), Luteolibacter (1.41%), and *Bacillus* (1.29%). The comparison showed that Hydrogenophaga and Mythylocystis were absent in the control fish but present in both treatment groups. In the TPB2, six genera, namely, *Chlamydia*, Neochlamydia, *Micrococcus*, Novosphyngobium, *Streptomyces*, and Sulfurospirillium, were absent. Therefore, a total of 38 genera could be detected in this group, whereas TPB1 showed 46 genera. A dose-dependent increase in the population of bacteria was noted in the genus *Bacillus*, Rhodobacter, and Fimbriglobus, whereas a reduction was recorded in Cetobacterium, Reyranella, *Pseudomonas*, and *Acinetobacter* treatment groups. The treatment with a higher dose of prebiotics further reduced the population of *Legionella*, Luteolibacter, and *Mycobacterium* but enhanced the *Staphylococcus* and Singulisphaera genus of gut microbes (Figure 2E).

To understand the microbiome diversity, we also calculated Shannon and Chao1 diversity indices. Results of one-way ANOVA and Tukey posthoc HSD analysis revealed statistically significant differences in the mean Shannon Diversity index (F>Fcrit; $p < 0.001$) and Chao1 index (F>Fcrit; $p < 0.001$) when compared among samples.

3.2 Presence absence analysis

To understand the differential abundance of OTUs among samples, we drew Venn diagrams for various taxonomic levels (Figures 2A–E). Closer scrutiny of the figures revealed an almost uniform OTU distribution for samples TPB1 and TCON. However, considerable differences were observed in the OTU distributions for sample TPB2.

3.3 Prediction of functionality

In terms of carbohydrate metabolism pathways, dose-dependent enhancement was recorded for glycolysis V, which is the super pathway of glycol metabolism and degradation, glucose and xylose degradation, glycolysis II, and sulfoglycolysis. Glycogen biosynthesis I and glycolysis III were downregulated compared to the control group (TCON). Some of the other pathways, such as glycogen degradation, glucose degradation, and glycogen degradation II, were found to be activated by a lower dose (TPB1) of prebiotics, followed by reduced activity with a higher dose (TPB2).

Upregulating trends in arginine, ornithine, and proline conversion and degradation of L-arginine and L-ornithine, putrescine, and the 4-aminobutanoate pathway in a dose-dependent manner were noticed. Heme biosynthesis from glycine, L-arginine biosynthesis I, II, III, IV, L-serine, glycine biosynthesis I, and glycine betaine degradation I were upregulated at a lower dose compared to the control group and a higher dose of prebiotic treatment.

Aromatic amino acid biosynthesis, L-arginine biosynthesis, II arginine, polyamine biosynthesis, branched amino acid biosynthesis, mixed acid fermentation, and tetrapyrrole biosynthesis II were decreased in a dose-dependent manner.

The thiamine diphosphate biosynthesis pathway was found to be downregulated in a dose-dependent manner, whereas the rest of the pathways, including Tricarboxylic acid cycle (TCA) cycle I, IV, VI, and VIII, and reductive TCA cycle I and II, were upregulated in the lower dose of prebiotics followed by downregulation at higher doses. A dose-dependent decreasing trend of pathway activities was recorded for lipid IVA biosynthesis and phospholipid biosynthesis I, whereas a reverse trend was seen for the super pathway of 2-lipid A biosynthesis. The lower dose group had a greater upregulation of the lipid IVA III pathway than the higher dose and control groups. The vitamin E biosynthesis pathway was found to be downregulated in both treatment groups when compared to the control group (Figure 3).

4 Discussion

The gut microbiome has been identified as a physiological and immune modulator, producing thousands of metabolites, and thus

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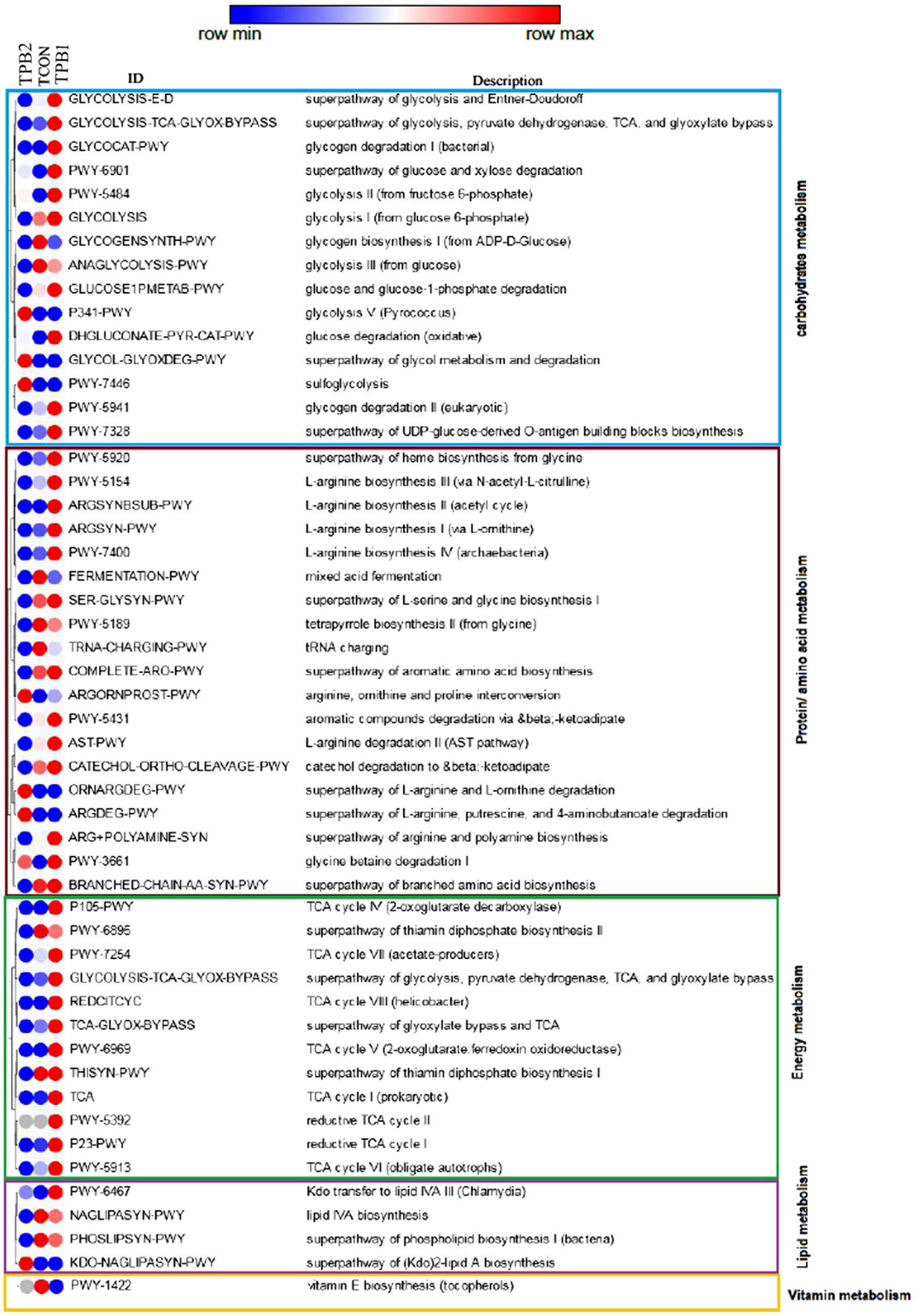

FIGURE 3
Comparison on prediction functionality of various metabolic pathways of gut microbiome in *O. niloticus* treated with prebiotics. [TCON–Control diet; TPB1—0.5% Aloe vera extract in the feed & TPB2—0.75% Aloe vera extract in the feed].

regulating the host's health and sustenance. The diverse growth environment of terrestrial and aquatic animals causes a striking difference in the composition and structure of microbiota in well-studied mammals and other animals (Ni et al., 2014; Xiong et al., 2019). In contrast to terrestrial vertebrates, aerobic, facultative, anaerobic, and obligate anaerobic bacteria are the principal

colonizers of the gastrointestinal tract of fishes (Opiyo et al., 2019). Bacterial cell count and concentration may also vary among human, rodent, and piscine populations. It is reported that fish gut harbors 10^7–10^{11} bacteria g^{-1} of intestinal content (Nayak, 2010).

The impact of the presence and absence of some specific bacteria and their role in the innate immune system of a particular host is the functional dimension of the host-microbe interaction. The influence of gut microbiota in fish is reported to be similar to that in mammals concerning feeding behavior and physiology, secretion of enzymes, hormones, and metabolites (Yang and Chiu, 2017), such as butyrate, acetate, indole, and short-chain fatty acids (Duca et al., 2012; Zhang and Davies, 2016). Previous studies on fish gut microbiota and its diversity were reported in Lactobacillus-supplemented zebrafish, which showed a reduced appetite (Falcinelli et al., 2016), whereas Japanese flounders supplemented with *Bacillus clausii* showed enhanced growth rates with improved feed efficiency (Ye et al., 2011). The metabolism of carbohydrates, amino acids, and lipids is found to be influenced by the compositional changes of the gut microbiota of grass carp (Ni and Yu, 2013). Diet-induced alterations in gut microbiota composition also influence fatty acid absorption by the epithelium of zebrafish (Forsatkar et al., 2017).

The host's stress adaptation and its associated sequelae are also mediated to a greater extent by the implication of its gut microbiota through energy homeostasis (Dehler et al., 2017). Fish with upset gut microbiota had disrupted energy homeostasis and enhanced stress hormones, as found in rainbow trout and goldfish (De Pedro et al., 1997; Ortega et al., 2013; Garcia-Reyero, 2018).

Previous studies (Ray et al., 2017; Zhu et al., 2020) have investigated how environmental factors and dietary composition affect the gut microbiota structure of Nile tilapia when fed with microbial community-fed diets supplemented with Previda® and Saponin. Various water parameters, such as salinity and temperature, play an essential role in the gut microbiota composition of fish (Al-Harbi and Uddin, 2004; Ringø and Song, 2016). Intestinal dysbiosis biases, reduced immunity, and increased susceptibility to pathogenic organisms are associated with a wide range of pollutants such as heavy metals, pesticides, and antibiotics in the aquatic system (Li et al., 2016; Stephens et al., 2016; Butt and Volkoff, 2019).

Recently, a few studies have been carried out to investigate the gut microbiome structure of tilapia at different life stages (Giatsis et al., 2014; Koo et al., 2017). One study reported the microbiome profile of tilapia (Genetically Improved Farmed Tilapia, GIFT) in phylum categories predominantly belonging to Fusobacteria, Bacteroidetes, Proteobacteria, and Firmicutes. The role of gut microbiota in fish reproduction was recorded (Fan et al., 2017; Ray et al., 2017) where the phyla Proteobacteria, Firmicutes, and Actinobacteria with the highest count of *Fusobacterium* (≥81%) were found dominating during the peak breeding period.

In our current study, we discovered that Proteobacteria was the most dominant phylum, with Verrucomicrobia and *Chlamydia* as minor phyla. Our finding is consistent with that of Li et al. (2017), who reported Proteobacteria, Firmicutes, Fusobacteria, and Bacteroidetes as the dominant phyla in the gut of a large yellow croaker (*Pseudosciaenacrocea*).

The proper functioning of the host immune system is the result of healthy host-microbiome interaction, which otherwise when incurred by unfavorable environmental challenges, causes mild to severe diseases. Gut microbiota and the gut mucosal immune system work together to promote immunity in fish by maturing and developing gut-associated lymphoid tissue and secreting antimicrobial peptides (Kim et al., 2017; Wang et al., 2018).

Prebiotics and probiotics can play an indispensable role in maintaining optimal health and treating chronic diseases as a consequence of variations in the gut microbiota composition (Lin et al., 2014). During physical and biological stress, intensively cultured fish compromise their innate immunity and become easily vulnerable to diseases. This can be effectively addressed by using suitable feed supplements, such as prebiotics and probiotics, instead of any growth-promoting agents or antibiotics. Prebiotics are generally considered as feed ingredients capable of favoring the growth of beneficial microbes in the gut environment (probiotics) of the animal host. The supplemented diet can not only help to overcome diseases but also modulate the immune system for better growth of the host organism (Amenyogbe et al., 2020). These are mostly oligosaccharides of plant or microbial origin. The compounds enhance the probiotics' population in the gut and also potentiate the immune system. Thus, supplementation with a suitable combination of prebiotics and probiotics is believed to be beneficial for fish health and growth by manipulating their gut microbiota. The storage polysaccharide, acetylated glucomannan, present mostly in the leaf of *A. vera* has numerous immunomodulatory, antimicrobial, anti-inflammatory and anti-cancer, properties (He et al., 2005; Jani et al., 2007). The acetylated β-(1→4)-D-mannosyl residues present in the mannans of *A. vera* counts significantly in the therapeutic constituents of the plant (Boudreau and Beland, 2006). Lesser amounts of arabinose, galactan and galactogalacturan units are the other biologically active polysaccharides present in the leaf gel of the plant (Ni et al., 2004).

The present investigation recorded that prebiotics at a dose of 0.5% of feed, showed two more numbers of the bacterial genus (Hydrogenophaga) and three more numbers of species (*Bacillus*, Circulans, Gemmata *spp*., and *Pedicoccuspentosaceus*) that were absent in the control group of tilapia fish. The higher dose of prebiotics resulted in a reduced number of bacteria at the phylum, class, family, genus, and species levels with an increase in the number of beneficial bacteria, which indicated that the selection of a suitable dose of prebiotics is necessary for the optimal manipulation of gut microflora to achieve beneficial effects. Conventional culture methods employed for the investigation of the GI microbiota of fishes were found to have limitations due to their dependency on culture conditions and the type of media used (Spanggaard et al., 2000). Besides, these methods consume more time with less accuracy in identifying bacterial isolates. Due to the low cultivability of the fish gut microbiome, less than 0.1% of the total microbial community could be detected in some fish (Zhou et al., 2014; de Souza et al., 2020). However, recent advances in molecular-based technologies and bioinformatics analysis have substantially redefined gut microbiome studies, with a wide array of data showing the elaborate elucidation and interaction of the structure, distribution, and diversity of bacterial phyla within the fish gut (Parma et al., 2016; Wang et al., 2018).

Microbial metabolic pathway analysis using PICRUSt is mostly suggested in association with carbohydrates, protein, amino acids, energy metabolism, membrane transport, nutrient digestion, immune function, and xenobiotic metabolism within the fish gut

(García-Márquez et al., 2022). Feeding habits and trophic levels of fish are important factors to determine parts of the gut microbe community. Hence, variation in feeding strategies could be an important approach to achieving the beneficial effects of gut microbes on fish health. Prebiotics have been shown to modulate the microbial community, which improves feed digestion and metabolism of important nutrients and molecules (Pratoomyot et al., 2010). Our findings suggest that plant polysaccharide-based prebiotics can modulate the microbial population of the tilapia gut microbiome and subsequently regulate various metabolic pathways involved in physiology, homeostasis, health, immunity, and disease resistance. Treatment with prebiotics could also improve carbohydrate metabolism through gaining firmicutes and bacilli. These two groups of bacteria are responsible for the breakdown of complex polysaccharides and carbohydrates (Maji et al., 2022). The KEGG pathway analysis also indicated an increase in carbohydrate metabolism for glycolysis V sulfoglycolysis and glycol metabolism. Feeding insect meals to fish was previously reported to induce pentose and glucuronate interconversion (Panteli et al., 2021). Most of the amino acid and protein metabolism pathways were found to be upregulated at a lower dosage of the prebiotics, indicating an optimum balance of the microbial community at a certain dosage level. The same observation was also recorded for energy metabolism, including the TCA cycle. As recorded earlier, Cetobacterium and Bacteroidetes are generally linked with protein digestion and synthesis (Liu et al., 2016; Xu et al., 2019), whereas *Lactobacillus* and *Bacteroides* contribute to glucose and lipid metabolism. In the present study, a decrease in the abundance of Proteobacteria and Gamma Proteobacteria in the higher dose group could be the cause of downregulation in some protein and amino acid synthesis pathways.

5 Conclusion

Taken together, our current investigation revealed the structure of the gut microbiome of *O. niloticus* under Indian farming conditions, which are mostly tropical in nature. Moreover, the study highlighted the potential for modulation of the gut microbiome of tilapia through polysaccharide-based prebiotic feeding for efficient nutritional and gut health management and enhanced productivity. The modulated microbial abundance further reflected various metabolic pathways, hence explaining the effective biochemical mechanism.

Data availability statement

The data presented in the study are deposited in the NCBI database repository, accession number PRJNA977195. The data can be accessed via the link: http://www.ncbi.nlm.nih.gov/bioproject/977195.

Ethics statement

The animal study was reviewed and approved by the Institutional Ethics Committee of ICAR–Central Inland Fisheries Research Institute (protocol code IAEC/2021/07; DATE: 10 12 2021).

Author contributions

AB: conducting the experiment, sample collection, processing, draft manuscript preparation; HC: conducting the experiment, manuscript editing; SG: analysis of results and manuscript editing; RM: sample collection processing and conducting laboratory work; NC: conducting laboratory work, data analysis and manuscript preparation; HC: bioinformatics analysis; HS: rearing of fish, conducting experiments and sample collection; MH: conducting feeding experiment and manuscript editing and BD: generating idea, plan of waor, overall supervising the research and final editing of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2023.1168284/full#supplementary-material

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The effects of replacing fish meal or soy protein concentrate with wheat gluten on growth, whole-body composition, and the retention and apparent digestibility coefficient of amino acids in Japanese seabass (*Lateolabrax japonicus*)

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The aim of this study was to evaluate the effects of replacing fish meal (FM) or soy protein concentrate (SPC) with wheat gluten on growth performance, feed utilization, and nutrient digestibility and retention in Japanese seabass (*Lateolabrax japonicus*). Seven isonitrogenous (441–456 g kg^{-1} crude protein) and isocaloric (21.5–22.0 MJ kg^{-1} gross energy) diets were produced to replace 0%, 33.3%, 66.7% and 100% of FM or SPC with a mixture of wheat gluten, wheat, and taurine (GWT, 77.5% wheat gluten, 20.5% wheat and 2.0% taurine). The gradual replacement of protein in FM with GWT had no significant effects on feed intake, whole-body composition, and the hepatosomatic and viscerosomatic indices, but resulted in a linear decrease in the weight gain rate, feed efficiency, and retention of nitrogen, energy, and essential amino acids (Arg, His, Ile, Leu, Lys, Met, Phe, Thr, and Val). The apparent digestibility of most essential amino acids (Cys, His, Leu, Lys, and Phe) and total amino acids increased linearly. Replacement protein in SPC with GWT had no significant effects on feed intake, growth, the feed conversion ratio, whole-body composition, and the hepatosomatic index, but resulted in a linear decrease in nitrogen, energy, and Met retention; the digestibility of Cys and Met increased linearly. Overall, wheat gluten is a more effective alternative for replacing protein in SPC than FM.

KEYWORDS

wheat gluten, fish meal, soy protein concentrate, Japanese seabass, methionine requirement

1 Introduction

The production of high-quality (LT) fish meal is highly standardized in terms of raw material freshness, separation, and the drying process. LT fishmeal has a high concentration of digestible protein and a balanced amino acid composition (Anderson et al., 1997). The lipid portion of LT fishmeal consists of triglycerides and phospholipids and high levels of n-3 fatty acids, which can provide essential fatty acids for fish (Olsen and Hasan, 2012). LT fishmeal is produced with presscake and stickwater, which contribute to the biological value of protein and the physical characteristics of extruded feed. However, the limited amount of fish meal and growing demand have led to higher prices (Tacon and Metian, 2008). Thus, it is necessary to find alternative protein sources to meet the protein requirement for fish growth in intensive aquaculture.

Wheat gluten is a proteinaceous material obtained from wheat, with a crude protein (CP) content of up to 80% (Apper-Bossard et al., 2013). It is a digestible binder in extruded feeds and has a protein digestibility close to 100% in the feed of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) (Storebakken et al., 2000a; 2015a). In addition, it contains a relatively high concentration of sulfur-containing amino acids, including 1.8% Met and 2.6% Cys, which is considerably higher than in soy protein concentrate (Apper-Bossard et al., 2013). Wheat gluten is virtually devoid of antinutritional factors due to the low content of non-starch polysaccharides and other indigestible carbohydrates (Tusche et al., 2012). A study in salmon showed that wheat gluten can efficiently replace high-quality (LT) fish meal (Storebakken et al., 2015a). Another finding indicated that wheat gluten could improve intestinal health in salmon, which is probably related to the high glutamine content (35%–40% of CP) (Apper et al., 2016).

Soy protein concentrate (SPC) is a major protein source in fish. It contains a higher protein concentration than soybean meal, especially Arg, which can support the rapid growth and efficient feed conversion of fish (Storebakken, 2000b; Zhang et al., 2012b). Furthermore, antinutritional factors, such as protease inhibitors, lectins, soluble non-starch polysaccharides, and indigestible oligosaccharides, have been inactivated or removed (Storebakken, 2000b). However, the main challenge for SPC is phytate, which can be concentrated during the extraction process. Phytate has potentially negative effects on freshwater eutrophication due to its low availability. In warm-water fish, it is feasible to overcome this challenge by applying phytase in feed (Denstadli et al., 2007).

Japanese seabass *Lateolabrax japonicus*, known as black-spotted bass, is one of the most important aquaculture species in China. Studies on fish meal substitution in Japanese seabass have been widely reported. Juvenile Japanese seabass adapted to a 50% dietary soybean meal (IBW of 6.7 g) but required additional crystalline methionine supplementation (Zhang et al., 2016; Zhang et al., 2018). However, its tolerance to canola rapeseed meal is lower at less than 20% (Cheng et al., 2010). Men et al. (2014) found that replacing 60% fishmeal with corn gluten meal did not hinder the growth of Japanese seabass. Hu et al. (2013) evaluated the effects of different qualities of fish meal in larger Japanese seabass (IBW of 76 g), and the results indicated that prime steam-dried fishmeal was superior to standard steam-dried fishmeal in terms of feed intake and growth rate. Therefore, the aim of this study was to evaluate the effects of replacing LT fishmeal or SPC with wheat gluten in Japanese seabass, which could contribute to further understanding the potential use of wheat gluten as a considerable alternative protein source.

2 Materials and methods

2.1 Ingredients and diets

Seven isonitrogenous (441–456 g kg^{-1} crude protein) and isocaloric (21.5–22.0 MJ kg^{-1} gross energy) diets were formulated to compare the nutritional value and combination effects of wheat gluten, LT fishmeal, and SPC. The chemical compositions of wheat gluten, FM, and SPC are presented in Table 1. A mixture (GWT) consisting of 77.5% wheat gluten, 20.5% wheat, and 2.0% taurine was produced. The V0, VF1, VF2, and VF3 diets were formulated with GWT gradually replacing 0, 33.3, 66.7, and 50% FM, respectively. Similarly, GWT gradually replaced 33.3, 66.7, and 100% SPC to form the VS1, VS2, and VS3 diets, respectively (Table 2). Based on previous studies (Mai et al., 2006; Li et al., 2012; Hu et al., 2013), the V0 diet could meet the nutrient requirements of Japanese seabass. To maintain the same level of dietary essential amino acids (EAA), the VS1, VS2, and VS3 diets were supplemented with crystalline Lys, Arg, and Thr, and the VF1, VF2, and VF3 diets were supplemented with crystalline Lys, Arg, Thr, and Met. In addition, monocalcium phosphate was also added to the diets, and yttrium oxide (Y_2O_3) was used as an inert marker for digestibility assessment (Austreng et al., 2000; Zhang et al., 2006). The formulation, proximate compositions, and amino acid profiles of the diets are presented in Table 3, 4.

2.2 Feed manufacturing and physical quality analysis

Diets were produced at the Feed Technology Laboratory of the Feed Research Institute, Chinese Academy of Agricultural Sciences in Beijing. All dry ingredients were ground in a hammer mill through a 0.18-mm screen, then mixed, preconditioned, and extruded in a twin-screw extruder (MY56X2A, Muyang, Jiangsu, China) with a 2.0 mm die plate (MY56A 12-03/02 XL 09 11). The goal of the extrusion process was to achieve a bulk density higher than 516 g L^{-1} prior to drying to facilitate the slow sinking of feed after drying and lipid coating. All extruded pellets were dried to 950 g kg^{-1} dry matter at ambient temperature. Fish oil and soy lecithin were coated into pellets using a vacuum coater (ZJB-100). Pellet length and diameter were determined using a digital caliper. Durability and breaking point were estimated for uncoated pellets using a pellet tester (ST-136, Shengtai Instrument Co., Ltd., Jinan, China) and hardness tester (ST-120B, Shengtai Instrument Co., Ltd., Jinan, China), respectively. Water stability and sinking rate were determined according to the methods described by Baeverfjord et al. (2006) and Sørensen et al. (2012), respectively.

2.3 Fish rearing and experimental conditions

A 72-day feeding trial followed by a 10-day digestibility evaluation experiment were conducted in an indoor recirculation system at the Haid Group research station, Seagull Island, Panyu, Guangzhou, China. Freshwater-acclimated Japanese seabass (*L. japonicus*) juveniles were obtained from a hatchery in Fujian,

TABLE 1 Compositions of the three main ingredients in the experiment (dry matter basis).

Ingredient	Fish meal[a]	Wheat gluten[b]	Soy protein concentrate[c]
Composition, kg^{-1}			
Dry matter, g	936	946	925
Crude protein, g	744	872	694
Crude fat, g	117	44	47
Starch, g	-	74	-
Ash, g	122	10	58
Gross energy, MJ	21.78	22.02	20.08
Essential amino acids (EAAs), g (16 g N)$^{-1}$			
Arg	5.28	3.43	7.46
His	2.26	1.83	2.74
Ile	4.07	3.54	4.77
Leu	6.97	6.82	7.85
Lys	7.40	1.65	6.43
Met	2.67	2.02	1.33
Phe	3.71	5.89	5.10
Thr	3.98	2.67	4.00
Trp	0.61	0.80	1.32
Val	4.96	3.90	4.96
Total EAAs	41.9	32.6	45.9
Total non-essential AAs[d]	40.6	61.5	50.5
Total AAs[d]	82.5	94.1	96.4

[a]Triple nine®, low-temperature dried fish meal, Esbjerg, Denmark.
[b]AMYGLUTEN 110, Syral, Aalst, Belgium.
[c]Yihai®.Wilpromil, Glodensea Grain and Oil Industry Co., Ltd, Wilmar, Qinhuangdao, China.
[d]Tyr excluded.

TABLE 2 Inclusion rate of GWT, FM, and SPC in the experiment diets.

Diet	GWT, %	FM, %	SPC, %
V0	0	50	50
VF1	16.7	33.3	50
VF2	33.3	16.7	50
VF3	50	0	50
VS1	16.7	50	33.3
VS2	33.3	50	16.7
VS3	50	50	0

GWT, a mix of wheat gluten, wheat, and taurine; FM, fish meal; SPC, soy protein concentrate.

China and fed a commercial diet for 1 month to adapt to the laboratory condition. Before the experiment, 630 fish with an initial weight of 10.73 ± 0.33 g were fasted for 24 h, anaesthetized with MS-222 (90 mg L^{-1}; Hangzhou Dongbao, Hangzhou, China), batch-weighed, and then randomly assigned to 21 circular tanks, with each diet being assigned triplicates and 30 fish per tank. All fish were fed four meals per day by hand (07:30, 10:30, 13:30, and 16:30 h), with each meal lasting 60 min. Fish were fed 10% in excess based on average feed intake over the previous 3 days. All uneaten feed was immediately sieved and dried to a constant weight at 95°C and reweighed. During the feeding experiment, the water temperature ranged from 28.5°C to 30.0°C, dissolved oxygen exceeded 5.0 mg L^{-1}, the pH value was 6.5–7.0, ammonia nitrogen and nitrite were less than 0.1 mg L^{-1}, and the photoperiod was 12D:12L.

2.4 Sampling

Before the experiment, 30 fish were euthanized by an overdose of MS-222 and stored at −20°C for whole-body composition analyses. At the end of the experiment, fish were anaesthetized with 90 mg L^{-1} MS-222 before sampling. Five fish from each tank were individually measured for weight and body

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TABLE 3 Feed formulations and analyzed chemical compositions (dry matter basis).

Diets	V0	VF1	VF2	VF3	VS1	VS2	VS3
Ingredients, g kg^{-1}							
GWT[a]	—	69.0	138.0	207.0	69.0	138.0	207.0
Fish meal[b]	200.0	133.0	66.0	—	200.0	200.0	200.0
Soy protein concentrate[c]	214.0	214.0	214.0	214.0	142.0	71.0	—
Soybean meal[d]	70.0	70.0	70.0	70.0	70.0	70.0	70.0
Peanut meal[d]	70.0	70.0	70.0	70.0	70.0	70.0	70.0
Krill meal[e]	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Wheat flour[f]	266.4	250.4	234.6	217.8	262.9	259.1	255.4
Fish oil	84.0	89.0	94.0	99.0	84.0	84.0	84.0
Soy lecithin	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Premix[g]	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Mono calcium phosphate	14.0	17.0	20.0	23.0	14.5	14.5	14.5
Choline Cl	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Y_2O_3	0.1	0.1	0.1	0.1	0.1	0.1	0.1
L-Lysine[h]	—	3.7	7.4	11.0	3.1	6.1	9.1
DL-Methionine[i]	—	0.4	0.8	1.2	—	—	—
L-Arginine[h]	—	1.1	2.1	3.1	2.1	4.2	6.2
L-Threonine[h]	—	0.8	1.5	2.3	0.8	1.5	2.2
Analyzed content, kg^{-1}							
Dry matter, g	951	954	957	956	957	957	957
Crude Protein, g	441	447	444	449	447	451	456
Crude Fat, g	135	136	139	142	140	141	131
Ash, g	76	69	61	54	72	68	64
Gross Energy, MJ	21.5	21.6	21.6	22.0	21.5	21.7	21.8

[a]Mixture of vital wheat gluten, wheat flour, and taurine (mixing ratio: 77.5%, 20.5%, and 2%).
[b]Triple 9®. Low-temperature dried fish meal, Esbjerg, Denmark.
[c]Yihai®. Wilpromil, Glodensea Grain and Oil Industry Co., Ltd, Wilmar, Qinhuangdao, China.
[d]Fengyuan®. Glodensea Grain and Oil Industry Co., Ltd, Wilmar, Qinhuangdao, China.
[e]QRILL™.Antarctic Krill Meal, Aker BioMarine, Oslo, Norway.
[f]Bluekey®. Beijing Grain and Oil Industry Co., Ltd, Wilmar, Beijing, China.
[g]Vitamin premix (mg kg^{-1} diet): vitamin A 20; vitamin B_1 12; vitamin B_2 10; vitamin B_6 15; vitamin B_{12} 8; niacinamide 100; ascorbic acid 1,000; calcium pantothenate 40; biotin 2; folic acid 10; vitamin E 400; vitamin K_3 20; vitamin D3 10; inositol 200; corn protein powder 150. Mineral premix (mg kg^{-1} diet): $CuSO_4 \cdot 5H_2O$ 10; $FeSO_4 \cdot H_2O$ 300; $ZnSO_4 \cdot H_2O$ 200; $MnSO_4 \cdot H_2O$ 100; KI (10%) 80; Na_2SeO_3 (10% Se) 67; $CoCl_2 \cdot 6H_2O$ (10% Co) 5; NaCl 100; zeolite 638. Vitamin premix: mineral premix = 2: 1.5.
[h]*L*-Lysine HCL, *L*-Arginine Base, *L*-Threonine, 98% feed grade, Siwei Development Group Ltd., Hangzhou, China.
[i]MetAMINO® *DL*-Methionine, 99% feed grade, Evonik-Degussa Antwerpen N.V, Antwerpen, Belgium.

TABLE 4 Amino acid compositions of the experimental diets, g (16 g N)$^{-1}$.

Diet	V0	VF1	VF2	VF3	VS1	VS2	VS3
Essential amino acids (EAAs)							
Arg	6.60	6.38	6.31	6.29	6.50	6.31	6.24
His	2.13	1.99	2.07	2.05	1.98	1.88	1.79
Ile	4.12	3.90	3.71	3.80	3.89	3.71	3.54
Leu	7.31	7.04	6.89	6.98	7.03	6.76	6.57
Lys	6.24	5.91	5.97	5.81	6.03	5.71	5.60
Met	1.58	1.53	1.39	1.42	1.72	1.75	1.80
Phe	4.72	4.61	4.84	4.84	4.57	4.43	4.39
Thr	3.90	3.74	3.64	3.64	3.79	3.66	3.60
Val	4.51	4.25	4.05	4.04	4.36	4.15	3.98
Total EAAs	41.1	39.4	38.9	38.9	39.9	38.4	37.5
Semi-EAAs							
Cys	0.91	1.03	1.08	1.24	1.05	1.10	1.14
Tyr	3.33	3.22	3.20	3.28	3.23	3.12	3.04
Total non-EAAs	52.9	53.4	54.6	57.4	53.3	53.5	54.5
Total AAs	94.0	92.8	93.5	96.3	93.2	91.9	92.0

length, then dissected to remove the whole viscera; the liver and carcass were weighed separately. Another six fish were randomly sampled from each tank and then kept at −20°C for analysis of whole-body and amino acid compositions. The remaining fish in each tank were assessed for the digestibility evaluation experiment. Faeces were collected by carefully dissecting the abdomen from the last 5 cm of the distal intestine, pooled by tank, and then stored at −20°C.

2.5 Chemical analysis

Initial and final whole-body samples from the same tank were cut into pieces and ground with a meat grinder, then autoclaved (YXQ-LS, Xunbo, Shanghai, China) at 120°C for 30 min, homogenized by a homogenizer (DS-1, Shanghai, China), and oven-dried (Jinghong, Zhejiang, China) at 80°C. Whole-body samples were finely ground with a pestle and mortar until all samples passed through a 0.9 mm screen. Dried whole fish and feed samples were analyzed for dry matter (105°C to constant weight), protein (Kjeldahl's method), lipids (Soxhlet

TABLE 5 Extrusion processing parameters and feed pellet physical quality.

Diet	V0	VF1	VF2	VF3	VS1	VS2	VS3
Extruder parameters							
Feeding rate, kg h^{-1}	125	125	125	125	125	125	125
Water addition in conditioner, %	8	12	16	16	14	16	16
Water addition in extruder, %	8	8	12	12	12	12	12
Die temperature, °C	117	87	86	86	88	85	85
Revolution screws, rpm	259	259	280	280	280	280	280
Cutter speed, rpm	2,300	2,400	2,350	2,350	2,400	2,450	2,400
Physical quality							
Length, mm	4.63	3.98	4.31	4.34	3.93	3.77	3.83
Diameter, mm	3.50	3.48	3.59	3.76	3.53	3.57	3.67
Expansion, %	74.9	74.1	79.3	87.9	76.7	78.7	83.4
Bulk density, g L^{-1}	518	561	571	563	561	561	550
Breaking point, N	24.7	37.1	35.0	34.7	32.4	34.0	35.4
Water stability, %	88.8	79.7	86.0	84.7	89.1	86.4	85.1
Sinking rate, cm s^{-1}	7.94	9.03	8.37	8.24	9.26	9.78	9.47

TABLE 6 Growth performance and feed utilization of Japanese seabass fed different experimental diets.

Parameters	Diets				Pooled S.E.M[a]	*P*	Regression model	R^2
	V0	VF1	VF2	VF3				
Feed intake (FI), g DM $fish^{-1}$	86.0	81.1	83.1	85.9	6.16	0.65	—	—
Weight gain (WGR), %	795[a]	725[ab]	646[b]	627[b]	63.14	0.019	786–1.75 x	0.66
Specific growth rate (SGR), %/d	3.04[a]	2.93[ab]	2.79[b]	2.75[b]	0.118	0.027	3.03–3.06 * 10–3 x	0.63
Feed conversion ratio (FCR), g FI (g WG)$^{-1}$	1.07[b]	1.11[b]	1.28[a]	1.36[a]	0.065	<0.001	1.05 + 3.08 * 10^{-3} x	0.83
Nitrogen retention, %	34.8[a]	33.6[a]	29.2[b]	26.9[c]	1.28	<0.001	35.3–0.0843 x	0.89
Energy retention, %	40.2[a]	38.0[a]	33.4[b]	32.5[b]	2.46	<0.01	40.2–0.0837 x	0.73
	V0	**VS1**	**VS2**	**VS3**				
FI, g DM $fish^{-1}$	86.0	85.3	84.9	87.5	5.78	0.92	—	—
WGR, %	795	757	747	766	56.82	0.67	—	—
SGR, %/d	3.04	2.98	2.97	3.00	0.091	0.67	—	—
FCR, g FI (g WG)$^{-1}$	1.07	1.12	1.13	1.13	0.051	0.39	—	—
Nitrogen retention, %	34.8[a]	32.5[b]	32.2[b]	31.9[b]	0.90	<0.01	34.7–0.0723 x + 4.56 * 10^{-4} x^2	0.73
Energy retention, %	40.2[a]	37.4[b]	37.0[b]	37.4[b]	1.02	<0.01	40.2–0.0977 x + 7.07 * 10^{-4} x^2	0.75

Pooled standard error of means. Data are means and pooled S.E.M. different superscript letters [a,b,] and[c] indicate significant ($p < 0.05$) differences among treatments.

Extraction System, Jingke, Shanghai, China), amino acids (amino acid analyzer, L-8900, Hitachi, Japan), ash (550°C, overnight), and energy (Phillipson Microbomb Calorimeter, Gentry Instruments Inc., Aiken, SC, United States) based on the previous study (Zhang et al., 2022). Faeces were freeze-dried at −50°C (Labonco Freezon 4.5, Kansas City, United States) and ground with a pestle and mortar. Yttrium oxide in feed and faeces was determined based on the previous study using inductively coupled plasma mass spectrometry (ICP-MS) (Zhang et al., 2012a). Briefly, approximately 50 mg of freeze-dried sample was weighed into a digestion tube, and then 6 ml HNO_3 and 2 ml hydrogen peroxide were added. After microwave digestion, the sample was diluted to a constant volume of 50 ml, aspirated 1 ml diluent, and then re-diluted to 10 ml for on-board testing.

2.6 Calculations and statistical analysis

Apparent digestibility (ADAA, %) of individual amino acids was calculated as $100 \times (1 - AA_f \times AA_d^{-1} \times Y_d \times Y_f^{-1})$, where AA_f and AA_d represent the concentration of individual amino acids in faeces and the diet and Y_f and Y_d represent the concentration of yttrium in faeces and the diet. Retention of nitrogen and energy (%) was calculated as

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TABLE 7 Morphologic index of Japanese seabass fed different experimental diets.

Somatic indices	Diets				Pooled S.E.M.	*P*	Regression model	R^2
	V0	VF1	VF2	VF3				
Hepatosomatic index (HSI), %	1.52	1.66	1.55	1.38	0.263	0.57	—	—
Viscerosomatic index (VSI), %	17.3	17.3	17.8	17.5	0.64	0.64	—	—
Condition factor (CF), g cm^{-3}	1.78	1.79	1.70	1.66	0.116	0.36	—	—
	V0	**VS1**	**VS2**	**VS3**				
HSI, %	1.52	1.51	1.57	1.36	0.348	0.84	—	—
VSI, %	17.3^{a}	17.0^{a}	16.6^{ab}	16.1^{b}	0.48	0.031	17.4–0.0125 x	0.64
CF, g cm^{-3}	1.78	1.74	1.79	1.74	0.069	0.69	—	—

Data are means and pooled S.E.M. different superscript letters [a] and [b] indicate significant (*P* 0.05) differences among treatments.

TABLE 8 Whole-body compositions of Japanese seabass fed different experimental diets.

Whole-body composition, kg^{-1}	Diets				Pooled S.E.M.	*P*
	V0	VF1	VF2	VF3		
Moisture, g	659	664	664	655	8.9	0.44
Crude protein, g	164	166	164	163	3.4	0.68
Crude fat, g	123	119	119	131	7.2	0.16
Ash, g	44.0	43.4	42.2	41.5	1.36	0.11
Gross energy, MJ	8.95	8.78	8.83	9.23	0.307	0.23
	V0	**VS1**	**VS2**	**VS3**		
Moisture, g	659	670	666	664	12.4	0.69
Crude protein, g	164	161	163	164	5.8	0.92
Crude fat, g	123	118	117	123	8.0	0.66
Ash, g	44.0	42.1	42.5	41.2	1.90	0.30
Gross energy, MJ	8.95	8.67	8.74	8.90	0.378	0.69

Data are means and pooled S.E.M.

$100 \times (N_1 \times FBW - N_0 \times IBW) \times (N_d \times FI)^{-1}$, where N_0 and N_1 represent the nutrient or energy in the initial and final whole body and N_d represents the nutrient or energy in the diet. Feed intake (FI, %/d) was quantified by subtracting uneaten feed from the amount of feed on a dry matter basis. The feed conversion ratio (FCR) was calculated as $FI \times (\text{weight gain, g})^{-1}$. The weight gain rate (WGR, %) was calculated as 100 × (FBW–IBW)/IBW, where FBW and IBW represent final body weight and initial body weight, respectively. The hepatosomatic and viscerosomatic indices (%) were expressed as organ weight as a percentage of body weight, and the condition factor (CF, g/cm^3) was calculated as $100 \times (\text{fish weight, g}) \times (\text{body length, cm})^{-3}$.

All data are presented as means ± pooled S.E.M. (n = 3) and assessed by one-way ANOVA (IBM, SPSS Statistics 20.0), with the level of significance set at $p < 0.05$. Moreover, a follow-up trend analysis using orthogonal polynomial contrasts was performed to determine whether the significant effects were linear and/or quadratic. Quadratic regressions were only presented when the regression coefficient of the second degree component was statistically significant ($p < 0.05$). Maxima or minima in quadratic regressions were calculated by setting the first derivative of the equation to 0. Significant differences in the ANOVA were ranked by the Pdiff routine under LSMEANS and indicated by different superscript letters [a, b, c].

3 Results

3.1 Extrusion parameter and physical pellet quality

The processing parameter and pellet physical quality are presented in Table 5. The V0 diet differed from the other diets, and the total amount of process water was limited to 20% to achieve

TABLE 9 Apparent digestibility coefficient (ADC) of amino acids of Japanese seabass fed different experimental diets.

ADC, %	Diets				Pooled S.E.M.	*P*	Regression model	R^2
	V0	VF1	VF2	VF3				
Arg	93.7	94.2	95.2	94.3	1.52	0.60	—	—
Cys	77.6^{b}	81.7^{ab}	84.8^{a}	86.5^{a}	3.42	0.027	78.2 + 0.0893 x	0.64
His	90.2^{b}	92.0^{ab}	93.3^{a}	93.5^{a}	1.45	0.042	90.6 + 0.0337 x	0.57
Ile	84.5	89.2	89.8	88.3	2.36	0.053	—	—
Leu	87.0^{b}	91.4^{a}	92.0^{a}	90.9^{a}	2.08	0.036	$87.1 + 0.160\ x - 1.23 * 10^{-3}\ x^2$	0.63
Lys	93.2^{b}	94.3^{ab}	95.0^{a}	94.9^{a}	0.68	0.020	93.5 + 0.0175 x	0.57
Met	66.8	71.6	70.3	69.3	3.42	0.30	—	—
Phe	89.1^{b}	92.5^{a}	93.2^{a}	92.8^{a}	1.52	0.017	$89.2 + 0.121\ x - 8.60 * 10^{-4}\ x^2$	0.69
Thr	82.4	86.8	87.7	87.0	2.46	0.055	—	—
Tyr	86.0	90.1	91.0	89.4	2.21	0.057	—	—
Val	84.5	89.1	89.6	88.3	2.45	0.069	—	—
EAAs	87.4^{b}	90.5^{a}	91.3^{a}	90.5^{a}	1.67	0.041	$87.4 + 0.120\ x - 8.93 * 10^{-4}\ x^2$	0.62
Total AAs	87.3^{b}	90.4^{a}	91.5^{a}	91.4^{a}	1.70	0.023	88.1 + 0.0408 x	0.52
	V0	**VS1**	**VS2**	**VS3**				
Arg	93.7	95.0	94.2	95.5	1.63	0.44	—	—
Cys	77.6^{b}	87.7^{a}	86.2^{a}	90.0^{a}	2.90	0.001	80.0 + 0.107 x	0.61
His	90.2	92.7	91.5	92.8	2.01	0.29	—	—
Ile	84.5	90.4	87.7	88.5	4.65	0.41	—	—
Leu	87.0	92.2	89.9	91.4	3.82	0.30	—	—
Lys	93.2	94.9	94.9	95.8	1.09	0.054	—	—
Met	66.8^{b}	74.7^{a}	73.2^{a}	76.7^{a}	3.85	0.031	68.6 + 0.0843 x	0.47
Phe	89.1	93.1	91.3	92.8	2.69	0.22	—	—
Thr	82.4	88.7	86.4	88.8	3.80	0.13	—	—
Tyr	86.0	91.1	88.3	89.9	3.93	0.35	—	—
Val	84.5	90.4	87.4	89.3	4.24	0.29	—	—
EAAs	87.4	91.6	89.8	91.4	2.88	0.22	—	—
Total AAs	87.3	91.6	90.1	92.1	2.49	0.092	—	—

Data are means and pooled S.E.M. different superscript letters ^{a} and^{b} indicate significant ($p < 0.05$) differences among treatments.

a uniform flow through the extruder. The combined effect of dietary composition and restricted water addition resulted in a lower bulk density, breaking point, and slower sinking rate compared with the other diets. The diametric expansion of all diets ranged from 74% to 88% and increased with increasing dietary wheat gluten. All diets had similar durability, with VS1 being the most water-stable and VF1 the least.

3.2 Growth performance and feed utilization

No significant differences were found in FI (Table 6). WGR linearly and significantly decreased from 795% to 627% with increasing dietary GWT. Nitrogen retention significantly decreased from 34.8% to 26.9%, while energy retention decreased from 40.2% to 32.5%. FCR linearly increased from 1.07 to 1.36 as GWT gradually replaced FM. No significant differences were found in WGR or FCR with GWT gradually replacing SPC. The retention of nitrogen and energy linearly decreased with the increase in GWT supplementation.

3.3 Whole-body composition and somatic index

Moisture, protein, lipid, ash, gross energy in the whole body, and the hepatosomatic index and condition factor were not significantly

TABLE 10 Retention of essential amino acids (EAAs) of Japanese seabass fed different experimental diets.

Retention of digestible EAAs, %	Diets				Pooled S.E.M.	*P*	Regression model	R^2
	V0	VF1	VF2	VF3				
Arg	31.7^{a}	31.7^{a}	27.0^{b}	25.4^{b}	1.31	<0.001	32.5–0.0712 x	0.80
His	31.9^{a}	32.5^{ab}	26.2^{bc}	24.4^{c}	1.30	<0.001	33.1–0.0866 x	0.79
Ile	35.0^{a}	34.1^{ab}	30.3^{b}	27.9^{b}	1.36	<0.001	35.6–0.0760 x	0.86
Leu	35.1^{a}	33.7^{ab}	29.2^{bc}	27.0^{c}	1.32	<0.001	35.6–0.0868 x	0.89
Lys	41.5^{a}	42.1^{a}	35.2^{b}	33.5^{ab}	1.60	<0.001	42.7–0.0919 x	0.77
Met	86.7^{a}	81.2^{ab}	77.6^{bc}	71.4^{c}	4.08	<0.01	86.6–0.148 x	0.78
Phe	31.4^{a}	30.0^{ab}	24.1^{bc}	22.4^{c}	1.13	<0.001	31.9–0.0988 x	0.89
Thr	42.2^{a}	40.6^{ab}	35.1^{b}	32.8^{b}	1.71	<0.001	42.7–0.101 x	0.87
Val	36.3^{a}	35.5^{ab}	31.5^{bc}	29.6^{c}	1.47	<0.001	36.8–0.0716 x	0.83
EAA	37.2^{a}	36.5^{ab}	31.2^{bc}	29.1^{c}	1.40	<0.001	37.9–0.0887 x	0.86
	V0	**VS1**	**VS2**	**VS3**				
Arg	31.7	29.6	30.3	29.8	1.12	0.11	—	—
His	31.9	31.0	32.8	33.5	1.31	0.11	—	—
Ile	35.0	32.3	34.5	35.5	2.39	0.32	—	—
Leu	35.1	32.1	33.8	33.8	1.89	0.25	—	—
Lys	41.5	39.2	40.9	40.7	1.31	0.17	—	—
Met	86.7^{a}	66.3^{b}	66.0^{b}	60.5^{b}	4.39	<0.001	85.4–0.572 x + 3.35 * 10^{-3} x^2	0.84
Phe	31.4	28.9	30.0	29.3	1.34	0.12	—	—
Thr	42.2	37.6	39.4	38.6	2.22	0.084	—	—
Val	36.3	32.6	35.0	35.3	2.15	0.18	—	—
EAA	37.2	34.1	35.7	35.5	1.67	0.16	—	—

Data are means and pooled S.E.M. different superscript letters ^{a,b}, and^{c}.indicate significant ($p < 0.05$) differences among treatments.

affected by different dietary GWT supplementation (Tables 7, 8). The viscerosomatic index linearly decreased from 17.3% to 16.1% with the increase of GWT supplementation.

3.4 Apparent digestibility coefficient (ADC) of amino acids

No significant differences were found in the ADCs of Arg, Ile, Met, Thr, Tyr, and Val (Table 9). As GWT gradually replaced FM, the ADC of Cys significantly increased from 77.6% to 86.5%. The ADCs of His, Lys, and total AAs followed a similar pattern and increased with the increase in GWT supplementation. The ADCs of Leu, Phe, and total EAAs increased in a curvilinear mode with increasing proportions of GWT. Except for Cys and Met, the ADCs of essential and total amino acids were not significantly affected by the gradual substitution of SPC by GWT. The ADC of Cys significantly increased from 77.6% to 90.0%, while the ADC of Met significantly increased from 66.8% to 76.7%. The ADCs of Cys and Met were lower in the V0 diet than in the other diets.

3.5 Retention of essential amino acids

The retention of individual and total EAAs significantly decreased in a linear manner (Table 10). The retention of Met was twice or more than the values observed in the other EAAs. The replacement of SPC with GWT had no significant effect on the retention of Arg, His, Ile, Leu, Lys, Phe, Thr, Val, and total EAAs. The retention of Met followed a U-shaped pattern, with the highest value being found in the V0 diet.

4 Discussion

The bulk density of the pellets was optimized by adjusting the amount of water in the preconditioner and extruder so that they slowly sunk into the water (Zhang et al., 2012b). A minimum amount of water (16%) was added during the extrusion of diet V0 (control diet). Diet VF1 had 20% water supplementation and the other diets had 26%–28% water supplementation. The low water supplementation during the extrusion of the V0 diet reduced the bulk density, sink rate, and breaking force of the pellets, which was

different from the VF1 diet. The increase in dietary GWT resulted in greater diametric expansion due to the viscoelastic nature of wheat gluten. This is in accordance with previous findings reported by Draganovic et al. (2011), who found that an increased proportion of wheat gluten to soy protein concentrate led to increased dietary hardness. Additionally, wheat gluten affects extrusion characteristics and physical qualities, including pellet hardness and durability (Day et al., 2006; Wieser, 2007; Apper-Bossard et al., 2013). However, an opposite trend of hardness was observed in diets VF1, VF2, and VF3 when GWT replaced FM, which may be associated with the degree of expansion and water addition (Sørensen et al., 2012; Storebakken et al., 2015b).

In this study, the SGR and FCR of fish fed the high fishmeal diet (V0 diet) were 3.04%/d and 1.10. This growth performance is similar to the previous growth responses (SGR = 3.52), with 52% fish meal-based diets being fed to comparably sized juvenile Japanese seabass (Cheng et al., 2010). Similarly, a study by Xu et al. (2010) reported that Japanese seabass fed a diet with 45% fish meal showed the SGR and FCR were 2.84%/d and 0.99, respectively. Studies have shown that a diet containing 41% protein and 12% lipids is optimal for Japanese seabass juveniles with an initial body weight (IBW) of 6.26 g (Ai et al., 2004). The current results showed that the growth performance of seabass fed 20% fish meal was similar to those fed 40% fish meal, which illustrated the potential of using plant protein in Japanese seabass. This is in keeping with studies in European sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata* L.) reported by Messina et al. (2013); Kissil and Lupatsch (2004), respectively. The lack of significant feeding stimulation in high GWT supplementation suggests that the incorporation of 5% krill meal is needed to provide sufficient marine attractant to ensure the high feeding intake of Japanese seabass. This result is consistent with previous observations in Atlantic salmon (Storebakken et al., 2000a), Atlantic halibut (Helland and Grisdale-Helland, 2006), rainbow trout (Storebakken et al., 2015a), and Asian seabass (Apper et al., 2016), suggesting that small amounts of marine components are required to ensure high feed intake when providing a high level of wheat gluten.

Despite rapid growth and high feed conversion, Japanese seabass fed the control diet showed signs of essential amino acid deficiency. The same situation was also found in fish that were fed diets in which fish meal and SPC were gradually replaced with GWT. The availability of Met by growth mainly depends on digestibility, bioactivity, and catabolism. Low water supplementation during extrusion of the V0 diet resulted in lower digestibility and bioactivity of sulfur-containing amino acids, which led to lower digestibility of Met (66.8%) and Cys (77.6%) in the V0 diet. The digestibility of amino acids in wheat gluten was higher than in LT fish meal, especially Cys, which is consistent with previous findings in Atlantic salmon (Storebakken et al., 2000a). Dietary concentrations of Cys and Met are often reported together mainly due to the fact that sufficient Cys stimulates the downregulation of Met catabolism. However, no significant difference was found in the regression of digestible Cys and Met on nitrogen retention (R_2 = 0.43) in this study. The ratio of digestible Met and Cys (DMDC) was linearly correlated with protein retention (Ret_N = 13.6 + 14.6 * DMDC, R_2 = 0.93). This linear relationship can be expressed by replacing GWT with fish meal (DMDC = 1.49–1.17 * 10^{-2} * Rep, R_2 = 0.973). Regression analyses showed that the highest nitrogen retention was obtained in the diets with a low replacement of protein from FM with GWT. Replacement of SPC with GWT also resulted in increased concentrations and digestibility of Cys and Met. Additionally, the retention of digestible methionine decreased with increasing GWT supplementation, indicating methionine deficiency.

5 Conclusion

The results of the present study indicated that replacing protein in fish meal with wheat gluten reduced growth, feed efficiency, and the retention of nitrogen, energy, and essential amino acids. Replacement protein in soy protein concentrate with wheat gluten had no significant effects on feed intake, growth, and the feed conversion ratio, but resulted in a lower retention of nitrogen, energy, and Met. Moreover, wheat gluten increased amino acid digestibility compared with soy protein concentrate and fish meal. This study highlights that a moderate amount of wheat gluten is a promising dietary protein alternative for Japanese seabass.

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 performed in strict accordance with the Laboratory Animal Welfare Guidelines of China (Decree No. 2 of Ministry of Science and Technology, issued in 1988).

Author contributions

BS: software, validation, writing–review and editing. LW and ZD: software, writing–original draft. SC, LH, and JH: writing–review and editing, supervision. TS: conceptualization, writing–review and editing, supervision. YZ: validation, resources, writing–review and editing, supervision, funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author JH was employed by Dongguan Yihai Kerry Syral Starch Technology Co., Ltd.

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

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Effects of fucoidan on growth performance, immunity, antioxidant ability, digestive enzyme activity, and hepatic morphology in juvenile common carp (*Cyprinus carpio*)

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Fucoidan with its excellent biological activities such as growth promotion, antioxidant and strong immunity, is widely used in animal production. The present study was conducted to investigate the influences of feeding fucoidan on growth performance, biochemical indices, immunity, the antibacterial ability of plasma, the digestive enzyme activity of the intestine, antioxidant capacity, and the histological structure of liver in juvenile common carp. Five experimental diets added with 0 (Diet 1), 500 (Diet 2), 1,000 (Diet 3), 1,500 (Diet 4), and 2,000 (Diet 5) mg/kg fucoidan were fed to triplicate groups of 30 fish (35.83 ± 0.24 g) respectively for 8 weeks. The results showed that fish fed diets with a fucoidan supplementation of 1,666.67–1,757 mg/kg might have the best growth performance ($p< 0.05$). The levels of plasma total protein (TP) and albumin (ALB) in Diet 3, Diet 4, and Diet 5 were higher than those in Diet 1 and Diet 2 ($p< 0.05$). Moreover, the contents of plasma C3, LYZ, and IgM; the antibacterial ability of serum; and the activity of SOD, CAT, POD, and GPX in the liver, and ACP, AKP, LPS, AMS, and TRY in the intestine significantly improved; the contents of LPO and MDA in the liver were notably decreased in diets with fucoidan supplement ($p< 0.05$). Furthermore, the activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and the contents of total bilirubin (TB) and glucose (Glu) in Diet 5 were the highest among the groups. Meanwhile, proinflammatory factors (plasma IL-6 and IL-1β) had a higher expression, but anti-inflammatory factors (plasma IL-1) had a lower expression in Diet 5 ($p > 0.05$). It indicated that a higher dose (2,000 mg/kg) of fucoidan may induce inflammation and metabolic disorders. Interestingly, histological results of liver also indicated that dietary fucoidan intake in certain amounts (500–1,500 mg/kg) could ameliorate hepatic morphology, but the high dosage (2,000 mg/kg) probably damaged the liver. To the best of our knowledge, this is the first

study on the application of fucoidan as a functional additive to juvenile common carp. The results of the present study can be used to guide the application of fucoidan in healthy aquaculture and can further reveal the effect and mechanism of fucoidan on the nutritional physiology of aquatic animals.

KEYWORDS

fucoidan, common carp, growth, immunity, antioxidant

Introduction

Feed supplements are usually used in the aquaculture industry to improve fish growth rate and production. Fucoidan is a type of natural active polysaccharide mainly extracted from marine brown algae (Pomin, 2015) containing sufficient L-fucose and sulfate eater groups (Li et al., 2008). Fucoidan has been found to be effective at growth promotion (Dawood et al., 2018; Fazio et al., 2019) and gastrointestinal function regulation (Cui et al., 2020; Liu et al., 2020), and to have anti-inflammatory (Kirindage et al., 2022; Wang et al., 2022), anti-oxidation (Sony et al., 2019; Abdel-Daim et al., 2020; Mahgoub et al., 2020), anti-tumor (Han et al., 2015a; Han et al., 2015b; Jin et al., 2022), anti-bacterial (Yu et al., 2015; Zhu et al., 2021), and immunomodulatory (Wang et al., 2019; Ikeda-Ohtsubo et al., 2020) properties. Recently, fucoidan has attracted the attention of aquatic animal researchers. However, an extreme limitation is that fucoidan research on aquatic animals has mainly focused on a few species, such as *Penaeus monodon* (Chotigeat et al., 2004; Sivagnanavelmurugan et al., 2014), *Marsupenaeus japonicus* (Traifalgar et al., 2010), *Pelteobagrus fulvidraco* (Yang et al., 2014), *Litopenaeus vannamei* (Sinurat et al., 2016), *Labeo rohita* (Mir et al., 2017; Adnan et al., 2018), *Pagrus major* (Sony et al., 2019), *Carassius auratus* (Cui et al., 2020), and *Oreochromis niloticus* (Abdel et al., 2021), and it has been found to improve the growth rate and stimulate the immune system. Therefore, fucoidan is considered as a promising feed additive to enhance the growth and immunity in aquatic animals.

As a freshwater economic fish, common carp (*Cyprinus carpio*), is one of the key aquaculture species in the world. Common carp production provides an economical, tasty, hyperproteic, healthy food supplement for people around the world. Hence, researchers have focused on improving the growth (Mohammadian et al., 2021; Khorshidi et al., 2022; Zhang et al., 2022), disease resistance (Ahmadifar et al., 2022; Ghafarifarsani et al., 2022) and stress resistance (Banaee et al., 2022; Dawood et al., 2022; Xue et al., 2022b) of common carp. As mentioned earlier, fucoidan as feed additive has remarkable effects on improving growth and immunity, but its application in fish feed research is limited. To the best of our knowledge, there is no available information about the effect of fucoidan on common carp.

Therefore, the present study was conducted to determine the dietary fucoidan effects on growth performance, biochemical parameters, specific and non-specific immunity, antibacterial ability, antioxidant capacity, digestive enzyme activity, and the histological structure of the liver in juvenile common carp, and the results of the study help to determine the optimal amount of fucoidan that could be effectively used in carp culture.

Materials and methods

Diet formulation and preparation

The basal diet was based on soybean meal as carbohydrate sources and fish meal as protein sources. Five isonitrogenous (38%) and isolipidic (6.6%) experimental diets (Table 1) were formulated and supplemented with 0 (Diet 1), 500 (Diet 2), 1,000 (Diet 3), 1,500 (Diet 4), and 2,000 (Diet 5) mg/kg fucoidan (purity ≥98%, Xi 'an Risen Biotechnology Co., LTD., China). The selected dosages of fucoidan were based on the findings of earlier studies (Sony et al., 2019; Cui et al., 2020). All feed ingredients were crushed and sifted by a 60-mesh sieve, weighed, and mixed by a stepwise expansion method to make pellet feed with a diameter of 1 mm, which was dried naturally outside in a drafty place that is not exposed to direct sunlight and then stored at −20°C until feeding.

Experimental fish and design

Common carp juveniles were purchased from an aquafarm (Chongqing, China) and acclimated for 14 days in a cement breeding pond (6 m × 6 m × 0.8 m) feeding with the commercial feed (Tongwei Co. LTD., China) twice a day. After acclimation to the trial conditions, 450 healthy fish (35.83 ± 0.24 g) were randomly assigned to 15 cement breeding ponds (2.1 m × 1.2 m × 0.8 m), each with 30 fish species. The feeding trial was conducted outdoors for 8 weeks in the aquaculture base of Southwest University (Chongqing, China). Fish in triplicate groups were fed the respective experimental diets to apparent satiation twice daily at 8:00 and 17:00. Meanwhile, the feed intake and mortality of fish in each pond were recorded. During the trial, the dissolved oxygen was ≥6.5 mg/L and the water temperature ranged from 25 to 28°C. The total ammonia nitrogen and nitrites remained< 0.05 mg/L, and the pH was kept at 7.4–8.2. The natural photoperiod was applied.

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TABLE 1 Ingredients and proximate nutrient compositions of experimental diets (dry).

Items	Dietary fucoidan supplementations (mg/kg)				
	Diet 1 (0)	Diet 2 (500)	Diet 3 (1,000)	Diet 4 (1,500)	Diet 5 (2,000)
Ingredients (%)					
Fish meal	26	26	26	26	26
Bean pulp	35	35	35	35	35
Rapeseed meal	11	11	11	11	11
Bran	8	8	8	8	8
Flour	12	11.5	11	10.5	10
Soybean oil	4	4	4	4	4
$C_a(H_2PO_4)_2$	1	1	1	1	1
Choline chloride	1	1	1	1	1
Binder	0.50	0.50	0.50	0.50	0.50
1.5% premix	1.50	1.50	1.50	1.50	1.50
Nutrient content (%)					
Crude protein	38.05	38.10	37.81	38.13	37.96
Crude lipid	6.59	6.60	6.65	6.64	6.61
Moisture	9.12	9.14	9.01	9.15	9.06
Ash	16.71	16.59	16.64	16.38	16.50
Met + Cys	1.20	1.21	1.19	1.23	1.21
Lys	2.43	2.41	2.39	2.42	2.43
Thr	1.53	1.55	1.54	1.52	1.51

The compound premix provides vitamins and minerals per kg of feed as below: V_C = 200 mg, V_A = 30,000 IU, V_E = 600 mg, V_{D3} = 25,000 IU, V_{B1} = 50 mg, V_{B2} = 60 mg, V_K = 100 mg, niacin = 100 mg, V_{B12} = 0.2 mg, calcium pantothenate = 120 mg, folic acid = 20 mg, biotin = 7 mg, inositol = 250 mg, $CuSO_4{\cdot}5H_2O$ = 7.20 g, $MnSO_4{\cdot}H_2O$ = 5.16 g, $FeSO_4{\cdot}7H_2O$ = 15.56 g, Na_2SeO_3 = 2.10 g, KI = 6.58 g.

Sampling

At the end of the trial, carps were starved for 24 h, weighed and dissected, and then counted to calculate growth index. Twelve fish were caught randomly from each pond, and anesthetized by applying eugenol for sampling. Among them, six fish were selected for blood collection purposes; their body length and weight (body, viscera, and liver) were then recorded for the purpose of computing the somatic indices. The blood of three fish was drawn from their caudal vein using sterile disposable syringes (2 ml), rinsed with heparin sodium solution. After the centrifugation (3,500 rpm, 15 min, 4°C) of the sample, the plasma was separated and stored at −80°C for biochemical index detection. Non-heparinized blood of 3 fish was centrifuged (3,500 rpm, 15 min, 4°C) to obtain the serum for antibacterial activity test. Three fish from each pond were sacrificed to obtain liver and intestine collected in an ultralow-temperature refrigerator for analysis of enzyme activity. Moreover, three hepatic tissues from each pond were placed in 2-ml centrifuge tubes containing Bouin's solution for micromorphological analysis. After 24 h of fixation, the samples were washed and stored using 70% ethanol solution until the preparation of tissue section.

Determination of plasma biochemical parameters

The assay of plasma biochemical indices in this study comprised alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP), albumin (ALB), total bilirubin (TB), urea nitrogen (UN), creatinine (Cr), glucose (Glu), triglycerides (TG), and total cholesterol (TC), which were all measured by using a fully automated biochemical analyzer (Chemray 240, Shenzhen, China).

Immune, antioxidant, and digestive enzyme indices assay

The content of interleukin-6 (IL-6), interleukin-1β (IL-1β), interleukin-10 (IL-10), complement 3 (C3), lysozyme (LYZ), and immunoglobulin M (IgM) in the plasma; the activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), glutathione peroxidase (GPX), lipid peroxide (LPO), and malonaldehyde (MDA) in the liver; and the activities of acid phosphatase (ACP), alkaline phosphatase (AKP), lipase (LPS), amylase (AMS), and trypsin (TRY) in the intestine were determined by utilizing the

commercial kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). The above kits were carried out in accordance with the manufacturer's specifications.

Antimicrobial activity of serum *in vitro*

The serum from non-heparinized blood was used for antibacterial activity test *in vitro*. For this purpose, *Aeromonas hydrophila* (BBAh1) stored in the Key Laboratory of Freshwater Fish Reproduction and Development (Ministry of Education) (Li et al., 2019) was grown in tryptic soy broth for 24 h at 30°C. Bacterial cells were centrifuged and suspended in sterile PBS and adjusted to an optical density of 0.5 at 546 nm. The diluted bacterial suspension was mixed with serum homogenates for incubation at 37°C for 1 h. After incubation, the number of viable bacterial cells was counted (Eslami et al., 2022).

Liver histological processing

Hepatic samples were fixed in Bouin's solution. Then, the fish tissues were placed in plastic cassettes and processed (gradual dehydration in 70%–100% alcohol, clearing in xylene, and paraffin wax embedding). Five-micron-thick sections were cut on a microtome and then stained with hematoxylin and eosin (HE). Slides were examined with a light microscope (Nikon DXM1200).

Calculations and statistical methods

The growth performance parameters were calculated as follows (Geetanjali et al., 2020; Jayant et al., 2021):

$$\text{Initial body weight (IW, g);}$$

$$\text{Final body weight (FW, g);}$$

$$\text{Final body length (FL, cm);}$$

$$\text{Weight gain(WG, \%)} = (\text{FW} - \text{IW}) \times 100/\text{IW};$$

$$\text{Specific growth rate(SGR, \%/day)} = (\text{Ln FW} - \text{Ln IW}) \times 100/\text{day};$$

$$\text{Feed conversion ratio(FCR)} = \text{total diet fed}/\text{total wet weight gain};$$

$$\text{Hepatosomatic index(HI, \%)} = \text{liver weight} \times 100/\text{body weight};$$

$$\text{Viscera-somatic index(VSI, \%)} = \text{visceral weight} \times 100/\text{body weight};$$

$$\text{Condition factor(CF)} = \text{final weight} \times 100/(\text{final body length});$$

$$\text{Survival rate(SR, \%)} = \text{final quantity} \times 100/\text{initial quantity}.$$

All of the data were expressed as the mean ± SEM. The data were analyzed by one-way analysis of variance and Tukey's multiple range test using SPSS 19.0 (IBM, Chicago, USA). Differences were considered significant at $p< 0.05$. The optimal dietary requirement of fucoidan for juvenile carp was calculated by using broken line regression analysis (Robbins et al., 2006).

Results

Growth performance, feed utilization, and somatic indices

The growth performance, feed utilization, and somatic indices of juvenile common carp fed the experimental diets containing different levels of fucoidan were presented in Table 2. At the end of the 8-week culturing trial, Diet 3, Diet 4, and Diet 5 were higher in FW, WG, and SGR than Diet 1 and Diet 2 ($p< 0.05$). Moreover, FCR in Diet 3 and Diet 4 was significantly lower than in the other diets ($p< 0.05$). VSI in Diet 3 and Diet 4 was significantly higher than in Diet 1, and fish fed diet 1,500 had a higher VSI than fish fed diet 500. Nevertheless, there were nearly no significant changes in HI, CF, and SR among all the treatments ($p > 0.05$). Furthermore, the regression analysis between fucoidan supplementation and WG showed that the optimal dietary content of fucoidan was 1,757 mg/kg (Figure 1), and the regression analysis between fucoidan content and SGR showed that the optimal dietary supplementation of fucoidan was 1,666.67 mg/kg for juvenile common carp (Figure 2). Meanwhile, the survival rate of common carp fed with dietary fucoidan remained unaffected compared to the control group, which was 100% in all diets.

Plasma biochemical parameters

The effects of dietary fucoidan on plasma biochemical parameters of juvenile common carp are presented in Table 3. The highest levels of plasma ALT, AST, TB, and Glu were found in Diet 5, which were statistically higher than other diets ($p< 0.05$). Additionally, plasma TP and ALB in Diet 3, Diet 4, and Diet 5 were higher than that in Diet 1 and Diet 2 ($p< 0.05$), whereas nearly no statistically significant differences were observed in UN, Cr, TG, and TC for any of the treatment ($p > 0.05$).

Immune parameters and antibacterial activity of serum

Table 4 shows that dietary supplementation with fucoidan containing different levels in common carp impacted the immune parameters in plasma. The values of proinflammatory cytokines IL-6 and IL-1β showed a decreasing trend in Diet 2, Diet 3, and Diet 4, and an increasing trend in Diet 5 ($p< 0.05$). Conversely, the values of anti-inflammatory cytokine IL-10 showed an increasing trend in Diet 2 and Diet 3, and a decreasing trend in Diet 4 and Diet 5 ($p< 0.05$). Furthermore, compared with Diet 1, dietary addition of fucoidan significantly improved the C3, LYZ, and IgM content

TABLE 2 Effect of dietary fucoidan supplementations on growth performance, feed utilization, and somatic indices of juvenile *Cyprinus carpio* (N = 18).

Items	Dietary fucoidan supplementations (mg/kg)				
	Diet 1 (0)	Diet 2 (500)	Diet 3 (1,000)	Diet 4 (1,500)	Diet 5 (2,000)
Initial body weight (IW, g)	36.10 ± 0.46	35.27 ± 0.57	35.68 ± 0.75	35.90 ± 0.52	36.20 ± 0.43
Final body weight (FW, g)	129.16 ± 4.51^{a}	148.33 ± 6.13^{b}	170.00 ± 3.89^{c}	182.50 ± 7.50^{c}	178.33 ± 6.72^{c}
Weight gain (WG, %)	259.49 ± 15.52^{a}	322.41 ± 20.16^{b}	378.69 ± 14.48^{c}	409.53 ± 22.34^{c}	393.61 ± 19.58^{c}
Specific growth rate (SGR, %/day)	2.26 ± 0.08^{a}	2.55 ± 0.08^{b}	2.78 ± 0.05^{c}	2.88 ± 0.08^{c}	2.83 ± 0.07^{c}
Feed conversion ratio (FCR)	1.21 ± 0.01^{de}	1.18 ± 0.01^{ce}	1.11 ± 0.01^{ab}	1.07 ± 0.01^{a}	1.14 ± 0.01^{bc}
Hepatosomatic index (HI, %)	2.43 ± 0.19^{ab}	2.56 ± 0.18^{ab}	2.81 ± 0.23^{b}	2.16 ± 0.18^{a}	2.58 ± 0.20^{ab}
Viscera–somatic index (VSI, %)	9.01 ± 0.36^{a}	9.76 ± 0.38^{ab}	9.94 ± 0.45^{ac}	11.19 ± 0.72^{c}	10.80 ± 0.38^{bc}
Condition factor (CF)	2.75 ± 0.06^{ab}	2.67 ± 0.06^{a}	2.87 ± 0.05^{b}	2.71 ± 0.05^{ab}	2.83 ± 0.04^{ab}
Survival rate (SR, %)	100.00	100.00	100.00	100.00	100.00

Data expressed as mean ± SEM.
Different letters in the same line indicate that the indexes are significant differences ($p< 0.05$).

($p< 0.05$). Moreover, the highest value of C3 and LYZ appeared in Diet 3, and the highest value of IgM appeared in Diet 4. Quite noticeably, the number of *A. hydrophila* colonies notably diminished ($p< 0.05$) in the fish serum feeding Diet 3 (cfu = 226 ± 21), Diet 4 (cfu = 301 ± 12), and Diet 5 (cfu = 309 ± 18) compared to Diet 1 (cfu = 362 ± 17) and Diet 2 (cfu = 343 ± 12) (Figure 3).

Antioxidative parameters in liver

As displayed in Table 5, antioxidative parameters in the liver were also affected significantly by dietary fucoidan supplementation. Distinctly, the activity of enzymes in antioxidant systems SOD (in Diet 3 and Diet 4), CAT (in Diet 2 to Diet 5), and POD and GPX (in Diet 3 to Diet 5) was significantly increased by feeding fucoidan ($p< 0.05$). Meanwhile, the content of the peroxidation product LPO (in Diet 3 to Diet 5) and MDA (in Diet 2 to Diet 5) was dramatically decreased by the supplementation of fucoidan ($p< 0.05$).

Digestive enzyme activity in the intestine

The digestive enzyme activity in the intestine was also affected by feeding fucoidan (Table 6). Obviously, all the digestive enzyme activity in Diet 3, Diet 4, and Diet 5 was significantly elevated compared to Diet 1 ($p< 0.05$). Notably, the activity of LPS in Diet 5 was the highest ($p< 0.05$), and the activity of AMY and TRY in Diet 3 and Diet 4 was higher than the other Diets ($p< 0.05$).

Histological observation of liver

The effect of dietary supplementation of fucoidan on the hepatic morphology of juvenile common carp is presented in Figure 4. Compared to Diet 1 and Diet 5, the hepatic tissues of fish feeding Diet 2, Diet 3, and Diet 4 were obviously more intact: hepatocytes were arranged neatly, with a clear cellular boundary and the nucleus mainly in the cell center, and slight vacuolization was observed. In Diet 5, hepatocytes were arranged irregularly, with a blurred cellular

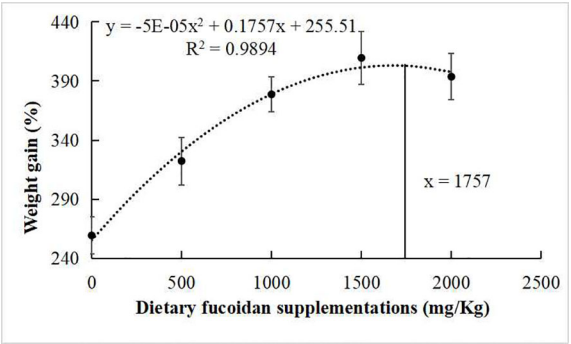


FIGURE 1
Broken line regression analysis between dietary fucoidan supplementation and weight gain of juvenile *Cyprinus carpio*.

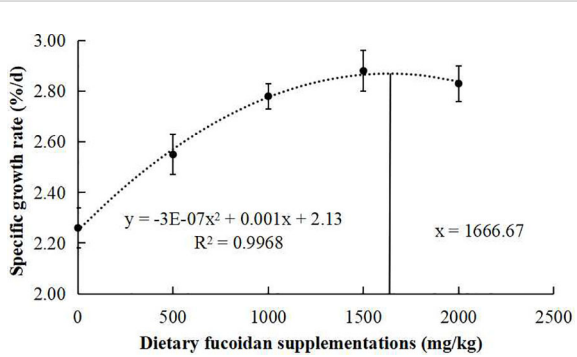


FIGURE 2
Broken line regression analysis between dietary fucoidan content and specific growth rate of juvenile *Cyprinus carpio*.

TABLE 3 Effect of dietary fucoidan supplementations on plasma biochemical indexes of juvenile *Cyprinus carpio* (N = 9).

Items	Dietary fucoidan supplementations (mg/kg)				
	Diet 1 (0)	Diet 2 (500)	Diet 3 (1,000)	Diet 4 (1,500)	Diet 5 (2,000)
Alanine aminotransferase (ALT, U/L)	30.00 ± 0.57^{a}	31.66 ± 3.71^{a}	36.00 ± 1.15^{a}	50.00 ± 1.73^{a}	264.33 ± 32.83^{b}
Aspartate aminotransferase (AST, U/L)	223.66 ± 15.30^{bc}	228.66 ± 1.76^{c}	195.66 ± 10.58^{a}	199.66 ± 2.02^{ab}	294.66 ± 1.20^{d}
Total protein (TP, g/L)	27.00 ± 1.00^{a}	27.00 ± 0.57^{a}	31.33 ± 1.33^{b}	31.33 ± 0.33^{b}	29.66 ± 1.76^{ab}
Albumin (ALB, g/L)	11.33 ± 0.33^{a}	11.33 ± 0.33^{a}	13.66 ± 0.33^{b}	13.66 ± 0.33^{b}	13.33 ± 0.88^{b}
Total bilirubin (TB, μmol/L)	1.56 ± 0.26^{a}	1.50 ± 0.25^{a}	1.43 ± 0.24^{a}	1.66 ± 0.13^{a}	1.90 ± 0.20^{b}
Urea nitrogen (UN, μmol/L)	33.13 ± 0.49^{a}	33.26 ± 0.20^{a}	33.23 ± 0.74^{a}	31.76 ± 0.21^{a}	32.53 ± 0.72^{a}
Creatinine (Cr, μmol/L)	29.66 ± 3.17^{ab}	31.33 ± 1.76^{b}	24.66 ± 1.45^{ab}	20.66 ± 1.20^{a}	27.66 ± 5.69^{ab}
Glucose (Glu, μmol/L)	4.61 ± 0.33^{a}	5.96 ± 0.99^{a}	5.65 ± 0.41^{a}	4.25 ± 0.28^{a}	7.89 ± 0.67^{b}
Triglyceride (TG, μmol/L)	1.95 ± 0.77^{a}	1.52 ± 0.39^{a}	1.43 ± 0.30^{a}	1.99 ± 0.37^{a}	1.16 ± 0.18^{a}
Total cholesterol (TC, μmol/L)	6.43 ± 0.56^{ab}	6.70 ± 0.15a^{b}	6.90 ± 0.80^{b}	6.33 ± 0.31^{ab}	5.20 ± 0.26^{a}

Data expressed as mean ± SEM.
Different letters in the same line indicate that the indexes are significant differences ($p < 0.05$).

boundary and the nucleus located at the cellular periphery (or even disappeared), cellular swelling, severe vacuolization, and even melanin macrophage and lymphocyte infiltration.

Discussion

Fucoidan as a functional supplement with various benefits, such as improved growth rate, feed utilization, immunity, and antioxidant capacity, was used in aquatic animals (Pomin, 2015; Yu et al., 2015). In the current study, the effect of fucoidan, a type of sulfated polysaccharide extracted from brown algae, as a feed additive in the soy protein-based diet for juvenile common carp has been evaluated.

The effect of fucoidan on growth

The growth rate (FW, WG, and SGR) of juvenile common carp supplemented with fucoidan were enhanced compared with the control group in the present study. Similarly, fucoidan additive in the diets of rohu (*L. rohita*) (Mir et al., 2017; Adnan et al., 2018), red sea bream (*P. major*) (Sony et al., 2019), gible carp (*C. auratus*) (Cui et al., 2020), and Nile tilapia (*O. niloticus*) (Abdel et al., 2021) improved growth performance. Notably, the regression analysis between dietary fucoidan supplementation and SGR or WG showed that the optimal dietary content of fucoidan was 1,666.67 mg/kg or 1,757 mg/kg for the growth of juvenile common carp in this study. However, the best recipes of fucoidan in *P. major* (0.4%) (Sony et al., 2019), *C. auratus* (30 g/kg) (Cui et al., 2020), and *L. rohita* (2%) (Mir et al., 2017) are very different. Therefore, a study on the appropriate amount of fucoidan added to different fishes was very important and necessary.

The effect of fucoidan on immunoregulation

In the present study, dietary fucoidan could significantly improve the immunity of juvenile common carp by downregulating the level of proinflammation factors (IL-6 and IL-1β) and enhancing the specific (IgM) and non-specific (lysozyme and C3) immunity. Analogously, immune cells' activation and the

TABLE 4 Effect of dietary fucoidan supplementations on plasma immune parameters of juvenile *Cyprinus carpio* (N = 9).

Items	Dietary fucoidan supplementations (mg/kg)				
	Diet 1 (0)	Diet 2 (500)	Diet 3 (1,000)	Diet 4 (1,500)	Diet 5 (2,000)
Interleukin-6 (IL-6, ng/L)	24.06 ± 0.61^{d}	20.89 ± 0.51^{c}	16.73 ± 0.42^{b}	13.29 ± 0.48^{a}	27.66 ± 0.38^{e}
Interleukin-1β (IL-1β, ng/L)	76.78 ± 1.60^{c}	64.60 ± 1.51^{b}	40.08 ± 1.2^{a}	62.79 ± 1.61^{b}	119.69 ± 1.55^{d}
Interleukin-10 (IL-10, ng/L)	487.65 ± 6.72^{c}	566.16 ± 5.58^{d}	587.49 ± 4.90^{e}	381.85 ± 6.26^{b}	335.674 ± 6.16^{a}
Complement 3 (C3, μg/ml)	315.84 ± 5.10^{a}	334.02 ± 5.57^{b}	1082.69 ± 5.59^{e}	856.14 ± 5.85^{d}	792.31 ± 6.53^{c}
Lysozyme (LYZ, μg/ml)	34.22 ± 3.74^{a}	40.58 ± 3.22^{ab}	66.07 ± 3.83^{c}	46.26 ± 3.23^{b}	55.05 ± 3.16^{b}
Immunoglobulin M (IgM, μg/ml)	844.99 ± 6.92^{a}	848.34 ± 7.12^{a}	872.26 ± 5.92^{b}	896.91 ± 6.29^{c}	891.81 ± 5.66^{bc}

Data expressed as mean ± SEM.
Different letters in the same line indicate that the indexes are significant differences ($p < 0.05$).

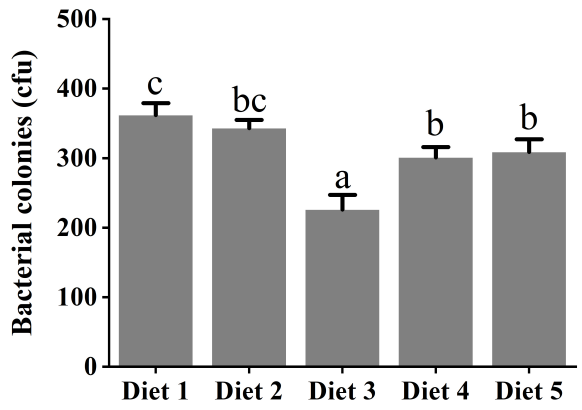


FIGURE 3
Challenge with *Aeromonas hydrophila* in serum of juvenile *Cyprinus carpio* fed dietary fucoidan. Different superscript letters indicate marked differences among the groups ($p< 0.05$). Diet 1, Diet 2, Diet 3, Diet 4, and Diet 5 refer to fish fed diets with varying levels of fucoidan (0, 500, 1,000, 1,500, and 2,000 mg/kg).

high expression of IgM, IgG, and IgA in plasma were observed in mice with dietary fucoidan (Makoto et al., 2019). The results of cytokines in this study showed that IL-6 and IL-1b decreased in Diets 2, 3, and 4, and increased in Diet 5. On the other hand, IL-10 increased in Diets 2 and 3, but decreased in Diets 4 and 5. Therefore, we could speculate that fucoidan might regulate the level of inflammation and have a proinflammatory effect at a high dose, which means that the dosage of fucoidan added in the feed should not be that high; otherwise, it will have adverse effects. Obviously, fucoidan could regulate the expression of inflammatory cytokines by activating inflammation-related signaling pathways, such as the NF-kB and eNOS signaling pathways (Wang and Chen, 2015; Caroline et al., 2019). Moreover, the survival rate of fry of *L. rohita* infected with *A. hydrophila* was significantly improved, and the content of lysozyme in plasma was also markedly increased (Adnan et al., 2018). Interestingly, we found that plasma lysozyme activity was negatively correlated with serum antimicrobial capacity in this study, which means that lysozyme activity in plasma is an important index to measure the antibacterial ability of the body (Kord et al., 2021).

The effect of fucoidan on antioxidant activity

The antioxidant system consisted of enzymes (SOD, CAT, POD, and GPX) and non-enzyme (glutathione and vitamins E and C) components, which jointly regulated the antioxidant level and maintained homeostasis (Sony et al., 2019; Abdel-Daim et al., 2020). In addition, the lipid peroxidation product LPO and its decomposition product MDA could directly reflect the rate and degree of lipid peroxidation in tissues (Mahgoub et al., 2020). In this regard, we evaluated the antioxidant capacity of the liver tissue in juvenile common carp fed with dietary fucoidan. The results showed that feeding with fucoidan significantly increased the activities of SOD, CAT, POD and GPX, and decreased the levels of LPO and MDA in common carp. Similarly, *P. fulvidraco* fed with dietary fucoidan could increase SOD and CAT activities and decrease MDA content (Yang et al., 2014). It was found that the antioxidant activity of fucoidan usually played a role through molecular pathways, such as MAPK (Kim et al., 2011; 2012), Keap1-Nrf2-ARE (Zhang et al., 2012), PI3K-Akt (Han et al., 2015a; 2015b), and the TLR-NFkB signaling pathway (Asanka et al., 2019).

The effect of fucoidan on hepatic function

The liver function could be regulated by using proper feed supplements in aquafeeds (Hemre et al., 2002), which would be reflected in antioxidant levels (Mohammadi et al., 2021), inflammation index (Yang et al., 2021), and the histological structure of the liver (Cui et al., 2020). ALT and AST levels in liver were much higher than those in plasma, but liver cell necrosis could lead to ALT and AST levels that are two times higher than plasma. Therefore, plasma ALT and AST could serve as indices to evaluate the degree of hepatic damage (Josekutty et al., 2013). Previous studies have documented that the activities of plasma ALT and AST restored the normal level in mice fed the diet containing 100 mg/(kg·day) fucoidan after BCG vaccine and lipopolysaccharide-induced immunological liver injury; it suggested that fucoidan could significantly reduce the necrosis of hepatocytes and protect the liver

TABLE 5 Effect of dietary fucoidan supplementations on liver antioxidant activities and oxidative stress of juvenile *Cyprinus carpio* (N = 9).

Items	Dietary fucoidan supplementations (mg/kg)				
	Diet 1 (0)	Diet 2 (500)	Diet 3 (1,000)	Diet 4 (1,500)	Diet 5 (2,000)
Superoxide dismutase (SOD, U/L)	44.70 ± 2.96^{a}	48.99 ± 2.51^{a}	61.75 ± 3.65^{b}	64.37 ± 3.16^{b}	50.77 ± 2.45^{a}
Catalase (CAT, U/L)	29.40 ± 1.91^{a}	34.70 ± 1.38^{b}	34.20 ± 1.10^{b}	36.20 ± 1.41^{b}	37.6 ± 1.46^{b}
Peroxidase (POD, U/L)	45.91 ± 2.34^{a}	45.40 ± 2.14^{a}	54.33 ± 2.16^{b}	57.68 ± 1.54^{b}	53.77 ± 1.62^{b}
Glutathione peroxidase (GPX, U/L)	63.50 ± 1.54^{a}	67.90 ± 1.52^{a}	101.00 ± 1.65^{c}	104.00 ± 1.61^{c}	74.80 ± 1.33^{b}
Lipid peroxide (LPO, U/L)	13.90 ± 0.72^{c}	13.30 ± 0.72^{c}	9.90 ± 0.65^{b}	6.99 ± 0.71^{a}	10.70 ± 0.40^{b}
Malonaldehyde (MDA, U/L)	16.30 ± 0.48^{d}	14.00 ± 0.54^{b}	7.19 ± 0.36^{c}	5.32 ± 0.43^{a}	13.60 ± 0.23^{b}

Data expressed as mean ± SEM.
Different letters in the same line indicate that the indexes are significant differences ($p< 0.05$).

TABLE 6 Effect of dietary fucoidan supplementations on digestive enzyme activity in the anterior intestine of juvenile *Cyprinus carpio* (N = 9).

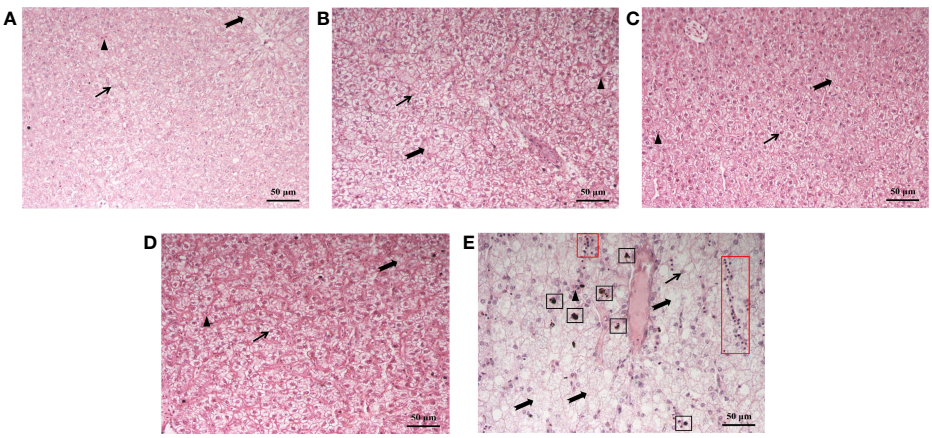
Items	Dietary fucoidan supplementations (mg/kg)				
	Diet 1 (0)	Diet 2 (500)	Diet 3 (1,000)	Diet 4 (1,500)	Diet 5 (2,000)
Acid phosphatase (ACP, U/mgprot)	0.39 ± 0.01^{a}	0.39 ± 0.01^{a}	0.45 ± 0.02^{b}	0.45 ± 0.01^{b}	0.45 ± 0.01^{b}
Alkaline phosphatase (AKP, U/mgprot)	0.60 ± 0.02^{a}	0.69 ± 0.03^{b}	0.71 ± 0.02^{b}	0.71 ± 0.03^{b}	0.69 ± 0.02^{b}
Lipase (LPS, U/mgprot)	0.07 ± 0.01^{a}	0.05 ± 0.01^{a}	0.12 ± 0.01^{b}	0.15 ± 0.01^{b}	0.21 ± 0.02^{c}
Amylase (AMS, U/mgprot)	0.73 ± 0.01^{a}	0.82 ± 0.02^{b}	0.90 ± 0.02^{c}	0.89 ± 0.03^{c}	0.88 ± 0.02^{bc}
Trypsin (TRY, U/mgprot)	216.30 ± 15.01^{a}	197.43 ± 14.43^{a}	361.86 ± 18.47^{c}	382.60 ± 13.27^{c}	283.48 ± 15.58^{b}

Data expressed as mean ± SEM.
Different letters in the same line indicate that the indexes are significant differences ($p < 0.05$).

(Xiao et al., 2017). Opposite results in the present study showed that the activity of plasma ALT and AST, and the content of TB and Glu were significantly increased in Diet 5 compared to other diets. It indicated that a higher dose (2,000 mg/kg) of fucoidan may induce liver injury and metabolic disorders. Moreover, hepatic micromorphology can be used to assess the healthy status of the liver. In our study, histological observation of the liver showed that the diet with 500–1,500 mg/kg fucoidan could ameliorate the morphology of hepatic tissue to a certain extent, such as reducing vacuolation, inflammatory response, and improving blood circulation in the liver. These findings clearly show that fucoidan may protect the liver, whose mechanism may be associated with the suppression of hepatocyte apoptosis, intrahepatic inflammation, and antioxidant activity (Ahmad et al., 2022; Xue et al., 2022a), whereas a high dosage of fucoidan, particularly at 2,000 mg/kg, led to the increase of plasma ALT and AST levels, as well as significant pathological changes in liver tissue. Thus, it indicated that the addition of low dosage of fucoidan could ameliorate the hepatic morphology of juvenile common carp, whereas the high dosage probably caused further damage to the liver.

The effect of fucoidan on digestive enzyme activity

ACP was one of the signature enzymes of lysosomes, and its activity could represent the intensity of intracellular digestion in the intestine (Maritza et al., 2012; Zacarias et al., 2013). AKP could directly participate in the transfer of phosphate groups and play a key regulatory role in metabolism. Furthermore, AKP was also an important immunoreactive enzyme, and its activity was often used as a key indicator to diagnose foreign pathogens and environmental pollution (Jean, 2020; Singh and Lin, 2021). In the present study, the activity of ACP, AKP LPS, AMS, and TRY in the intestine was notably enhanced in groups feeding fucoidan, which indicated that fucoidan could effectively improve the digestive ability of the intestine of juvenile common carp. Similar results were observed in *C. auratus* feeding fucoidan (Cui et al., 2020). Of course, the intestinal digestive function was closely related to intestinal microbial composition (Wu et al., 2019; Amenyogbe et al., 2022; Chang et al., 2023). Thus, future studies are needed to explore the effect of fucoidan on intestinal microorganisms of common carp.



Data availability statement

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

Ethics statement

The animal study was reviewed and approved by the Committee of Laboratory Animal Experimentation at Southwest University.

Author contributions

FL and HS conceived the ideas and designed the methodology. YL, DH, and CR caught and dissected fish individuals. FL and CZ collected data. FL and GL analyzed data. FL and HS led the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Growth, biochemical indices and transcriptomic profile of Chinese mitten crab (*Eriocheir sinensis*) respond to different ratios of dietary carbohydrates to lipids

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Introduction: Although carbohydrates and lipids are important energy substances for Chinese mitten crab (*Eriocheir sinensis*), little is known about their synergistic effect on the growth, energy utilization characteristics and mechanisms involved in this process.

Methods: A 58-d feeding experiment was conducted to investigate the effects of dietary carbohydrate to lipid ratio (C/L) on the growth performance, biochemical indices, and metabolism-related differential gene expression of juvenile *E. sinensis* in both intermolt (InM) and premolt (PrM) stages. Five experimental diets were formulated with increasing dietary C/L (1.34, 2.39, 3.59, 5.52 and 9.42).

Results: The results showed that the weight growth rate of juvenile *E. sinensis* was highest in dietary C/L3.59 group, which was significantly higher than that in the other groups. As dietary C/L increased, the hepatic glycogen contents increased, but triglyceride contents decreased in the hepatopancreas of E. sinensis in the InM. In both two molting stages, the activities of glycogen synthase and fatty acid synthase paralleled with their contents, respectively. Crabs in the InM showed higher contents of triglyceride and the activities of glycolytic rate-limiting enzymes but lower contents of hepatic glycogen than those in the PrM, especially in the C/L 1.34 and C/L 3.59 groups. In all dietary groups, the activities and transcription of gluconeogenesis and fatty acid synthesis related enzymes were significantly higher in the InM than those in the PrM. KEGG analysis showed that differential genes were enriched in fatty acid biosynthesis, fatty acid metabolism, oxidative phosphorylation pathway, pentose phosphate pathway, pyruvate metabolism and steroid biosynthesis between different dietary groups and molting stages.

Discussion: To conclude, the optimal dietary C/L was estimated to be 3.59 for juvenile *E. sinensis* based on the survival and growth performance. Compared to PrM, E. sinensis in the InM was more active in the carbohydrate metabolism (glycolysis and gluconeogenesis) and fatty acid synthesis, with more triglyceride and less glycogen accumulated in the hepatopancreas.This study could contribute to better understanding the carbohydrate and lipid metabolism

between different molting stages, and optimizing the precise feed formulation for juvenile *E. sinensis*.

KEYWORDS

carbohydrate to lipid ratio, *Eriocheir sinensis*, growth performance, biochemical indices, transcriptomic analysisprofile

1 Introduction

Because of high nutritional value and delicious taste, Chinese mitten crab (*Eriocheir sinensis*) has been very popular among Chinese consumers for centuries. In recent years, there is a rapid development of crab aquaculture in China, the production of Chinese mitten crab has been steadily increasing, with the China Fisheries Yearbook reporting that it has reached 800 thousand tons per year (Song et al., 2019). Molting is necessary for the growth, development, and reproduction of *E. sinensis* Panganiban et al., 1995; Jung et al., 2013; Huang et al., 2015).

Protein is an important nutrient for sustaining the normal growth and physiological process of aquatic animals (Johnston et al., 2003). The ever-increasing price of protein ingredients such as fish meal and soybean meal seriously limits the sustainable development of aquaculture industry (Moreira et al., 2008; Lee et al., 2012). As non-protein energy sources, carbohydrates and lipids have the characteristics of low price, easy access and low nitrogen pollution (Gao et al., 2010; Wang et al., 2014). Furthermore, carbohydrates and lipids are closely related to the nutritional metabolism and immunological regulation of aquatic animals (Nakano et al., 1998; Borba et al., 2006; Dong et al., 2018). However, excessive dietary lipids and carbohydrates can cause metabolic disorder, reduced growth rate and even threaten the health status of aquatic animals (Borges et al., 2009; Zhang et al., 2013; Qiang et al., 2017; Li et al., 2020).

It is considered that carbohydrates and lipids have an inseparable close relationship between each other, and an imbalance may negatively affect the growth, feed conversion, and body composition of aquatic animals (Chen et al., 2021; Miller et al., 2023). Carbohydrates are converted into lipid and stored in the body when their contents beyond the optimal requirement for energy supply (Chen et al., 2021). Similarly, lipids can replace carbohydrates for energy supply when the carbohydrate content is insufficient (Meng et al., 2013). Therefore, the steady state of carbohydrates and lipids metabolism is particularly important. It was previously found that the supplementation of carbohydrates or lipids can improve the growth performance and disease resistance of *E. sinensis* (Chen et al., 2016; Wen et al., 2021). Molting is an important biological process closely related to the growth of crustaceans. The molting cycle could be divided into three vital stages including intermolt (InM), premolt (PrM) and postmolt (PoM) (Gao et al., 2015). The premolt (PrM) is a preparation stage for upcoming molting and energy consumption, and the intermolt (InM) is the longest period in a molting cycle during which accumulates energy for next molting (Huang et al., 2015). However, to the best of our knowledge, little is known about the synergistic effect of carbohydrates and lipids on the growth and dietary administration and mechanisms involved in this process. Thus, this study was conducted to investigate the effects of the dietary carbohydrate to lipid ratio (C/L) on the survival, growth, biochemical indices in *E. sinensis*. Furthermore, digital gene expression (DGE) analysis was used as a transcriptome sequencing method to measure high-throughput relative gene expression, and to identify genes related to glucose metabolism (glycolysis, gluconeogenesis and glycogen synthesis) and lipid metabolism (fatty acid synthesis and fatty acid oxidation) in *E. sinensis* at different molting stages.

The goals of this study were to determine: i) the optimal C/L for juvenile *E. sinensis*; ii) the characteristics of energy utilization at different molting stages; and iii) preliminary mechanisms involved in carbohydrates and lipids metabolism in *E. sinensis*.

2 Materials and methods

2.1 Ethics statement

In this study, all the operational procedures were granted by ethical rules of Dalian Ocean University and relevant rules of China.

2.2 Experimental diets

The ratios of different C/L in the diets were designed according to Li et al. (2022). Five isoproteic and isoenergetic feeds with different ratios of C/L were formulated by adjusting the amounts of soybean oil and corn starch in the formulation (Table 1) (), which were named C/L1.34, C/L2.39, C/L3.59, C/L5.52, and C/L9.42, respectively.

The feeds were manufactured by following the procedures described by Luo et al. (2008). The solid ingredients (<150 μm) were first mixed evenly, which were then mixed well with the oil and water. After that, a twin screw granulator (Jinan Dingrun Machinery Company, Jinan, China) was used to produce feed pellets (1.5 mm ×1.0 mm). After dying, the feeds were cooled, packed, and stored at -20°C.

TABLE 1 Ingredients and nutrient composition of the experimental diets.

Ingredients	Dietary carbohydrate to lipid ratio				
	1.34	2.39	3.59	5.52	9.42
Fish meal[a]	23.00	23.00	23.00	23.00	23.00
Soybean meal[b]	20.00	20.00	20.00	20.00	20.00
Casein	12.00	12.00	12.00	12.00	12.00
Beer yeast[c]	5.00	5.00	5.00	5.00	5.00
Soybean lecithin	0.50	0.50	0.50	0.50	0.50
Mineral mixture[d]	2.00	2.00	2.00	2.00	2.00
Vitamin mixture[e]	2.00	2.00	2.00	2.00	2.00
Monocalcium phosphate	1.00	1.00	1.00	1.00	1.00
Choline chloride	0.20	0.20	0.20	0.20	0.20
Chromium sesquioxide	0.10	0.10	0.10	0.10	0.10
Calcium propionate	0.10	0.10	0.10	0.10	0.10
Ethoxyquin	0.01	0.01	0.01	0.01	0.01
Soybean oil	9.00	6.00	4.00	2.00	0.00
Corn starch[f]	10.50	18.00	23.50	28.50	33.70
Microcrystalline cellulose	14.59	10.09	6.59	3.59	0.39
Proximate analysis					
Crude protein	39.78	39.81	39.82	39.84	39.85
Carbohydrate	16.54	23.80	28.33	33.51	36.93
Crude lipid	12.34	9.96	7.89	6.07	3.92
Energy (MJ/kg)	17.53	17.49	17.53	17.50	17.50

[a]Fish meal: crude protein 68.1% dry matter, crude lipid 10.2% dry matter, Qingdao Qihao Biotechnology Company (Qingdao, Shandong Province, China).
[b]Soybean meal: crude protein 43.4% dry matter, crude lipid 1.9% dry matter, Qingdao Qihao Biotechnology Company (Qingdao, Shandong Province, China).
[c]Beer yeast: crude protein 42.6% dry matter, crude lipid 1.0% dry matter, Jinan Huamu Feedstuff Company (Jinan, Shandong Province, China)
[d]Mineral mixture (mg or g kg-1 diet): CuSO4·5H2O, 10 mg; Na2SeO3 (1%), 25 mg; ZnSO4·H2O, 50 mg; CoCl2·6H2O (1%), 50 mg; MnSO4·H2O, 60 mg; FeSO4·H2O, 80 mg; Ca (IO3)2, 180 mg; MgSO4·7H2O, 1200 mg; zeolite, 18.35 g.
[e]Vitamin mixture (mg or g kg-1 diet): vitamin D, 5 mg; vitamin K, 10 mg; vitamin B12, 10 mg; vitamin B6, 20 mg; folic acid, 20 mg; vitamin B1, 25 mg; vitamin A, 32 mg; vitamin B2, 45 mg; pantothenic acid, 60 mg; biotin, 60 mg; niacin acid, 200 mg; α-tocopherol, 240 mg; inositol, 800 mg; ascorbic acid, 2000 mg; microcrystalline cellulose, 16.47 g.
[f]Corn starch: carbohydrate 85.1% dry matter, Shenyang Leishi Starch Co. Ltd. (Shenyang, Liaoning Province, China).

2.3 Feeding procedures

Crabs were purchased from Jiangsu Haitong Aquatic Products Co. Ltd. (Nantong, China) and transported to the experimental base of Dalian Ocean University. After two weeks of acclimation, healthy and intact crabs (initial body weight: 1.09 ± 0.01 g) were randomly allocated to 15 plastic tanks (96L). Each tank was stocked with 25 individuals. Each diet was assigned to three tanks (25 crabs/tank) at random. Plastic tubes and nets were used as shelters to avoid cannibalism between individuals.

E. sinensis juveniles were fed to apparent satiation at 9:00 and 18:00 every day. At the beginning of feeding, a small number of feeds were thrown into the tanks to attract the attention of crabs. Crabs gathered quickly and ingest the feeds. When most of them dispersed, it indicated that crabs approached to the state of satiation.The residual feeds, feces, shells, and carcass in the tanks were cleaned up every day by syphoning. In total, 2/3 of the water was exchanged every two days. The following water conditions were maintained during the 58d-feeding experiment: temperature, 18–22°C; dissolved oxygen, above >8 mg/L; and ammonia-N, below 0.05 mg/L.

2.4 Sampling procedures

Experimental animals were counted and weighed following a 24h period of starvation. Before sampling, food intake and activity of animals were monitored every day. Crabs with vigorous food intake were thought to be at the intermolt (InM). When the food intake gradually decreased and then stopped, they were thought to be at the intermolt (InM).

In each tank, three crabs in intermolt (InM) and premolt (PrM) were chosen out and placed in an ice box for anesthesia. Subsequently, hepatopancreas were dissected and pooled into the sterile centrifuge tube. The hepatopancreas were used to determine the contents of biochemical indices, activities of metabolic enzymes,

and high-throughput relative gene expression. All tubes with samples were frozen by liquid nitrogen and then stored at -80°C.

2.5 Proximate analysis

The contents of moisture, crude protein, lipid and ash were analyzed following the AOAC (1995). All the samples were dried to constant weight at 105°C to calculate moisture contents. Then, Kjeldahl method was used to determine the protein contents. Soxhlet method was used to determine the lipid contents. Ash was determined by calculating the remaining weight of samples after they were burned at 550°C. Finally, the contents of carbohydrates in a sample were calculated by subtracting the weight of moisture, protein, lipid and ash.

2.6 Determination of biochemical indices and metabolic enzymes activities of hepatopancreas

The hepatopancreas was mixed with freezing saline (0.85% NaCl) at a ratio of 1/9, which was then homogenated under the ice-water bath. Then, the homogenate was centrifuged (9000 g) at 4°C for 10 min. After that, the supernatant was separated and transferred into new centrifuge tubes. The supernatant was then analyzed for the biochemical indices and metabolic enzymes.

The concentration of biochemical indices including hepatic glycogen (HG) and triglyceride (TG), and the activities of metabolic enzymes including glycogen synthase (GS), hexokinase (HK), pyruvate kinase (PK), fatty acid synthesis (FAS), acetyl-CoA carboxylase (ACC), phosphoenolpyruvate carboxykinase (PEPCK), and carnitine palmitoyltransferase (CPT) were measured by following the instructions of the kits of Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.7 RNA-Seq and differential expression analysis

The transcriptome sequencing of the hepatopancreas of *E. sinensis* at the InM and PrM stages in the C/L1.34, C/L3.59 and C/L9.42 groups was performed by using the Illumina Nova seq 6000 (Biomarker Technologies, Beijing, China). The transcriptome assembly was done with DIAMOND, and the assembled unigenes were then annotated based on multiple databases, including Nr (NCBI non-redundant protein sequences), Swiss-Prot (a manually annotated and reviewed protein sequence database), KOG/COG (clusters of orthologous groups of proteins), GO (Gene Ontology) and Pfam (a large collection of protein families). Q30 was used as an indicator to measure the quality of sequencing data (Kozich et al., 2013). The unigenes were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to annotate their potential metabolic pathways. Differentially expressed gene sets were obtained from the different samples using the DESeq2 software. To identify differentially expressed genes (DEGs) across samples, the fold change (the ratio of expression levels between two samples) ≥1.5 and p value<0.05 were set to be the thresholds.

2.8 Real-time PCR of related genes

Trizol (TIANGEN, China) was used to extract total RNA from the hepatopancreas of *E. sinensis*, and then the gDNA Eraser (Takara, Japan) was used to remove gDNA contamination in the first reaction of cDNA synthesis. The first strand of cDNA was synthesized by using 1 μg total RNA as template and oligo dT-adaptor as primers according to the protocol of manufacturer (TaKaRa, China). The synthesis reaction was performed at 37°C for 15 min, and terminated by heating at 85°C for 5 s. After the integrity was checked, total RNA was reverse transcribed to cDNA, which was used for the templates of RT-PCR. Fast Start Essential DNA Green Master was used to prepare the reaction system by following the instructions. The primer sequences can be referred in Table 2. A LightCycler®96 (Roche group, Basel, Switzerland) was used to perform the RT-PCR, which was programmed as follows: 95°C (10 min); 95°C (15 s), 60°C (60 s) for 40 cycles; 95°C (10 s), 65°C (60 s); and 97°C, 1 s. The $2^{-\Delta\Delta CT}$ method (Dhanasekaran et al., 2010) was used to calculate the relative mRNA expression levels.

2.9 Formulas and statistical analysis

$$\text{Weight growth rate (WGR, \%)} = \left(W_f - W_i\right) \times 100/W_i$$

$$\text{Survival rate (SR, \%)} = N_f \times 100/N_i$$

Where W_i and W_f are the initial and final average weights of crabs in each tank, respectively. N_i and N_f are the initial and final numbers of crabs in each tank, respectively.

The interaction effects between dietary C/L and molting stage were analyzed by a two-way analysis of variance (ANOVA) in SPSS 23.0 (Redmond, WA, USA) for Windows. All data was presented in the form of means ± standard error (n=3). If a statistical significance ($P < 0.05$) was detected, Tukey's multiple range test was applied to compare the means between dietary groups. Statistical significance was considered when P values <0.05.

3 Results

3.1 Survival rate and growth performance

The SR of *E. sinensis* was higher than 90%, with no statistical significance observed between dietary groups ($P > 0.05$). The WGR was significantly affected by dietary C/L ($P < 0.05$). The highest WGR was observed in the C/L3.59 group (131%), which was significantly higher than that in the C/L1.34, C/L2.39, C/L5.52 and C/L9.42 groups ($P < 0.05$) (Table 3).

10.3389/fmars.2023.1176976

TABLE 2 Primer sequences of the genes used for real-time PCR.

Genes	Position	5′–3′ Primer sequence	Accession No.
FAS	Forward	AGGTTCACCACAATGCCAAAATTGG	VN_GLEAN_10001849
	Reverse	GCTTCCTTGAGAGTGTCTTCATG	
G6PD	Forward	GCAAGATCTGACCTTACCATTGAGC	*Eriocheir_sinensis*_newGene_23950
	Reverse	GGCTTTTTCCGTTCCAACCTTCG	
PEPCK	Forward	ACCCCAACTCCCGCTTCTGTAC	VN_GLEAN_10005379
	Reverse	CATGATGACCTTGGCCTTGTGTTC	
Ndufa6	Forward	CCCCAAGAATGAGAGAAGATGGAC	VN_GLEAN_10003252
	Reverse	GTGACTATTCTTTCCTCTGCCGC	
CPT	Forward	TGTTGAAGCCTGACCTTCCA	MH037158
	Reverse	GGTTGTAGCAGCAGCCATAC	
ACAA2	Forward	CACCCTACGCTGTCAGGAACATTC	VN_GLEAN_10001117
	Reverse	CAGACTCCATTCACTACTGAACAAGC	
Elovl6	Forward	TACTTCGTACTGTTCGCTCGCTT	KT779219
	Reverse	TTACCCTTGGTGCTCTTTCCTT	
Aco	Forward	CTCAGAAGCGTTCAATGCGTTAAGG	*Eriocheir_sinensis*_newGene_17770
	Reverse	GGTGAGGAGACCAAAACTGTACC	
Acly	Forward	CAAACTGTCCTGTCCACTCATACG	VN_GLEAN_10005945
	Reverse	GGGGATAGGTGTTTGGAAGATTGTG	
β-actin	Forward	GCATCCACGAGACCACTTACA	KM244725.1
	Reverse	CTCCTGCTTGCTGATCCACATC	

FAS, fatty acid synthesis; G6PD, glucose-6-phosphatedehydrogenase; PEPCK, phosphoenolpyruvate carboxykinase; Ndufa6, NADH, ubiquinone oxidoreductase subunit A6; CPT, carnitine palmitoyltransferase; ACAA2, acetyl-coenzyme A acyltransferase 2; Elovl6, elongase of very long chain fatty acid 6; Aco, acetyl coenzyme A oxidase; Acly, ATP citrate lyase.

3.2 Glycogen and triglyceride contents in the hepatopancreas

There was no significant interactive effect ($P > 0.05$) between dietary C/L and molting stage on the contents of hepatic glycogen and triglyceride in the hepatopancreas of *E. sinensis*. In the two molting stages, the hepatic glycogen contents significantly increased with increasing dietary C/L ($P < 0.05$). The highest hepatic glycogen contents were observed in dietary C/L9.42 groups in the two molting stages, which were significantly higher than that in the C/L1.34 group ($P < 0.05$). In dietary C/L3.59 groups, hepatic glycogen contents in the InM were significantly higher than that in the PrM ($P < 0.05$) (Figure 1A).

As the dietary C/L increased, triglyceride contents in the InM showed a decreased tendency ($P > 0.05$). In all dietary C/L groups, triglyceride contents in InM were all higher than that in PrM, with statistical significance only observed in the C/L3.59 group ($P < 0.05$) (Figure 1B).

3.3 Metabolic enzymes activities of hepatopancreas

There was a significant interaction between dietary C/L and molting stage on the activities of HK, PK and FAS in juvenile *E.*

TABLE 3 Effects of different dietary carbohydrate to lipid ratio (C/L) on weight gain rate (WGR) and survival rate (SR) of juvenile *E. sinensis*.

Dietary C/L	W0	W1	WGR (%)	SR (%)
1.34	1.09 + 0.00	2.30 + 0.02	112.11 + 1.34^{b}	92.00 + 0.00
2.39	1.10 + 0.01	2.27 + 0.17	108.27 + 2.97^{ab}	96.00 + 2.31
3.59	1.10 + 0.01	2.54 + 0.02	131.34 + 0.21^{c}	97.33 + 0.21
5.52	1.10 + 0.01	2.23 + 0.05	111.47 + 2.35^{b}	92.00 + 4.62
9.42	1.10 + 0.02	2.19 + 0.13	100.75 + 6.61^{a}	96.00 + 2.31

Values are presented as means ± standard error (SE) (n=3). Values with different superscript letters in the same column are significantly different at P<0.05.

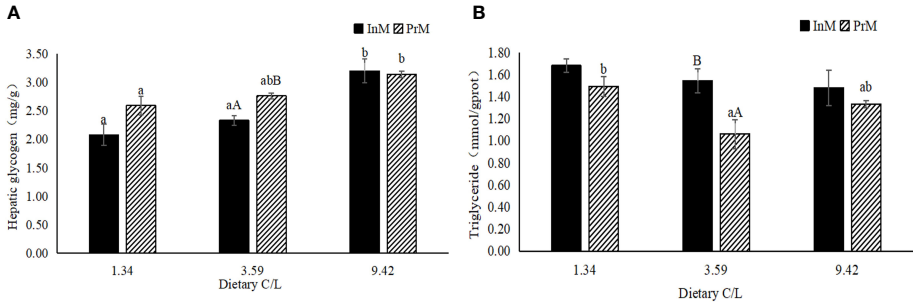

FIGURE 1
Effects of different dietary carbohydrate to lipid ratio on biochemical criterion of hepatopancreas of juvenile *E. sinensis* in different molting stage. Values are presented as means ± standard error (SE) (n=3). Bars with different upper-case letters differ significantly from each other in the same dietary C/L groups (P<0.05). Bars with different lower-case letters differ significantly from those of other dietary C/L groups in the same molting stage (P<0.05). Hepatic glycogen contents **(A)**, Triglyceride contents **(B)**.

sinensis ($P < 0.05$). At both molting stages, the activities of GS significantly increased with increasing dietary C/L ($P < 0.05$), but no statistical significance was observed between InM and PrM in all dietary groups ($P > 0.05$) (Figure 2A).

As the dietary C/L increased, the activities of HK significantly increased ($P < 0.05$) in the PrM but decreased in the InM ($P > 0.05$) (Figure 2B). The highest activities of PK in the InM were observed in the C/L3.59 group, which were significantly higher than those in the other groups ($P < 0.05$) (Figure 2C). In dietary C/L1.34 and C/L3.59 groups, HK and PK activities in the InM were higher than those in the PrM (Figures 2B, C).

As the dietary C/L increased, the activities of FAS significantly decreased in the InM ($P < 0.05$). FAS activities in the C/L9.42 group in the InM were significantly lower than those in the PrM (Figure 2D).

The activities of ACC, PEPCK and CPT were not significantly affected by different dietary C/L, with higher values observed in the InM than those in the PrM in all dietary groups (Figures 2E–G).

3.4 Transcriptome analysis between dietary C/L groups or molting stage

A total of 118.16 Gb of Clean Data was obtained from transcriptome analysis of 18 samples. Q30 base percentage of all samples in this study were above 91.86%, which showed that all data were qualified. The DEG number between each two different groups at the same dietary C/L level in different molting stages was analyzed, and the results were shown in Table 4. In the comparison among these nine groups, the DEG number between C/L1.34 of InM and C/L1.34 of PrM was the most, and the DEG number between C/L3.59 of PrM and C/L9.42 of PrM was the least. Feeding with dietary C/L1.34 has more DEGs between InM and PrM than that feeding with dietary C/L3.59 and C/L9.42. The number of DEGs was higher between feeding dietary C/L3.59 and C/L9.42 in InM, and higher between C/L1.34 and C/L3.59 in PrM. To further assign the putative functions to DEGs, KEGG analysis was performed. KEGG enrichment results showed that the DEGs were mainly enriched in biological processes, such as metabolic process (GO:0008152), cellular process (GO:0009987) and

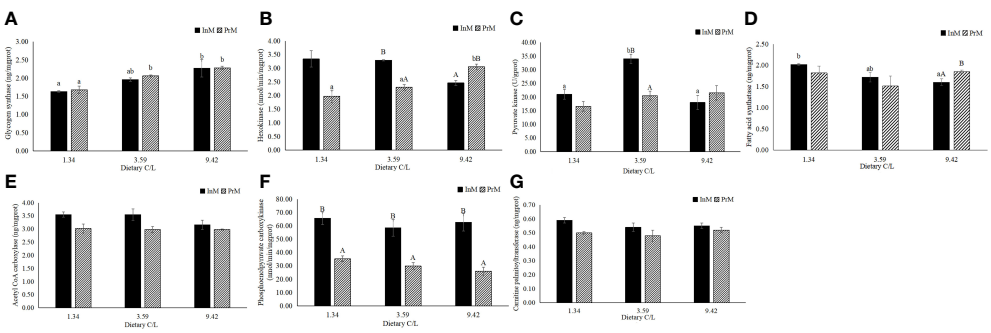

FIGURE 2
Effects of different dietary carbohydrate to lipid ratio on metabolic enzymes activities of hepatopancreas of juvenile *E. sinensis* in different molting stage. Values are presented as means ± standard error (SE) (n=3). Different upper-case letters on the bars represent a significance in the values between the two molting stages within the same dietary group (P<0.05). Different lower-case letters on the bars represent a significance in the values between dietary groups with the same molting stage (P<0.05). GS: glycogen synthase **(A)**, HK, hexokinase **(B)**, PK, pyruvate kinase **(C)**, FAS, fatty acid synthesis **(D)**, ACC, acetyl-CoA carboxylase **(E)**, PEPCK, phosphoenolpyruvate carboxykinase **(F)**, CPT, carnitine palmitoyltransferase **(G)**.

10.3389/fmars.2023.1176976

TABLE 4 Effects of different dietary carbohydrate to lipid ratio and molting cycle on DEG number and analysis of KEGG pathway in juvenile *E. sinensis*.

Different groups	DEG Number	Up-regulated	Down-regulated	KEGG ID	Description of KEGG pathway	EnrichmentScore	Pvalue
C/L1.34 of InM vs C/L1.34 of PrM	3615	1965	1650	ko00190	Oxidative phosphorylation	-0.602	0.002
				ko01212	Fatty acid metabolism	-0.576	0.004
				ko00590	Arachidonic acid metabolism	-0.572	0.015
				ko00061	Fatty acid biosynthesis	-0.719	0.021
				ko01040	Biosynthesis of unsaturated fatty acids	-0.612	0.041
C/L3.59 of InM vs C/L3.59 of PrM	498	238	260	ko00500	Starch and sucrose metabolism	-0.625	0.002
				ko00190	Oxidative phosphorylation	0.459	0.002
				ko00052	Galactose metabolism	-0.538	0.004
				ko00590	Arachidonic acid metabolism	-0.533	0.004
				ko00100	Steroid biosynthesis	-0.758	0.004
C/L9.42 of InM vs C/L9.42 of PrM	1302	515	787	ko00531	Glycosaminoglycan degradation	-0.738	0.002
				ko00511	Other glycan degradation	-0.710	0.002
				ko00500	Starch and sucrose metabolism	-0.599	0.003
				ko00620	Pyruvate metabolism	-0.651	0.005
				ko00590	Arachidonic acid metabolism	-0.554	0.009
C/L1.34 of InM vs C/L3.59 of InM	2675	1575	1100	ko01200	Carbon metabolism	-0.505	0.002
				ko00190	Oxidative phosphorylation	-0.740	0.002
				ko00071	Fatty acid degradation	-0.545	0.017
C/L1.34 of InM vs C/L9.42 of InM	1590	719	871	ko00190	Oxidative phosphorylation	-0.622	0.002
				ko01212	Fatty acid metabolism	-0.526	0.060
				ko04070	Phosphatidylinositol signaling system	-0.497	0.082
C/L3.59 of InM vs C/L9.42 of InM	2540	974	1566	ko00500	Starch and sucrose metabolism	-0.617	0.002
				ko00190	Oxidative phosphorylation	0.574	0.003
				ko00010	Glycolysis/Gluconeogenesis	0.541	0.013
				ko00052	Galactose metabolism	-0.515	0.024
				ko00100	Steroid biosynthesis	-0.624	0.028
				ko00561	Glycerolipid metabolism	-0.474	0.031
				ko01200	Carbon metabolism	0.364	0.046
C/L1.34 of PrM vs C/L3.59 of PrM	1291	773	518	ko00061	Fatty acid biosynthesis	0.747	0.007
				ko00770	Pantothenate and CoA biosynthesis	0.594	0.065
				ko00020	Citrate cycle (TCA cycle)	-0.485	0.100
				ko00590	Arachidonic acid metabolism	-0.438	0.103
C/L1.34 of PrM vs C/L9.42 of PrM	1745	1126	619	ko00040	Pentose and glucuronate interconversions	-0.528	0.035
				ko00500	Starch and sucrose metabolism	-0.475	0.041
				ko00600	Sphingolipid metabolism	-0.451	0.069

(Continued)

TABLE 4 Continued

Different groups	DEG Number	Up-regulated	Down-regulated	KEGG ID	Description of KEGG pathway	EnrichmentScore	Pvalue
C/L3.59 of PrM vs C/L9.42 of PrM	440	269	171	ko01212	Fatty acid metabolism	-0.548	0.002
				ko00500	Starch and sucrose metabolism	-0.548	0.007
				ko00561	Glycerolipid metabolism	-0.482	0.010
				ko00600	Sphingolipid metabolism	-0.509	0.010
				ko00061	Fatty acid biosynthesis	-0.680	0.018
				ko00564	Glycerophospholipid metabolism	-0.394	0.031
				ko01040	Biosynthesis of unsaturated fatty acids	-0.540	0.040

Values are presented as the P value=0.05, $\log^2$FC=1.5 (FC, fold change) (n=3).

biological regulation (GO:0065007). With the enrichment of KEGG, fatty acid biosynthesis (ko00061), fatty acid metabolis (ko01212), moxidative phosphorylation (ko00190), pentose phosphate pathway (ko00030) and pyruvate metabolism (ko00620) have enriched more DEGs. For C/L 1.34, C/L 3.59 and C/L 9.42, the DEGs between InM and PrM were enriched in oxidative phosphorylation process (ko00190), steroid biosynthesis (ko00100) and pyruvate metabolism (ko00620), respectively. For In InM, the more DEGs were found between C/L 1.34 and C/L 3.59, which were mostly enriched in oxidative phosphorylation process (ko00190), and lower DEGs were found between C/L 1.34 and C/L 9.42. While, the C/L 1.34 and C/L 3.59 group had more DEGs in PrM, which were enriched in starch and sucrose metabolism (ko00500) and fatty acid metabolism (ko01212) (Table 4).

3.5 The mRNA expression of metabolism related genes expression in E. sinensis

There was a significantly interactive effect ($P< 0.05$) between dietary C/L and molting stage on the mRNA expression levels of all selected genes in *E. sinensis* (Figure 3). It appears that the mRNA expression levels of *FAS, G6PD, PEPCK, Ndufa6, CPT, ACAA2, Elovl6, Aco* and *Acly* vary significantly depending on the dietary C/L ratio. The expression levels of *Fas*, *G6PD*, *PEPCK*, *Ndufa6* and *Acly* at the InM were higher than that at the PrM (Figures 3A-D, I). The expression levels of *CPT* at the InM post the dietary C/L1.34 and C/L3.59 treatment were lower than that in the PrM stage, and the trend in the C/L9.42 group was the opposite (Figure 3E). In each dietary group, the expression level of *Aco* at the InM was lower than that at the PrM (Figure 3H).

In the InM, the mRNA expression levels of *Elovl6* and *Aco* in the dietary C/L1.34 group were significantly higher than those in dietary C/L3.59 and C/L9.42 groups ($P < 0.05$). The mRNA expression levels of *FAS*, *G6PD*, *CPT* and *Acly* in the dietary C/L3.59 group were significantly higher than those in the C/L1.34 and C/L9.42 groups ($P < 0.05$). The expression levels of *PEPCK*, *Ndufa6* and *ACAA2* in the dietary C/L9.42 group were significantly higher than those in the dietary C/L1.34 and C/L3.59 groups ($P < 0.05$) (Figures 3A-H).

In the PrM, the mRNA expression levels of *G6PD*, *Ndufa6*, *Elovl6* and *Aco* post dietary C/L1.34 treatment were significantly higher than those in the C/L3.59 and C/L9.42 groups ($P < 0.05$). The mRNA expression levels of *FAS*, *CPT* and *ACAA2* post the dietary C/L3.59 treatment were significantly higher than those in the C/L1.34 and C/L9.42 groups ($P < 0.05$). Besides, the mRNA expression levels of *PEPCK* post the dietary C/L9.42 treatment were significantly higher than those in the dietary C/L1.34 and C/L3.59 groups (Figures 3A-H).

4 Discussion

Carbohydrates and lipids are widely used as non-protein energy sources in the formulated feeds (Xie et al., 2017; Dong et al., 2018; Liu et al., 2020). Survival, growth performance, and feed cost are usually taken into consideration when estimating the optimal dietary lipids and carbohydrates in the diets. The present study showed that, post the 58-day feeding trial, the SR of *E. sinensis* was above 90% and was hardly affected by dietary C/L. Carbohydrates and lipids can be effectively used to achieve ideal growth performance by most crustaceans, such as *Jasus edwardsii*, *Cherax quadricarinatus* (Zhu et al., 2013), *E. sinensis* (Bao et al., 2020; Wen et al., 2021). In the present study, the optimal dietary C/L for juvenile *E. sinensis* was estimated to be 3.59 based on WGR. This was close to the optimal requirement of C/L for other aquatic animals, such as blunt snout bream (*Megalobrama amblycephala*) (Li et al., 2013), large yellow croaker (*Larmichthys crocea*) (Zhou et al., 2016), bullfrog (*Rana (Lithobates) catesbeiana*) (Zhang et al., 2016) and hybrid grouper (♀ *Epinephelus fuscoguttatus* × ♂ *E. lanceolatus*) (Chen et al., 2021), but was higher than that for *Jasus edwardsii* (Johnston et al., 2003) and *Scylla paramamosain* (Dong et al., 2018), which was estimated to be 2.0 and 1.39-2.08, respectively. Excessive carbohydrates or insufficient lipids reduced feed palatability (Chen et al., 2021) and negatively affected the normal metabolism of several aquatic animals, such as *M. salmoides*

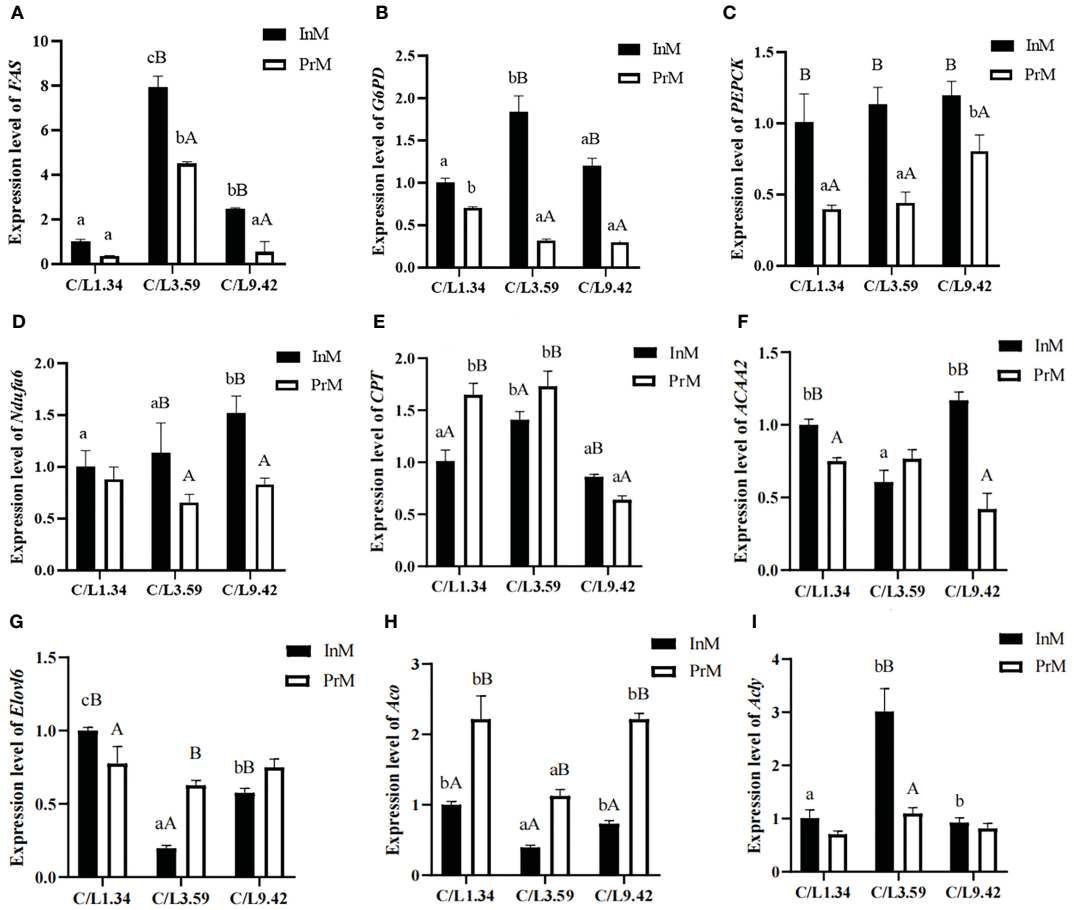

FIGURE 3
Effects of different dietary carbohydrate to lipid ratio on the mRNA expression of antioxidative genes of juvenile *E. sinensis* in different molting stage. Values are presented as means ± standard error (SE) (n=3). Bars with different upper-case letters differ significantly from each other in the same dietary C/L groups ($P<0.05$). Bars with different lower-case letters differ significantly from those of other dietary C/L groups in the same molting stage ($P<0.05$). FAS relative mRNA expression **(A)**, G6PD relative mRNA expression **(B)**, PEPCK relative mRNA expression **(C)**, Ndufa6 relative mRNA expression **(D)**, CPT relative mRNA expression **(E)**, ACAA2 relative mRNA expression **(F)**, Elovl6 relative mRNA expression **(G)**, Aco relative mRNA expression **(H)**, Acly relative mRNA expression **(I)**.

(Ma et al., 2019) and *C. quadricarinatus* (Zhu et al., 2013). In this study, the lipid level in the C/L9.42 was only 3.92%, which was lower than the estimated requirement for *E. sinensis* (Li et al., 2013; Zhang et al., 2016; Wen et al., 2021).

In this study, hepatic glycogen contents in the hepatopancreas significantly increased, while triglyceride (TG) contents decreased with increasing dietary C/L. Similar results have also been reported orange-spotted grouper (*E. coioides*) (Wang et al., 2017), tilapia (*O. niloticus*) (Xie et al., 2017) and red swamp crayfish (*Procambarus clarkii*) (Li et al., 2022). Consistently, activities of glycogen synthase (GS) increased while those of fatty acid synthesis (FAS and ACC) decreased with increasing dietary C/L. CPT located in the mitochondria is the rate-limiting enzyme for fatty acid oxidation, and it plays an important role in the oxidation of fatty acids in *E. sinensis* (Liu et al., 2018). In this study, the activities of CPT were not significantly affected by the dietary C/L. On one hand, it could be the decreased fatty acid synthesis that accounted for the decreased contents of TG in the hepatopancreas of *E. sinensis* as observed in this study. On the other hand, the increasing C/L could decrease the lipid transport efficiency that resulted in the decreased retention of TG (Du et al., 2005; Gao et al., 2010).

Molting is an indispensable and ongoing physiological process in the life-history of all crustaceans, especially for sustaining normal growth, development and reproduction (Panganiban et al., 1995; Jung et al., 2013; Huang et al., 2015). *E. sinensis* accumulates substances and energy in the InM, which are used for the formation of new exoskeletons in the PrM (Huang et al., 2015). In the present study, the glycogen content increased while the TC content decreased in PrM compared with that in InM. Glycolysis and gluconeogenesis are two important activities of glucose metabolism (Zhang et al., 2019). In this study, it was found that the activities of glycolytic rate-limiting enzymes (HK and PK) in the InM were promoted by low or moderate dietary C/L (1.34-3.59) but were inhibited by the highest dietary C/L. This was consistent with the findings of Chen et al. (2021) who found that glycolytic ability of juvenile hybrid grouper was suppressed by excessive carbohydrates in the diets. Notably, more carbohydrates were used for glycolysis in the InM among the two molting stages at low or moderate C/L

levels. While in the highest C/L group, more carbohydrates were used for glycolysis in the PrM than InM. This indicated that the amounts of carbohydrates participating into glycolysis were not only affected by dietary C/L, but also were affected by molting stages. Feeding activity decreases and even stops during PrM and molting, and begins again postmolt when the crustaceans are rigid enough to handle food (Li et al., 2022). Thus, crustaceans rely mainly on the internal nutrients reserved in the hepatopancreas during PrM and molting (Niu et al., 2012). The energy released by lipid oxidation is much higher than that of carbohydrates because the relative contents of carbon and hydrogen in lipids are higher than those of carbohydrates. Thus, lipids are more suitable substances for instant and high demand of energy, especially for the premolt and molting crabs. Since crabs in the InM can ingest food normally, thy are prone to utilize glucose through glycolysis and save lipids for later use in PrM stage. It was postulated that steroids may be related to the regulation of glycolysis. The role of PEPCK is to catalyze the conversion of oxaloacetate to phosphoenolpyruvate, which is a key rate-limiting enzyme in the gluconeogenesis pathway (Lu et al., 2018). In this study, PEPCK in the PrM significantly decreased in both mRNA levels and activities than those in the InM in all dietary groups. This indicated that gluconeogenesis is more active in the InM of the *E. sinensis*. G6PDH is a key enzyme involved in the production of NADPH in the pentose phosphate pathway, and NADPH is necessary for lipogenesis (Enes et al., 2009; Guerrero-Zárate et al., 2019; Liu et al., 2020). In this study, the mRNA levels of G6PDH were higher in the InM, which was consistent with the increased TC contents in this stage of all dietary groups.

The information obtained from transcriptome analysis can provide some molecular basis for crustaceans (Hu et al., 2015). In this study, DEGs was used to perform transcriptome analysis on the expression profile in the hepatopancreas of *E. sinensis* fed diets with increasing C/L. The results showed that metabolic process terms were over-represented in the InM and PrM, for instance, glycogen biosynthetic process (GO:0005978) and fatty acid biosynthetic process (GO:0006633). KEGG analysis demonstrated the top enriched pathways include fatty acid biosynthesis (ko00061), fatty acid metabolism (ko01212), oxidative phosphorylation (ko00190), pentose phosphate pathway (ko00030) and pyruvate metabolism (ko00620). Energy metabolism has become an indispensable part of studying ion exchange and osmotic regulation in organisms (Hu et al., 2015). In this study, glycolysis/gluconeogenesis, citric acid cycle (TCA cycle) and fatty acid synthesis/degradation are abundant pathways related to energy metabolism. Our research has found some significant differentially expressed genes related to energy metabolism. The tendency of nine genes (Figure 3) was basically consistent with the transcriptome information after identification by RT-PCR. *FAS* controls the synthesis of fatty acids which catalyzes the lipid synthesis pathway by converting carbohydrates into fatty acids (Chirala and Wakil, 2004; Mashima et al., 2009). Compared with the PrM, the mRNA expression levels of *FAS* at the InM were significantly up-regulated, indicating that *E. sinensis* in the InM needs to accumulate more energy for utilization in the PrM (Huang et al., 2015). At the same time, FAS was also clearly responding to changes in the dietary C/L in the diets. This showed that the dietary C/L3.59 was more conducive to the accumulation of lipid for *E. sinensis*. It has also confirmed that lipids stored at the InM play an important role in the energy supply of other non-eating molting stages of *E. sinensis*. *G6PD* is a key gene involved in the pentose phosphate pathway (Yilmaz et al., 2006). The role of *PEPCK* is to catalyze the conversion of oxaloacetate to phosphoenolpyruvate, which is a key gene in the gluconeogenesis pathway (Lu et al., 2018). We have observed that these two genes involved in the conversion of carbohydrates and lipids were highly expressed at the InM. It is worth noting that as the dietary C/L increased, the expression levels of *PEPCK* showed an increasing trend, while *G6PD* was the opposite at the PrM. Combined with the content of hepatic glycogen, this showed that *E. sinensis* accumulated more carbohydrates at the PrM, in other words, carbohydrates were not used as the main energy source. The expression level of *PEPCK* was higher at the InM than at the PrM. This corresponds to the PEPCK activities, which indicates that gluconeogenesis is more active at the InM than at the PrM, with more glucose generated and then converted into glycogen. Both Aco and Acly are involved in the regulation of the Citrate cycle (TCA cycle) (ko00020). The *CPT* gene participates in the metabolic pathway of AMPK/ACC/CPT, by degrading fatty acids to avoid excessive liver lipid deposition (Liu et al., 2018; Tobita et al., 2018; Fang et al., 2019). Within the appropriate range of dietary C/L (1.34-3.59), the expression levels of *CPT* were significantly up-regulated at the PrM of *E. sinensis*. This indicated that lipid was broken down at the PrM when *E. sinensis* needs a lot of energy to prepare for molting (Huang et al., 2015). However, the mRNA expression level of *CPT* could be restricted by dietary high carbohydrate or low lipid. Taken together, these results indicated that *E. sinensis* utilizes carbohydrates as an energy source in the InM, while fatty acids and lipids are used in the PrM.

5 Conclusion

A moderate dietary C/L (3.59) achieved the best growth performance of juvenile *E. sinensis*. Dietary C/L increased glycogen synthesis but decreased lipid synthesis in the hepatopancreas. Compared to PrM, *E. sinensis* in the InM was more active in the carbohydrate metabolism (glycolysis and gluconeogenesis) and fatty acid synthesis, with more triglyceride and less glycogen accumulated in the hepatopancreas. This may indicate that juvenile crabs are prone to utilize carbohydrates for energy supply through glycolysis in the InM and store lipids for later energy use in the PrM. Moreover, the transcriptomic analysis showed that compared with C/L 1.34 and C/L 9.42, the differentially expressed genes between InM and PrM were enriched not only in energy metabolism, but also in steroid biosynthesis in C/L 3.59, which indicated that C/L 3.59 might promote the steroid biosynthesis at PrM stage contributing to growth performance. These results could be helpful for optimizing the feed formulation for this species in different molting stages.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: NCBI, PRJNA977214.

Author contributions

RZ: conceptualization, methodology, writing. BW: formal analysis, writing. YJ: manuscript revision. SH: data analysis. QY: supervision, project administration. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Fermented *Astragalus membranaceus* could promote the liver and intestinal health of juvenile tiger grouper (*Epinephelus fuscoguttatus*)

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In order to understand the effects of fermented *Astragalus membranaceus* (FAM) on the liver and intestinal health of tiger grouper (*Epinephelus fuscoguttatus*), this study was conducted. This study evaluates the effects of different levels of FAM on liver and intestinal tissue structure, serum biochemical parameters, intestinal digestive enzyme, and microbiota structure of tiger grouper. Fish were fed with diets (crude protein ≥ 48.0%, crude fat ≥ 10.0%) with five levels of FAM (L1:0.25%, L2: 0.5%, L3: 1%, L4: 2% and L5: 4%) in the experimental groups and a regular diet was used as the control (L0: 0%) for 8 weeks. Compared with AM, the protein content of FAM was significantly changed by 34.70%, indicating that a large amount of bacterial protein was produced after AM fermentation, and its nutritional value was improved. FAM had significant effects on the growth performance of tiger grouper ($p < 0.05$). The high-density lipoprotein cholesterol (HDL-C) was highest in L4 group, being significantly different from L0 group. The area and diameter of hepatocytes were lowest in L3 and L4, and the density of hepatocyte was highest in L4 group and relatively decreased in L5 group. The mucosal height and muscular thickness were highest in L3 group. The intestinal microbiota structure of tiger grouper was changed under the intervention of FAM. The lower abundance of potential pathogenic bacteria and higher abundance of probiotics colonization in the L4 group showed that the dose of FAM had the best effect on improving the health of intestinal microbiota. This study indicates that the addition of FAM in the feed contributes to liver health, improves intestinal morphology, and regulates the intestinal microbiota of tiger grouper. The addition ratio of 1%–2% is better for intestinal and liver health, and a high addition ratio will cause liver damage. Our work will provide a reference for the addition and management of FAM in the aquaculture industry.

KEYWORDS

feed additives, Chinese herbal medicine, probiotics fermentation, histology, intestinal microbiota, *Astragalus membranaceus*

1 Introduction

Tiger grouper is aquaculture-targeted specie as a result of its high commercial value and demand (Lim et al., 2016). However, the culture of tiger grouper presents many challenges due to its high susceptibility to infectious diseases and sensitivity to stress. The use of antibiotics and chemotherapeutants to control diseases of grouper as practiced by some farmers have raised several issues including high operational cost, emergence of drug-resistant bacteria, suppression of immunity, and food and environmental contamination (Apines-Amar et al., 2012). Since antibiotics and chemical drugs can promote antibiotic resistance in bacteria, they have a negative impact on the environment and human health (Meng et al., 2023), resulting in the quality of aquatic products cannot be guaranteed, which seriously affects the sustainable development of aquaculture. Although it is still widely used to alleviate diseases, some countries have restricted or banned the use of antibiotics and chemical drugs (Cabello, 2006; Meng et al., 2023). Due to the limited use of antibiotics and chemical drugs, finding alternative suitable substances to enhance the health of fish has become the focus of this study.

In order to reduce the use of antibiotics in aquaculture, alternatives such as antimicrobial peptides and probiotic feed have been proposed (Rajanbabu and Chen, 2011; Bidhan et al., 2014). In addition, Chinese herbal medicine (CHM) therapy can also enhance fish resistance, improve growth and feed efficiency, thereby enhancing the sustainability of aquaculture (Reverter et al., 2014). CHM is not only a rich source of nutrients, vitamins and minerals but also comprise several phytochemicals' constituents which are the bioactive components like alkaloids, steroids, flavonoids, saponins, glycosides, carotenoids, terpenoids, phytoandrogens, phytosterols, curcumin and so on (Faheem et al., 2020; Mulat et al., 2020). Because of the presence of above-mentioned components, medicinal plants naturally possess several pharmacological properties making them a promising candidate as fish feed additive (Faheem et al., 2022).

Studies have shown that CHM may maintain the balance of intestinal microbiota, improve the immunity of aquatic animals, and enhance the host's resistance to pathogen infection (Pu et al., 2017; Meng et al., 2023; Yao et al., 2023). Active components of herbs are believed to improve nutrient digestibility, absorption, assimilation capacity, and digestive enzyme secretion, as well as maintain healthy intestinal microflora in fish (Hoseinifar et al., 2020). A large number of studies have reported the beneficial effects of *Acanthopanax senticosus*, *Licorice root* and *Astragalus membranaceus* (AM) in aquaculture (Wu, 2020; Adineh et al., 2021; Meng et al., 2023). AM, a leguminous plant, is one of the most famous CHM that has various therapeutic effects such as anti-cancer, anti-virus, and immune regulation (Yao et al., 2023). Studies have shown that dietary AM supplementation can significantly improve the physiological and nutritional status of fish (Hoseinifar et al., 2020), such as catla (*Catla catla*) (Harikrishnan et al., 2022), *Channa argus* (Zhu et al., 2021), common carp (*Cyprinus carpio*) (Shi et al., 2022) and grass carp (*Ctenopharyngodon idellus*) (Shi et al., 2021).

Due to the complex structure of CHM, its active components cannot be completely absorbed and utilized by aquatic animals. The bioactivity of the active components could be seriously reduced by the traditional extraction method (Pu et al., 2017). Biological fermentation processing of traditional CHM is the use of microorganisms with strong ability to decompose and transform substances, and can produce abundant secondary metabolites. The process reaction conditions are mild, and the traditional CHM preparation is efficient, low toxicity and low residue. Therefore, the application of modern biotechnology fermentation transformation of traditional CHM has become a research hotspot (Li et al., 2004; Ruan et al., 2009). The study of Xie (2015) found that fermented CHM and CHM had obvious preventive and therapeutic effects on the hemorrhagic disease of crucian carp caused by *Aeromonas hydrophila*, and the effect of fermented CHM was better than that of unfermented CHM. Compared with liquid fermentation, solid fermentation is more and more used in clinic because of its low production cost, simple operation and no need for large instruments and equipment (Qiao et al., 2018a).

Probiotics fermentation can decompose CHM into useful components such as organic acids and polysaccharides. The fermentation products can improve the immunity, disease resistance and antioxidant capacity of the host. As an important organ of aquatic organisms, the intestine is accountable for nutrient digestion and absorption. In recent years, the intestinal microbiota has been verified that it plays a vital role in the health of the host, as it can control the proliferation of pathogenic bacteria present in the intestinal tract, regulate the host metabolism and physiology, regulate the absorption of nutrients and stimulate the immune system (Shi et al., 2022a). Hence, the intestine is not merely a vital organ responsible for the absorption of nutrients but also a major site of host immunity (Ran et al., 2020). Studies have shown that FAM can regulate the fecal microbiota of *Arbor acre*, improve its antioxidant properties and promote its growth (Qiao et al., 2018b). FAM water extract can improve the intestinal morphology and microenvironment of *Cyprinus carpio*, enhance its immune function and promote its growth (Shi et al., 2022a).

Hoseinifar et al. (2020) study found that the effects of herbal feed supplements are species-specific and must be considered cautiously. Adding 0.1% *Astragalus* compound CHM fermentation products to the feed can significantly improve the disease resistance of grass carp (*Ctenopharyngodon idella*) and significantly reduce the lethal rate of pathogenic *Aeromonas hydrophila* to it (Li and Zhou, 2018). The growth performance and survival rate of juvenile *Palea steindachneri* were significantly improved by adding 0.4% FAM to the diet, and the feed utilization rate was improved. The weight gain rate, specific growth rate and antioxidant properties of *Litopenaeus vannamei* can be significantly improved by adding 1%–4% compound probiotics FAM to the basic feed of *Litopenaeus vannamei* (Qi et al., 2018). The addition of 0.25%–2% FAM feed could significantly improve liver antioxidant performance, significantly reduce liver malondialdehyde (MDA) content and serum glutamic oxalic aminotransferase (GOT) activity of tiger grouper (Xiao et al., 2023). The addition of compound CHM fermentation preparation powder to largemouth bass (*Micropterus salmoides*) feed can improve immunity, enhance antioxidant capacity and promote fat metabolism, and the addition amount below 0.5% will not affect the production performance (Peng, 2019). The addition of 5‰–10‰ FAM feed to hybrid sturgeon can significantly improve its ability to resist streptococcal infection, and 10‰ FAM can improve its non-specific immune function

(Dai, 2022). According to the application of fermented CHM in other aquatic animals and pre-experiments, we selected the inclusion levels.

Zhao et al. (2021a) and Englezos et al. (2019)' research showed that it was difficult for a single strain to complete numerous biochemical reaction processes in the fermentation process, so mixed bacterial fermentation gradually developed, and more studies were conducted on Chinese herbal compounds. *Bacillus subtilis* can produce many enzymes such as cellulase, protease and hemicellulase during its growth (Liu et al., 2012). In the early stage of fermentation, *Bacillus subtilis* grows and multiplies rapidly and consumes oxygen. With the reduction of oxygen, *Lactobacillus plantarum* begins to grow and multiply, produce lactic acid, and gradually reduce the pH value, and effectively control the infection of other miscellaneous bacteria (Jung et al., 2012; Li et al., 2021a). The growth conditions of *Saccharomyces cerevisiae* are extensive and easy to cultivate. It can use the fermentation products of *Bacillus subtilis*, such as hexose and pentose (Xie, 2015), to produce protein and multiple vitamins, and remove the product effect. *Lactobacillus plantarum* and *Enterococcus faecalis* have a good symbiotic relationship (Qiao et al., 2018a). The former provides the latter with essential peptides and amino acids, such as glycine, histidine, valine, leucine, glutamic acid, tryptophan and isoleucine (Bassaganya-Riera et al., 2012), while the latter produces formic acid and CO_2 to stimulate the proliferation of the former. Among many species of *Aspergillus fungi*, *Aspergillus flavus* can produce toxic substances and cannot be used in microbial transformation. *Aspergillus Niger* has a variety of highly active enzyme systems, does not produce toxins, can produce pectinase, mannanase, protease, amylase, cellulase, hemicellulase, lipase, glucosidase and other enzymes, it is widely used in the conversion of traditional Chinese medicine (Jin et al., 2016). In addition to *Aspergillus Niger*, other Aspergillus enzymes such as *Aspergillus oryzae* have also been applied, but they are far less than the application of *Aspergillus Niger*. So in this experiment, *Astragalus* was fermented by mixed bacteria (*Aspergillus Niger spores*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Lactobacillus plantarum* and *Entero-coccus faecalis*) solid-state fermentation process. In aquaculture, the use of probiotics and dietary enhancement have been recognized as alternative methods of health management. In particular, nutritional status has been increasingly acknowledged as a crucial factor in host defense against pathogens. As such, use of feed supplements aiming to improve not only the growth but also the health of aquaculture species has gained widespread interest and acceptance. Our work will provide reference for the addition and management of FAM in the aquaculture industry.

2 Materials and methods

2.1 Fermented *Astragalus membranaceus* (FAM) and diet preparation

2.1.1 Fermentation process

Chinese herbal medicine AM produced in Gansu and fermented by mixed bacteria (*Aspergillus Niger spores*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Lactobacillus plantarum* and *Entero-coccus faecalis*) solid-state fermentation process. The fermentation method was improved according to Xie (2015)'s method and previous studies (Liu et al., 2017; Qiao et al., 2018a; Li et al., 2021a). AM, corn, soybean meal and wheat bran were pulverized and passed through a 100mesh sieve, and then mixed at 75: 10: 10: 5 as the initial material. In addition, 5% molasses, 0.2% ammonium sulfate, 0.05% potassium dihydrogen phosphate, 0.1% dipotassium hydrogen phosphate, 0.07% sodium chloride and 0.01% magnesium sulfate heptahydrate were added. Drying at 55°C for 24 h before mixing. The substrate was sterilized by 121°C high temperature and high-pressure steam (SHENAN, LDZX-30KBS, China) for 20min and then added into the sterile fresh water of equal quality. *Aspergillus Niger* spores' powder (2×10^{10} CFU·g^{-1}), *Bacillus subtilis* powder (2×10^{11} CFU·g^{-1}), *Saccharomyces cerevisiae* powder (2×10^{10} CFU·g^{-1}), *Lactobacillus plantarum* powder (1×10^{10} CFU·g^{-1}) and *Enterococcus faecalis* powder (1×10^{11} CFU·g^{-1}) were used for fermentation, the inoculum concentration of each strain was 2×10^{7} CFU·g^{-1}. FAM preparation conditions were as follows: aerobic fermentation at 35°C for 24°h, anaerobic fermentation at 35°C for 72°h, drying at 55°C for 24°h, and grinding to particle size < 0.15°mm.

2.1.2 Feed preparation

The basic feed is commercial grouper feed, produced in Santong Bio-engineering (Weifang) CO., Ltd. (http://www.santonghaitong.com/). The nutritional levels of feed were as follows: crude protein ≥ 48.0%, crude fat ≥ 10.0%, crude ash ≤ 16.0%, crude fiber ≤ 3.0%, lysine ≥ 2.5%, moisture ≤ 10.0%. Commercial complete crushed to particle size < 0.28mm, 1.5% adhesive sodium alginate was added. FAM or microcrystalline cellulose (the total addition was 4%) were added to make granules, drying at 55°C for 24 h. The diet was stored at −20°C.

The control group (L0) was fed commercial complete feed with no added FAM. Five treatments were involved. The treatment group (L1, L2, L3, L4 and L5) was fed commercial complete feed supplemented with FAM (0.25%, 0.5%, 1%, 2% and 4%, respectively). For each group, three replicates were used. During the experimentation period, 30% water was exchanged in each tank. The water parameters were measured daily and were maintained at pH 7.8, dissolved oxygen > 6.0 mg/L, and water temperature 26.5°C ± 1.5°C.

2.2 Chemical characterization analysis

Astragalus dry powder and FAM were weighed three equal samples to be tested. Protein content was determined by Kjeldahl nitrogen determination method (GB 5009.5-2016). Polysaccharide was extracted by water extraction and alcohol precipitation method (Qiao, 2020), total sugar was determined by phenol-sulfuric acid method (Zhao, 2015), and reducing sugar was determined by 3, 5-dinitrosalicylic acid colorimetric method (Zhang et al., 2017). Polysaccharide extraction rate (%) = (total sugar mass - reducing sugar mass)/sample mass×100% (Chen, 2020). The relative molecular mass of polysaccharide was determined by gel permeation chromatography (GPC) method (Yang et al., 2023).

The content of total saponins was determined by sulfuric acid-vanillin method and total flavones by sodium nitrite—aluminum

nitrate-sodium hydroxide method (Zhang et al., 2021). The content of Astragaloside A was determined by HPLC-ELSD method according to Pharmacopoeia of the People's Republic of China (2020 edition). The content of effective substances in FAM containing the same amount of AM = the content of effective substances in FAM ÷ 70.85%.

2.3 Experimental fish and feeding management

Juvenile tiger grouper individuals (bodyweight 44.48 ± 2.06 g) were raised at the Tropical Fisheries Research and Development Center, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Sanya, China. We randomly divided 540 fish among 18 tanks (300 L each, 30 fish per tank). Fish were allowed to acclimate for 7 days and were only fed commercial complete feed during this period. Upon completion of the acclimation period, experimentation began and lasted 8 weeks. Fish were fed *ad libitum* twice daily at 08:30 and 16:30.

2.4 Fish performance and feed efficiency

At the end of the feeding trial, following a 24 h starvation period and anesthetized with 150 mg/L Eugenol CAS:97-53-0, all the fish were measured and weighed. In addition, 3 fish from each tank were randomly collected before sampling. The calculation formulas of fish performance and feed efficiency are as follows:

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

$$\text{Specific growth rate (SGR, \% \cdot d}^{-1}\text{)} = (\ln W_t - \ln W_0)/t \times 100$$

$$\text{Survival rate (SR, \%)} = N_f/N_i \times 100$$

$$\text{Feed coefficient (FC)} = F/(W_t - W_0)$$

In the formula, W_0 was the fish body weight (g) at the beginning of the experiment; W_t was the fish weight (g) at the end of the experiment; t was the test days (d); N_f was the number of terminal fish; N_i was the initial fishtail number; F was feed intake (g).

2.5 Sampling

The surface water was wiped with paper towels, and a 1-mL sterile syringe was used to extract blood from the tail veins of tiger grouper. After standing for 4 h, blood samples were centrifuged for 10 min at 3500×g, 4°C, and then the supernatants were collected. The collected supernatant was stored at −80°C for further measurement and analysis. The intestines and livers of three fish from each tank were aseptically dissected; the intestinal contents were collected.

2.6 Physiology and biochemistry

Serum biochemical parameters were determined according to the instructions of the commercial kits (Nanjing Jiancheng Biological Co., Ltd., Nanjing, China), i.e., total protein (TP) (Item No. A045-4-2): BCA microplate method; total cholesterol (TC) (Item No. A111-1-1): cholesterol oxidase–peroxidase aminoantipyrine method; high density lipoprotein cholesterol (HDL-C) (Item No. F003-1-1): liquid precipitation separation method; and low-density lipoprotein cholesterol (LDL-C) (Item No. A113-1-1): double reagent direct method. All serum biochemical parameters were performed in triplicates.Intestine samples from each parallel were 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.

2.7 DNA extraction

The intestinal contents from the three fish were mixed, transferred to a sterile freezing tube, snap-frozen in liquid nitrogen, and stored at −80°C (Haier, DW-86L626, China) for DNA extraction. Total DNA was extracted from the intestinal contents using TIANamp Stool DNA Kits (Tiangen), following the manufacturer's instructions. A ultramicro biochemical spectrophotometer (Thermo Scientific, NanoDrop, 2000, China) and agarose gel electrophoresis (Beijing Liuyi Instrument Factory, DYY-6C, China) were used to determine DNA quantity and quality.

2.8 PCR amplification and 16S rRNA gene library construction

The V3–V4 hypervariable region of the bacterial 16S rRNA gene was PCR-amplified using universal primers (338F: 5′-ACTCCTACGGGA GGCAGCAG-3′, 806R: 5′-GGACTACHVGGGTWTCT AAT-3′). Indexed adapters were added to the ends of the 16S rRNA gene amplicons to generate indexed libraries for downstream NGS sequencing on the Illumina MiSeq platform. Sequencing adapters were also added to the termini of the PCR products to facilitate MiSeq sequencing. All PCR amplifications were performed in triplicate using TransStart FastPfu DNA Polymerase Kits (TransGen). Each 20ul PCR mixture contained 4ul of 5×FastPfu Buffer, 2.5 ul of dNTPs, 0.8 ul of each primer, 0.4 ul of FastPfu Polymerase, 0.2 ul of BSA, 10 ng of template DNA, and ddH2O to make 20ul. The thermal cycling conditions were as follows: initial denaturation at 95°C for 3min; 27 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 45 s; and a final extension at 72°C for 10 min. All of the PCR products were visualized on agarose gels (2% in TAE buffer) containing ethidium bromide and purified using DNA gel extraction kits (Axygen).

2.9 Bioinformatics analysis

After de-multiplexing the data and discarding certain reads, the remaining reads were converted to FASTQ format. In this study, 250-bp reads were truncated at any site receiving an average quality score of < 20 over a 10bp sliding window. Reads < 50bp were discarded. The minimum value of the overlap was 10bp when merging the reads; sequences whose barcodes did not match an expected barcode were also discarded.

Chimeric sequences were determined by UCHIME (Edgar et al., 2011). OTUs were defined with a threshold of 97% similarity by UPARSE (Edgar, 2013). Taxonomic richness and diversity estimators were determined for each library in Mothur. The mean of the estimated richness was used for comparisons among samples. The heatmap was constructed by using the heatmap 2 function of the R g-plots package based on the top 100 genera of the samples.

2.10 Histological analysis

The livers and intestines were collected and fixed in 4% paraformaldehyde. The fixed tissues were embedded in paraffin blocks and sliced into a series of transverse sections (4μm thick) using a Leica RM 2016 rotary microtome (Shanghai Leica Instrument Co., Ltd., China). A hematoxylin–eosin (HE) stain was used for general histological analysis. Each slide with tissue sections was mounted permanently using neutral balsam. The sections were scanned using a Panoramic 250/MIDI scanner (3D HISTECH Co., Ltd., Hungary), and Case viewer 2.0 (3D HISTECH Co., Ltd., Hungary) was used for image analysis and measurement. For each intestine sample, mucosal height and muscularis thickness were quantified by taking 10 measurements per intestinal section. For each liver sample, area, density and diameter of hepatocyte were quantified by taking 10 measurements per liver section.

2.11 Statistical analysis

Statistical analysis was performed using SPSS 19.0 statistical software package (SPSS Inc.). Excel 2016 for data processing and mapping. Microbioinformatics analysis and mapping were performed on the Meiji Biocloud platform (https://cloud.majorbio.com/) of Shanghai Meiji Biotechnology Co., Ltd. All of the values are presented as means ± standard deviation (mean ± SD). *t*-test was used to analyze the significant difference of *Astragalus* components before and after fermentation. One-way analyses of variance (ANOVAs) were used to analyze the data of growth performance, morphological index, serum biochemical index, digestive enzyme and intestinal flora α diversity test results. Comparisons between different groups were conducted by LSD test when there was a significant difference. We considered $p < 0.05$ statistically significant.

3 Results

3.1 Chemical characterization

3.1.1 Components

As shown in Table 1, compared with *Astragalus* herb, the total saponin content, polysaccharide extraction rate, total flavonoid content, astragaloside A and protein content of FAM were significantly different ($p < 0.05$), which were differed by 87.44%, 21.77%, 200%, 34.07% and 34.70%, respectively. After mass conversion, the total saponin content, polysaccharide extraction rate, astragaloside A content and total flavonoid content in FAM containing the same amount of AM were significantly different ($p < 0.05$), which were differed by 164.73%, 71.88%, 6.94% and 340%, respectively.

3.1.2 Relative molecular mass of polysaccharides

The molecular weight of the extracted polysaccharide of *Astragalus* herb and FAM was determined by GPC method. The polysaccharide with larger molecular weight entered the micropores of the filler less and was separated out first. The longer the retention time, the smaller the average molecular weight of the polysaccharide (Figure 1). The peak molecular weight (Mp), weight-average molecular weight (Mw), number-average molecular weight (Mn) and molecular weight (MW) distribution of polysaccharide samples on the chromatogram were calculated by GPC software through the viscosity and peak time of the samples (Tables 2, 3). The peak dispersion coefficient (Mw/Mn) data before and after fermentation showed that the molecular weight distribution of *Astragalus* polysaccharides before and after fermentation was uneven, and the distribution range was wide, including three different molecular weight components. However, the molecular weight distribution of *Astragalus* polysaccharides before and after fermentation was significantly different ($p < 0.05$). The highest molecular weight of the component represented by peak 1 after fermentation was 117634167 larger than that before fermentation, and the proportion of molecular weight above 1000000Da was 51.15%, which was also higher than that before fermentation, indicating that *Astragalus* promoted the dissolution of large molecular weight polysaccharides after fermentation. According to the difference in the relative proportion of the peak area of peak 3 to the total peak area before and after fermentation, it can be seen that the relative peak area of *Astragalus* polysaccharide peak 3 changed significantly after fermentation ($p < 0.05$), indicating that the fermentation of *Astragalus* promoted the degradation and utilization of large molecular weight polysaccharides by probiotics, which was converted into small molecular weight polysaccharides, resulting in an increase in the proportion of small molecular weight polysaccharides. The proportion of components represented by peak 3 with a molecular weight below 10000Da after fermentation also showed the same results.

3.2 Growth performance

Feed addition of FAM had no significant effect on the survival rate of tiger grouper. The WGR and SGR were highest in L4 group, being significantly different from all other treatments, including L0 group ($p < 0.05$). However, when the dose of FAM was the

TABLE 1 Composition changes of *Astragalus* membranaceus before and after fermentation.

Items	*Astragalus* herb	FAM	Effective substance content after mass conversion
Total saponin content/%	2.07 ± 0.05	3.88 ± 0.06*	5.48 ± 0.09*
Polysaccharide extraction rate/%	9.28 ± 0.06	11.30 ± 0.37*	15.95 ± 0.53*
Total flavone content/%	0.05 ± 0.00	0.15 ± 0.00*	0.22 ± 0.01*
Astragaloside A content/mg·kg^{-1}	1726.26 ± 25.39	1138.13 ± 22.08*	1606.39 ± 31.16*
Protein content/%	13.63 ± 0.12	18.36 ± 0.67*	—

Note: "*" indicates significant difference compared with *Astragalus* herb ($p < 0.05$, $n = 9$). FAM: fermented *Astragalus*.

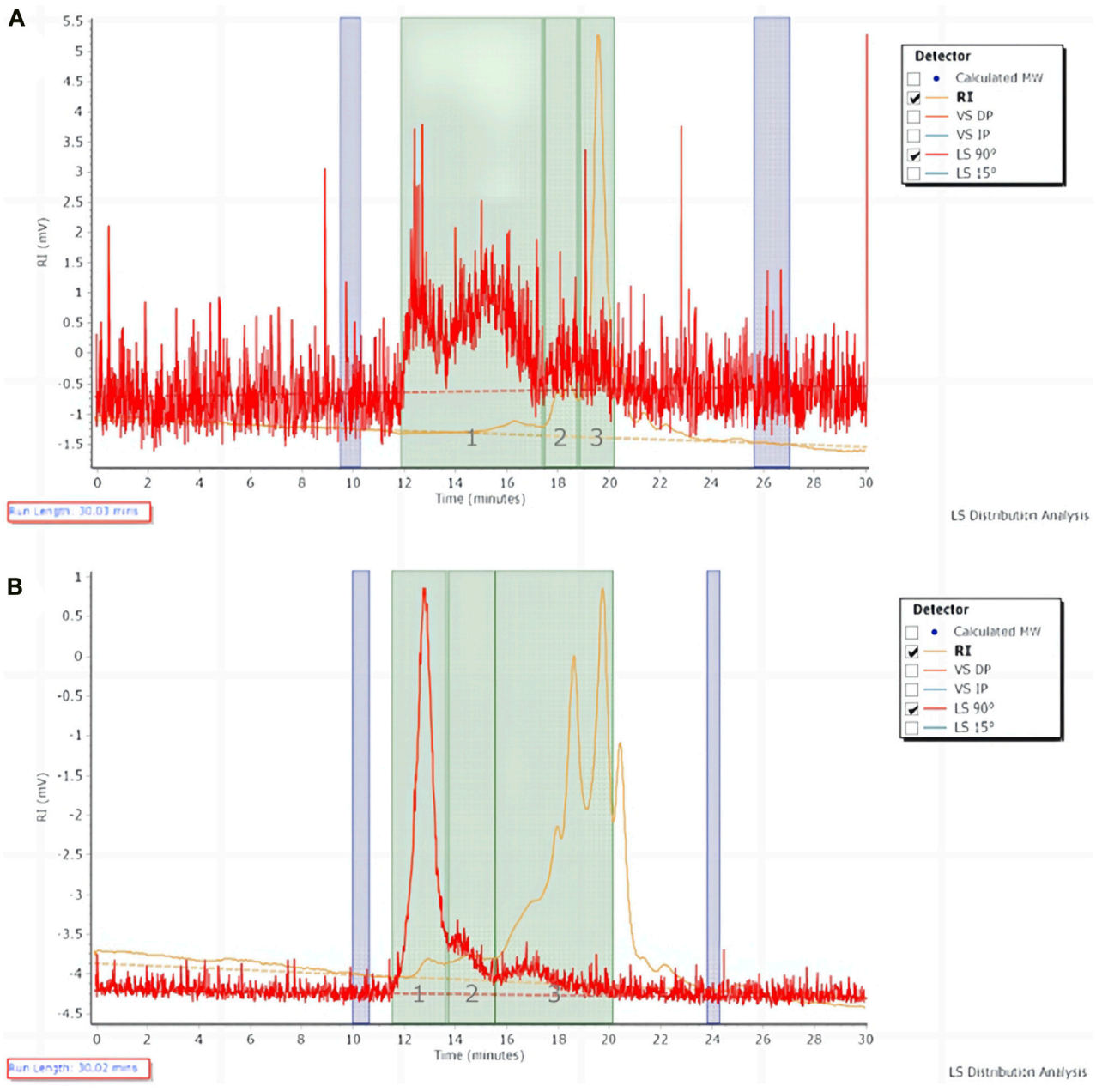

FIGURE 1
The chromatogram of polysaccharide in *Astragalus* herb and fermented *Astragalus*. **(A)** *Astragalus* herb; **(B)** Fermented *Astragalus*.

TABLE 2 Molecular weight determination result of polysaccharide in *Astragalus* herb and fermented *Astragalus*.

Samples	Peak number	Mp/Da	Mw/Da	Mn/Da	Mw/Mn
Polysaccharide of *Astragalus* herb	1	214819	364948	144580	2.524194
	2	54556	57901	57490	1.007149
	3	3578	39672	29834	1.329758
Polysaccharide of FAM	1	1669612	1679129	662840	2.533234
	2	59647	68658	52987	1.295752
	3	6511	11602	9467	1.22552

Note: FAM, fermented *Astragalus*; Mp, peak molecular weight; Mw, weight-average molecular weight; Mn, number-average molecular weight.

TABLE 3 Molecular weight distribution of *Astragalus* herb and fermented *Astragalus*.

Peak number		MW		MW		Percent/%	
		High limit MW/Da		Low limit MW/Da			
Astragalus herb	FAM	*Astragalus* herb	FAM	*Astragalus* herb	FAM	*Astragalus* herb	FAM
1	1	96741174	117634167	1000000	1000000	7.03	51.15
1	1	1000000	1000000	500000	500000	11.36	20.54
1	1	500000	500000	300000	300000	13.74	13.86
1	1	300000	300000	200000	200000	16.55	10.81
1	1	200000	200000	100000	175512	26.14	3.63
1		100000	—	50000	—	21.12	—
1		50000	—	43241	—	4.06	—
2	2	70060	155622	50000	100000	99.47	19.97
2	2	50000	100000	49909	50000	0.53	40.63
	2	—	50000	—	30000	—	28.01
	2	—	30000	—	24453	—	11.39
3	3	151226	36971	100000	30000	3.54	2.08
3	3	100000	30000	50000	20000	16.84	8.92
3	3	50000	20000	30000	10000	39.27	37.35
3	3	30000	10000	20000	5431	23.83	51.65
3		20000	—	10000	—	16.47	—
3		10000	—	9971	—	0.04	—

Note: FAM, fermented *Astragalus*; MW, molecular weight.

highest (L5 group), the WGR and SGR again decreased. The FC was lowest in L3 and L4 group, being significantly different from all other treatments, including L0 group ($p < 0.05$). However, when the dose of FAM was the highest (L5 group), the FC again increased (Table 4).

3.3 Serum biochemical parameters

The HDL-C was highest in L4 group, being significantly different from all other treatments, including L0 group ($p < 0.05$). Feed addition of FAM had no significant effect on TP, TC and LDL-C of tiger grouper (Table 5).

3.4 Digestive enzymes

The AMS was lowest in L0, being significantly different from L1, L4 and L5 ($p < 0.05$). The TRYP was lowest in L0, being significantly different from L5 ($p < 0.05$). The LPS was highest in L2 and L3, being significantly different from all other treatments, including L0 group ($p < 0.05$) (Figure 2).

3.5 Hepatic histology

The area and diameter of hepatocyte were lowest in L3 and L4, being significantly different from L1 ($p < 0.05$). The density of

TABLE 4 Effects of fermented *Astragalus* on growth performance of tiger grouper.

Items	L0	L1	L2	L3	L4	L5
SR/%	98.89 ± 1.92	98.89 ± 1.92	98.89 ± 1.92	100 ± 0.00	100 ± 0.00	100 ± 0.00
WGR/%	75.09 ± 6.38^{c}	84.27 ± 5.65^{bc}	77.76 ± 8.16^{bc}	90.18 ± 9.53^{b}	105.00 ± 7.10^{a}	79.42 ± 5.13^{bc}
SGR/%·d^{-1}	1.00 ± 0.06^{c}	1.09 ± 0.06^{bc}	1.03 ± 0.08^{bc}	1.15 ± 0.10^{b}	1.28 ± 0.06^{a}	1.04 ± 0.05^{bc}
FC	1.31 ± 0.17^{a}	1.23 ± 0.18^{a}	1.40 ± 0.18^{a}	0.93 ± 0.03^{b}	0.93 ± 0.07^{b}	1.24 ± 0.04^{a}

Note: The control group (L0) was fed commercial complete feed with no added FAM, The treatment group (L1, L2, L3, L4 and L5) was fed commercial complete feed supplemented with FAM (0.25%, 0.5%, 1%, 2% and 4%, respectively). SR, survival rate; WGR, weight gain rate; SGR, specific growth rate; FC, feed coefficient. In the same row, values with different small letter superscripts mean significant difference ($p < 0.05$, $n = 9$).

TABLE 5 Effects of fermented *Astragalus* on serum biochemical indexes of tiger grouper.

Items	L0	L1	L2	L3	L4	L5
TP/g·L^{-1}	11.55 ± 1.05	11.87 ± 0.73	12.25 ± 0.67	12.15 ± 0.44	12.33 ± 1.20	11.08 ± 0.75
TC/mmol·L^{-1}	3.27 ± 0.25	3.23 ± 0.14	3.19 ± 0.13	3.18 ± 0.11	3.04 ± 0.26	3.19 ± 0.33
HDL-C/mmol·L^{-1}	4.61 ± 0.16^{b}	5.06 ± 0.51^{b}	4.89 ± 0.72^{b}	5.02 ± 0.73^{b}	5.53 ± 0.42^{a}	5.22 ± 0.20^{b}
LDL-C/mmol·L^{-1}	1.80 ± 0.24	1.72 ± 0.49	1.66 ± 0.17	1.70 ± 0.17	1.59 ± 0.15	1.62 ± 0.07

Note: The control group (L0) was fed commercial complete feed with no added FAM, The treatment group (L1, L2, L3, L4 and L5) was fed commercial complete feed supplemented with FAM (0.25%, 0.5%, 1%, 2% and 4%, respectively). TP, total protein; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. In the same row, values with different small letter superscripts mean significant difference ($p < 0.05$, $n = 9$).

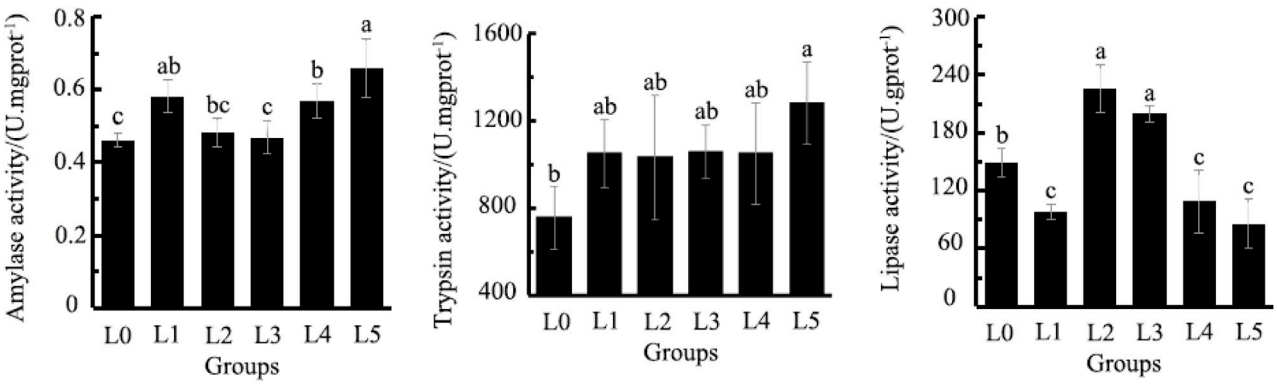

FIGURE 2
Effects of Fermented *Astragalus* on intestinal amylase activity, trypsin activity and lipase activity of tiger grouper. Note: The control group (L0) was fed commercial complete feed with no added FAM. The treatment group (L1, L2, L3, L4 and L5) was fed commercial complete feed supplemented with FAM (0.25%, 0.5%, 1%, 2% and 4%, respectively). Different superscript letters indicate significant differences among treatments ($p < 0.05$).

hepatocyte was highest in L4 group, being significantly different from all other treatments, including L0 group. However, when the dose of FAM was the highest (L5 group), the density of hepatocyte again decreased (Table 6).

The results of HE staining of liver tissue sections of tiger grouper was shown in Figure 3. In the L0 group, many hepatocytes were swollen and vacuolated, some nuclei were deviated, and the number of nuclei in the field of view was less than other groups (from density of hepatocyte). After the addition of FAM, the migration of hepatocyte nucleus in L1 group was reduced, but the cell swelling and vacuolization were not improved. In the L2 group, the number of hepatocyte nuclei increased significantly, a small number of nuclei shifted, and cell swelling and vacuolization were improved. Cell swelling, vacuolization and nuclear migration in the L3 group were further improved. In the L4 group, only a very few hepatocytes showed vacuolization and nuclear deviation, cell swelling was significantly improved, clear nuclei were visible in the center of the cells, and the number in the field of vision was significantly increased. The hepatocytes were evenly distributed and closely arranged, and the cell membrane contour was clear. The L5 group with a high proportion of FAM showed swelling and vacuolization, and there was a serious nuclear deviation. The number of nuclear deviation cells was significantly higher than that of other groups, showing a pathological feature.

TABLE 6 Effects of fermented *Astragalus* on liver morphological parameters of tiger grouper.

Items	L0	L1	L2	L3	L4	L5
Area of hepatocyte/um^2	63.23±	79.51±	72.00±	48.16±	47.98±	68.97±
	13.08^{ab}	7.14^{a}	1.69^{ab}	8.74^{b}	11.57^{b}	31.07^{ab}
Density of hepatocyte/(Number/mm^2)	3249.39±	3582.26±	3989.97±	3849.22±	4590.92±	3653.75±
	182.63^{d}	66.01^{c}	26.88^{b}	209.82^{bc}	220.97^{a}	275.12^{bc}
Diameter of hepatocyte/um	8.75±	9.71±	9.12±	7.95±	7.75±	8.87±
	0.75^{ab}	0.37^{a}	0.10^{ab}	0.54^{b}	0.67^{b}	1.48^{ab}

Note: The control group (L0) was fed commercial complete feed with no added FAM, The treatment group (L1, L2, L3, L4 and L5) was fed commercial complete feed supplemented with FAM (0.25%, 0.5%, 1%, 2% and 4%, respectively). In the same row, values with different small letter superscripts mean significant difference ($p < 0.05$, $n = 9$).

3.6 Intestinal histology

The measurement results of tiger grouper intestinal tissue sections in each group were shown in Table 7. The mucosal height was highest in L3 group, being significantly different from L0, L1, L2 and L4 groups ($p < 0.05$). The muscularis thickness was highest in L3 and L5 group, being significantly different from L0, L1 and L2 groups ($p < 0.05$). Feed addition of FAM had no significant effect on the mucosal number of tiger grouper.

At the same time, FAM affected the intestinal microstructure of tiger grouper, as shown in Figure 4. In the control group, the intestinal mucosa was sparse and disordered, and the columnar epithelial cells of the intestinal mucosa were dissolved and separated from the lamina propria. The nucleus at the base was disordered, and the intestinal mucosa microstructure was significantly damaged. Compared with the control group, the damage to the intestinal structure in each FAM group was alleviated, and the effect of the L3 group was better. The intestinal tissue structure was complete and clear. The intestinal mucosa was well developed, arranged closely and abundantly, and straight into the cavity. The nuclei of epithelial cells were closely and neatly arranged at the base, and the striated edges were neatly arranged.

3.7 Intestinal microbiota structure

3.7.1 Intestinal microbial OTU division and Alpha diversity comparison

The sequences with similarity higher than 97% were clustered into the same classification operation unit (OTU) for bioinformatics statistical analysis. From the OUT-classification level, draw the Venn diagram, as shown in Figure 5. A total of 336 OTUs were obtained from the test samples, of which the number of common OTUs was 90. The only OTUs in the L0-L5 group were 51, 25, 36, 45, 59 and 30, respectively. The total OTU values of each group were 141, 115, 126, 135, 149 and 120, respectively. Therefore, the ratio of the only OTU to the total OTU was 36.17%, 21.74%, 28.57%, 33.33%, 39.60% and 25.00%, respectively. The above results can be concluded that the structure of tiger grouper intestinal microbiota can be changed due to the intervention of FAM. The richness and diversity of intestinal microbial communities were analyzed by single sample diversity (Alpha diversity), including a series of statistical analysis indexes to estimate the species abundance and diversity of ecological communities. The Alpha diversity analysis of this experiment was estimated by five indexes. The results are shown in Table 8. At the level of 97% similarity, the sequencing coverage of each sample was higher than 99.8%. There was no significant difference in the Sob index, Shanno index, Simpson index, Ace index and Chao index between groups ($p > 0.05$).

3.7.2 Intestinal microbial community composition and difference analysis

As shown in Figure 6A, *Proteobacteria*, *Firmicutes*, Unclassified _ k __ norank _ d __ Bacteria, *Bacteroidota*, *Actinobacteria* and *Fusobacteriota* were the dominant phyla in tiger grouper at the phylum level. *Proteobacteria* (66.78%), *Firmicutes* (8.87%) and *Bacteroidota* (14.71%) were dominant in the L0 group. *Proteobacteria* (71.56%), *Firmicutes* (17.36%) and *Fusobacteriota* (8.15%) were dominant in the L1 group. The composition of dominant bacteria in L2-L4 groups was similar. The proportion of *Proteobacteria* in each group was 87.70%, 89.33%, 81.44% and 87.41%, respectively. The proportion of *Firmicutes* in each group was 2.81%, 2.28%, 4.77% and 1.91%, respectively. The proportion of Unclassified _ k __ norank _ d __ Bacteria in each group was 6.27%, 5.60%, 6.00% and 7.30%, respectively. In addition, the proportion of *Actinobacteria* in the L4 group was significantly higher than that in other groups, reaching 4.81%. The above shows that *Proteobacteria* was the first dominant phylum.

The relative abundance of tiger grouper intestine microbiota at the genus level is shown in Figure 6B. At the genus classification level, there were some differences in the dominant intestinal microbiota of each treatment group. The relative abundance of core microbiota was higher in *Ralstonia*, *Photobacterium*, *Vibrio*, Unclassified _ k __ norank _ d __ Bacteria and *Candidatus_ Cardinium*, which constituted the dominant genus in the intestinal microbiota of tiger grouper in this experiment. The abundance of *Ralstonia* and *Vibrio* were lowest in L0 group, being significantly different from all other treatments, except L4 group.

According to Figure 6C Partial Least Squares Discriminant Analysis (PLS-DA), the control group was significantly different from the experimental group in terms of species abundance, while the L4 group was more significantly different from the other groups, indicating that FAM had a specific effect on the intestinal microbiota of tiger grouper, and the L4 group had the most significant effect.

As shown in Figure 6D, according to the abundance information and species annotation of the samples at the level of intestinal bacterial genus, the genus with abundance in the top 50 was screened. According to the abundance information in each sample, the samples and species were clustered at two levels to

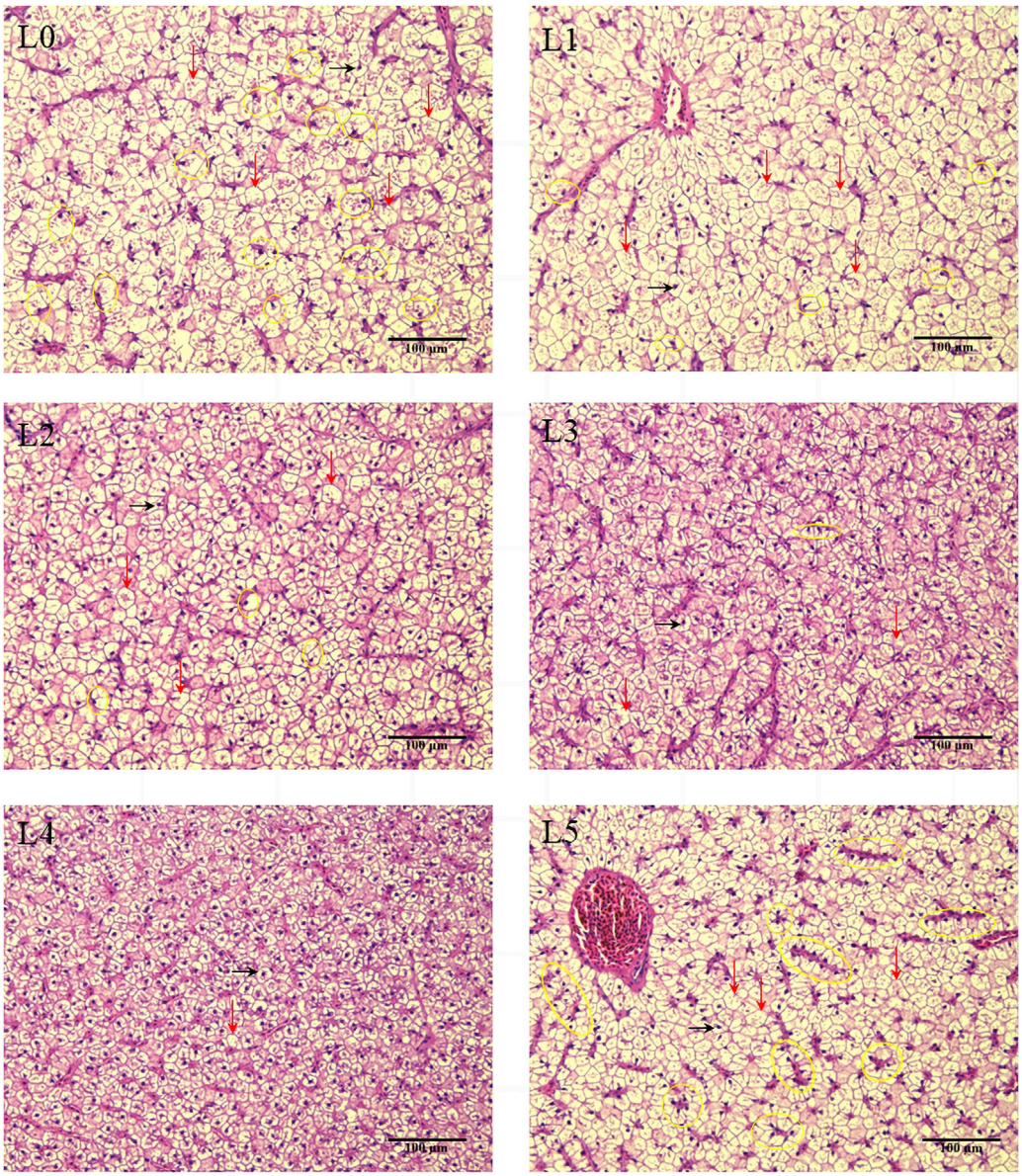

FIGURE 3
Effects of fermented *Astragalus* on hepatic tissue structure of tiger grouper. Note: The control group (L0) was fed commercial complete feed with no added FAM. The treatment group (L1, L2, L3, L4 and L5) was fed commercial complete feed supplemented with FAM (0.25%, 0.5%, 1%, 2% and 4%, respectively). The black arrow marks the liver nucleus, the black ellipse marks the shift of the liver nucleus, and the red arrow marks the hepatocyte swelling and vacuolation.

draw a heat map. The color gradient in the heat map analysis from blue to red indicates that the relative abundance is from low to high. The results showed that the intestinal microbial samples of each experimental group were first clustered into one branch, which was far away from the L0 group. The abundance of *Ralstonia* in the control group was lowest. In addition to the L4 group, the *Vibrio* abundance of each experimental group was also higher than that of the L0 group. The abundance of *Photobacterium*, *Pseudomonas*, *Wolbachia*, *Escherichia-Shigella* and *Shimwellia* in each experimental group was lower than that in the control group. The abundance of *Rhodococcus*, *Shinella*, *Lactobacillus*, *Achromobacter*, *Brucella*, *Brevibacterium*, *Acinetobacter* and *Brevundimonas* in the L4 group was higher than that in other groups. Except for the L3 group, the abundance of *Bacillus* in each experimental group was relatively higher than that in the L0 group.

4 Discussion

Modern pharmacological studies have shown that AM has anti-cancer, analgesic, anti-inflammatory, antibacterial, antioxidant and other pharmacological effects, and contains a variety of active ingredients such as saponins, flavonoids, polysaccharides, amino acids and trace elements. AM is one of the most widely used traditional herbs. The main functions of *Astragalus* polysaccharides and flavonoids are anti-oxidation and immunity

TABLE 7 Effects of fermented *Astragalus* on intestinal morphological parameters of tiger grouper.

Items	L0	L1	L2	L3	L4	L5
Mucosal height	355.60 ± 135.23b	346.64 ± 170.09^{b}	314.85 ± 144.72^{b}	447.79 ± 252.91^{a}	363.55 ± 149.26^{b}	402.38 ± 157.20^{ab}
Muscularis thickness	131.24 ± 27.12ab	113.93 ± 22.89^{b}	115.20 ± 34.51^{b}	154.85 ± 63.44^{a}	134.05 ± 43.47^{ab}	143.79 ± 29.01^{a}
Mucosal number	32.33 ± 3.06	30.33 ± 1.53	30.00 ± 2.65	34.00 ± 6.08	33.33 ± 8.50	31.33 ± 0.58

Note: The control group (L0) was fed commercial complete feed with no added FAM, The treatment group (L1, L2, L3, L4 and L5) was fed commercial complete feed supplemented with FAM (0.25%, 0.5%, 1%, 2% and 4%, respectively). Different superscript letters indicate significant differences among treatments ($p < 0.05$, $n = 9$).

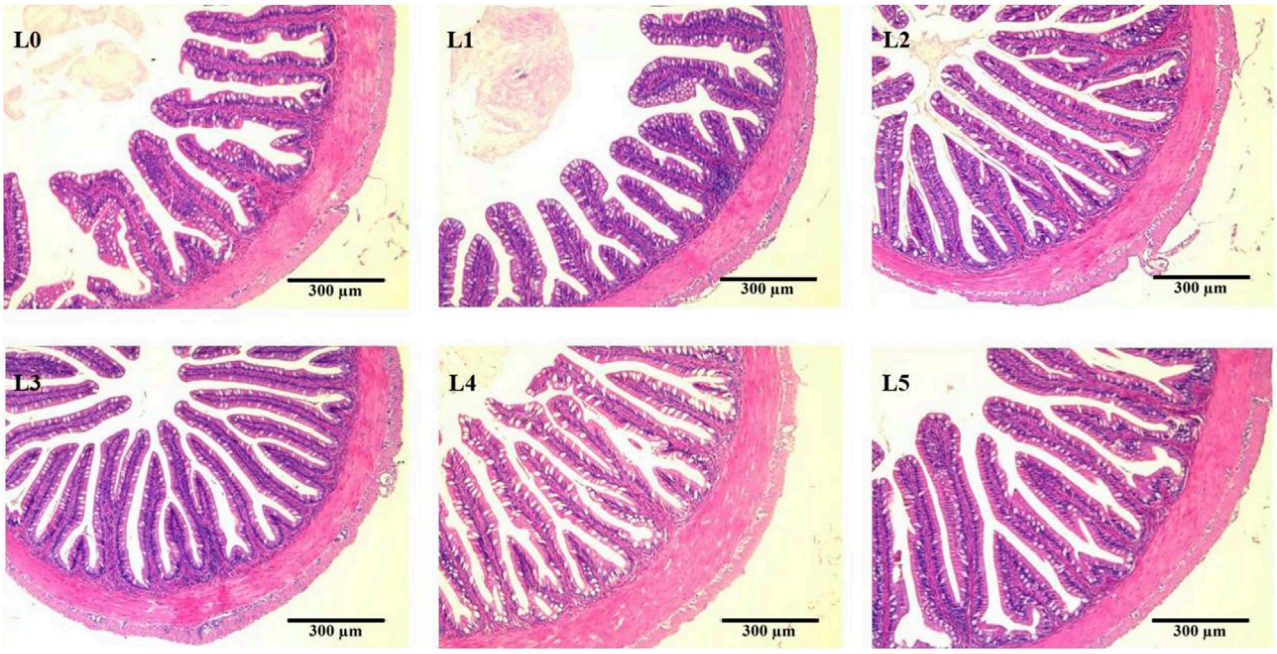

FIGURE 4
Effects of fermented *Astragalus* on intestinal morphology of tiger grouper. Note: The control group (L0) was fed commercial complete feed with no added FAM. The treatment group (L1, L2, L3, L4 and L5) was fed commercial complete feed supplemented with FAM (0.25%, 0.5%, 1%, 2% and 4%, respectively). h, mucosal height; t, muscularis thickness; black circle indicates the mucosa; red circle indicates the dissolution and shedding of columnar epithelial cells.

enhancement. Saponins have the functions of anti-tumor, immune regulation, and protection of the cardiovascular system. Astragaloside A is the main active ingredient of *Astragalus* saponins, which is a qualitative and quantitative index stipulated in the pharmacopoeia. In this experiment, AM was fermented by mixed bacteria solid-state fermentation process. After fermentation, it had a special fermented koji aroma. Compared with the crude drug AM, the protein content of FAM was significantly changed by 34.70%, indicating that a large amount of bacterial protein was produced after AM fermentation, and its nutritional value was improved. The total saponin content, polysaccharide extraction rate and total flavonoid content in FAM containing the same amount of AM were significantly changed by 164.73%, 71.88% and 340%, but the content of astragaloside A was significantly changed by 6.94%. Similar probiotic fermentation experiments of AM showed that the use of *Aspergillus Niger*, *Lactobacillus plantarum*, and *Saccharomyces cerevisiae* for mixed solid-state fermentation of AM, the polysaccharide extraction rate and total flavonoid content of AM increased by 11.93% and 43%, respectively (Zhou, 2018). The liquid fermentation of AM roots was carried out by *non-lactose streptococcus*, and the increase rates of polysaccharides, total saponins and total flavonoids were 177.46%, 68.50% and 55.67%, respectively (Su, 2017). Wang (2020) used liquid fermentation of AM by *Bacillus natto*. After fermentation, the polysaccharide content decreased by 28.49% compared with that before fermentation, the total saponin increased by about 9.21%, and the total flavonoid content increased by about 36.43% (Wang, 2020); Zhao (2015) used *lactic acid bacteria* to ferment AM by solid-state fermentation. The content of polysaccharide and astragaloside A were the highest on the 12th and 18th days, respectively, and the increase rates were 95.5% and 17.46% higher than those before fermentation, but lower than the control group in the later stage of fermentation (Zhao, 2015); Qin (2012) used the selected lactic acid bacteria to

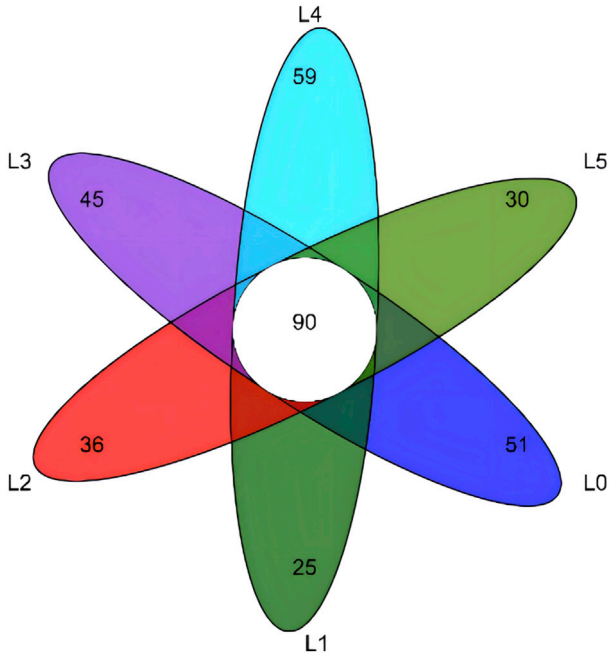

FIGURE 5
Venn diagram of intestinal microbiota of tiger grouper. Note: The control group (L0) was fed commercial complete feed with no added FAM. The treatment group (L1, L2, L3, L4 and L5) was fed commercial complete feed supplemented with FAM (0.25%, 0.5%, 1%, 2% and 4%, respectively).

ferment AM in liquid state, and the yield of polysaccharide increased by 59.34%, but the extraction rate of total saponins decreased by 17.20% after fermentation, and the content of astragaloside A decreased by 27.6% (Qin, 2012). The above results showed that different fermentation methods had different effects on the yield of effective components of AM. Under the action of powerful enzymes of microorganisms, dense structural components such as cellulose, hemicellulose and pectin in plant cell wall are decomposed and transformed, resulting in loose structure of plant cell wall, increased intercellular space and easy release of active components, so the content of active components is increased (Song et al., 2021). However, some chemical components may also be bio transformed by probiotics, thus changing the chemical composition of traditional Chinese medicine (Ai et al., 2019). In this study, the content of astragaloside A in FAM decreased, indicating that the fermented strain may have been bio transformed with this precursor. The specific transformation pathway and the new compounds generated need to be further studied. As one of the extremely important active ingredients of *Astragalus*, *Astragalus* polysaccharides have pharmacological effects such as anti-virus, regulating blood sugar, anti-oxidation and enhancing immunity (Wang et al., 2022b). The use of gel chromatography to detect the molecular weight changes of *Astragalus* polysaccharides before and after fermentation helps to evaluate its efficacy changes. In this experiment, the yield of polysaccharides increased after fermentation, and the proportion of polysaccharides in the low molecular weight section changed significantly after fermentation, indicating that the high molecular weight polysaccharides were degraded after fermentation, which was consistent with the results of Liang Zijing FAM polysaccharides (Liang, 2019). High molecular weight polysaccharides have poor water solubility, complex structure and conformation, and are difficult to cross the tissue barrier into the cell or attach to the receptor to play a role (Zhang, 2020). On the contrary, low molecular weight polysaccharides have higher solubility and lower viscosity than high molecular weight polysaccharides in water, so they are more easily absorbed by the body when they act in the body, have higher bioavailability, and have higher affinity with phagocytes, which contributes to immune activation (Li et al., 2020); In addition, polysaccharides with lower molecular weight also have higher antioxidant activity (Sun et al., 2009). The proportion of low molecular weight polysaccharides was significantly increased after fermentation of AM. The results provide a reference for the functional characteristics of FAM, such as immune regulation and intestinal microecology improvement.Enzymes produced in the process of microbial fermentation can effectively decompose plant cell wall, so that the effective components can be released from the cell to improve the medicinal effect of traditional Chinese herbal medicine (Hussain et al., 2016). A large number of studies had shown that fermented Chinese medicine can improve the growth performance of aquatic animals, such as common carp (*Cyprinus carpio*) (Zhao et al., 2017; Shi et al., 2022a), *Cyprinus carpio haematopterus* (Xie et al., 2015) and Ctenopharyngodon idella (Tang et al., 2021). During the experiment, the WGR and SGR were highest in L4 group, the FC was lowest in L3 and L4 group, being significantly different from all other treatments, including L0 group. The comprehensive performance of the L3 and L4 group

TABLE 8 Alpha-diversity indexes of gut microbiota of tiger grouper.

Groups	Sob index	Shanno index	Simpson index	Ace index	Chao index
L0	148.33 ± 69.30	2.11 ± 0.76	0.28 ± 0.11	165.25 ± 65.31	175.61 ± 66.65
L1	133.00 ± 13.53	2.25 ± 0.31	0.23 ± 0.14	146.10 ± 8.74	147.57 ± 12.22
L2	139.33 ± 13.58	1.98 ± 0.26	0.30 ± 0.07	143.18 ± 12.98	144.21 ± 11.87
L3	136.00 ± 10.15	1.76 ± 0.64	0.43 ± 0.18	141.60 ± 6.64	146.89 ± 6.60
L4	170.33 ± 3.21	2.53 ± 0.37	0.29 ± 0.09	176.37 ± 3.35	179.44 ± 7.85
L5	141.33 ± 28.57	1.87 ± 0.56	0.36 ± 0.13	146.18 ± 28.42	147.46 ± 30.23

Note: The control group (L0) was fed commercial complete feed with no added FAM, The treatment group (L1, L2, L3, L4 and L5) was fed commercial complete feed supplemented with FAM (0.25%, 0.5%, 1%, 2% and 4%, respectively).

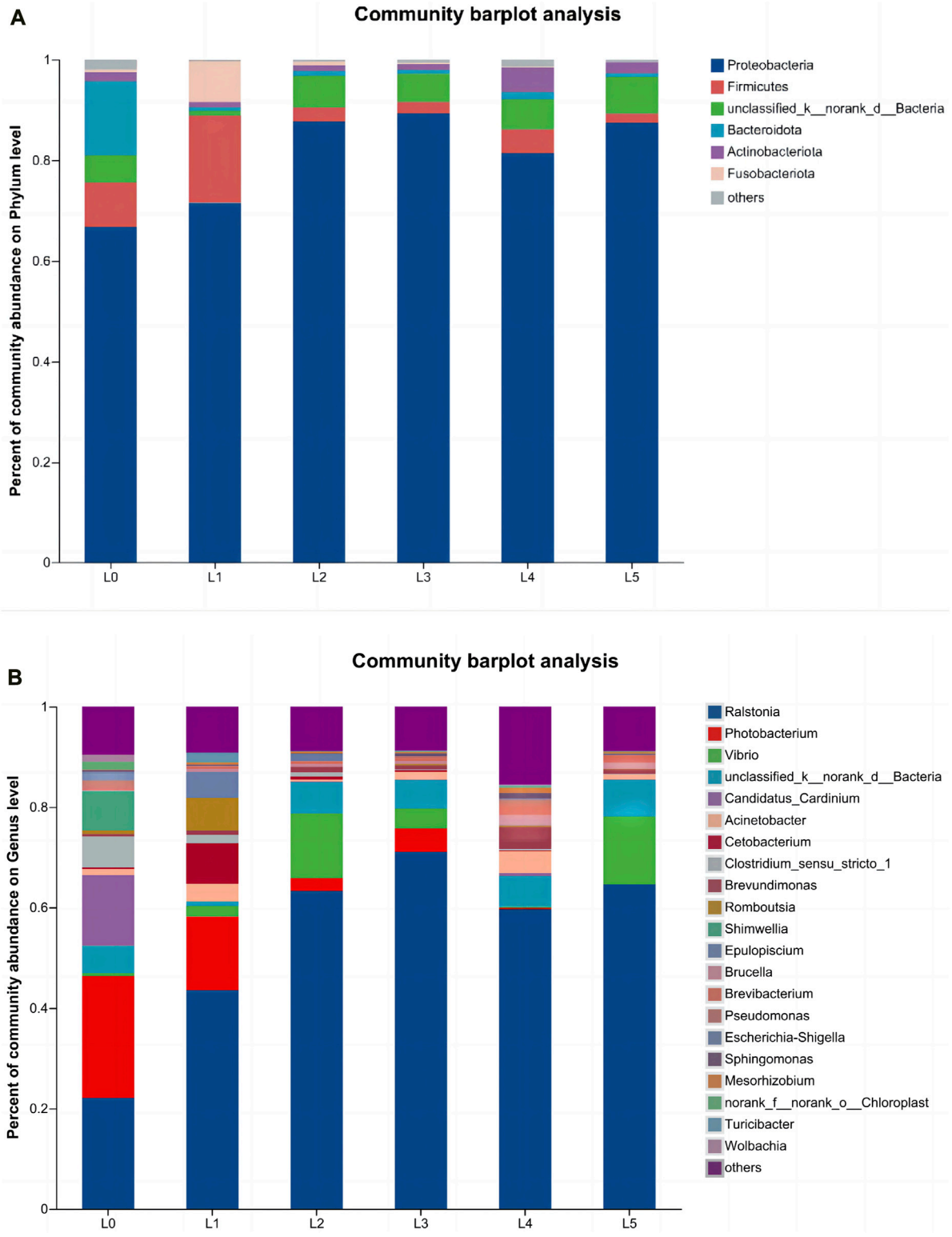

FIGURE 6
Intestinal microbiota of tiger grouper. **(A)** Community composition at phylum level; **(B)** Community composition at genus level; **(C)** PLS-DA **(D)** The clustering heat map analysis at the genus level. Note: The control group (L0) was fed commercial complete feed with no added FAM. The treatment group (L1, L2, L3, L4 and L5) was fed commercial complete feed supplemented with FAM (0.25%, 0.5%, 1%, 2% and 4%, respectively).

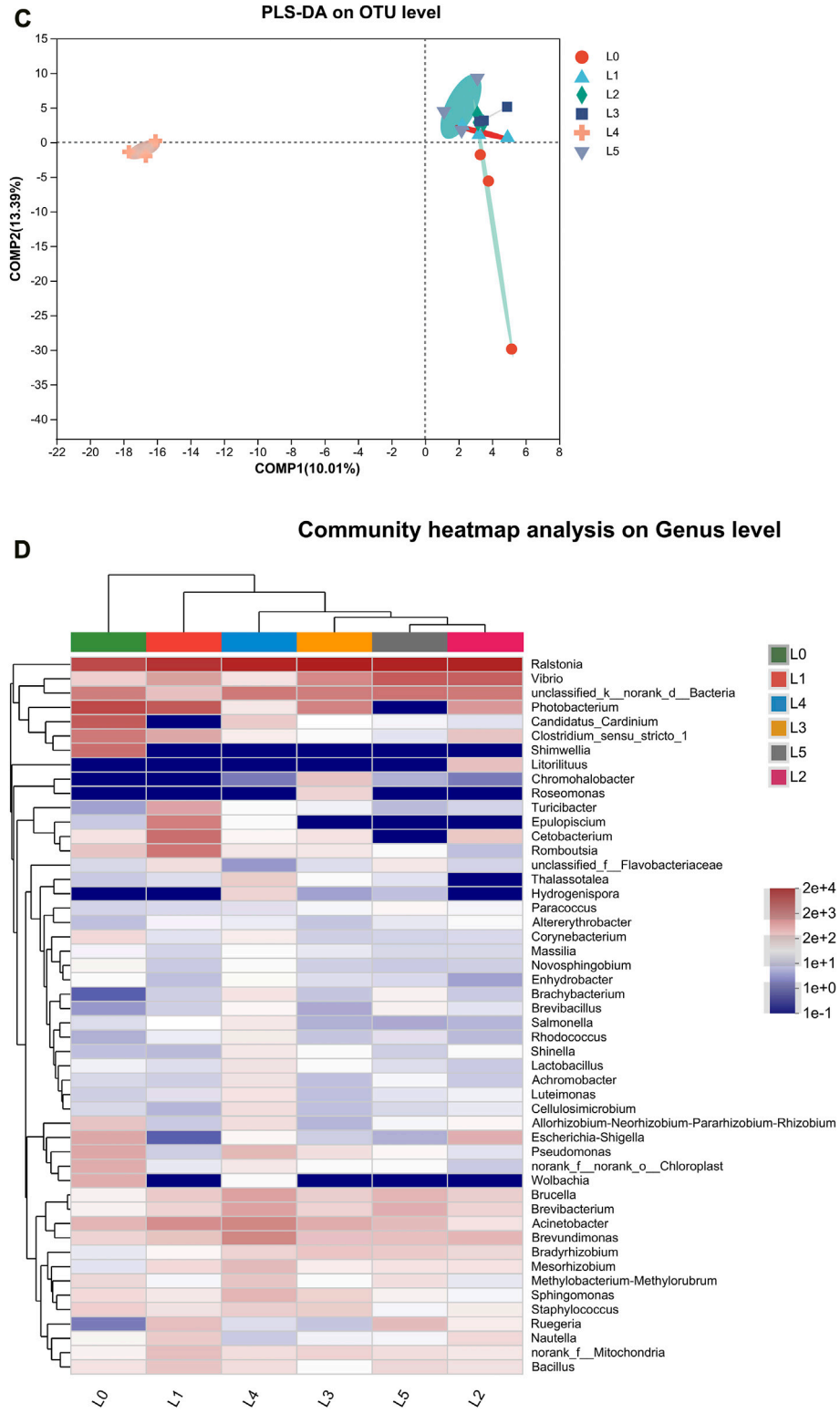

FIGURE 6
Continued.

was better, indicating that FAM can promote the digestion and absorption of nutrients in fish and promote the growth of tiger grouper, which was similar to the results of *Palea steindachneri* (Nong and Li, 2019) and common carp (*Cyprinus carpio*) (Shi et al., 2022a). The polysaccharide yield of Astragalus was improved after probiotic fermentation (Xiao et al., 2023). As one of the effective active ingredients of *Astragalus*, *Astragalus* polysaccharide can promote the growth of fish has been reported (Xiang et al., 2011;

Wang et al., 2018). In addition, the probiotics added during the fermentation process can secrete a variety of digestive enzymes after activation, growth and reproduction, thereby improving intestinal health and improving the digestion and absorption capacity of animals (Xiao et al., 2023). The nutritional factors such as bacterial protein, vitamins, and small peptides produced during the metabolic process may also have a positive effect on the growth of the test fish. Studies had shown that adding 1.5% *Astragalus* directly to *Pseudosciaena crocea* R. feed causes more than half of the mortality rate (Wei, 2014). In this experiment, the addition of FAM had no significant effect on the survival rate of tiger grouper, and the highest dose of the L5 group (4% FAM) also had no death, indicating that the fermentation treatment of AM may reduce the toxic and side effects of high-dose traditional Chinese medicine on fish. The reason is that the microbial population in fermentation decomposes and transforms the anti-nutritional components of traditional Chinese medicine or modifies the structure of toxic substances, thus improving the pharmacological characteristics of Chinese herbal medicine (Li et al., 2021b), which was also one of the advantages of fermented traditional Chinese medicine. In general, the addition of FAM at 1%–2% had a better effect on promoting growth and reducing feed ratio.

The metabolism and physiological and pathological conditions of fish can be reflected by serum biochemical indicators (Zhou et al., 2001). Liver is the main place for protein synthesis, and serum TP concentration can reflect the ability of liver protein synthesis and metabolism (He et al., 2017). Serum TC is mainly synthesized in the liver, reflecting the liver's metabolism of fat (Tan et al., 2018). Cholesterol transport depends on high-density lipoprotein and low-density lipoprotein. High-density lipoprotein helps transport cholesterol from peripheral tissues and plasma to the liver, degrades and removes excess cholesterol to maintain cholesterol homeostasis in the body (Meng et al., 2021). Low-density lipoproteins transport endogenous cholesterol synthesized in the liver to peripheral tissues for their use (Wang et al., 2021a). Feed addition of FAM had no significant effect on TP, TC and LDL-C of tiger grouper. The HDL-C was highest in L4 group, being significantly different from all other treatments, including L0 group. The above results indicate that FAM can promote the lipid transport in the liver of tiger grouper.

The liver is the main metabolic organ of fish and plays an important role in regulating physiological functions, such as digestion, nutrient storage, synthesis of new substances, detoxification of harmful chemicals and metabolic homeostasis (He, 2019; Zhong et al., 2021). In order to pursue the benefits of aquaculture, high-energy long-term feeding can lead to liver dysfunction in fish, which in turn induces fatty liver and metabolic disorders, eventually leading to slow growth of fish (Tan et al., 2019) Previous studies have shown that plants or plant extracts can increase liver lipid metabolism and improve liver morphology in fish (Tan, 2020; Sun et al., 2022). Compared with the control diet, *Pangasianodon hypophthalmus* juveniles fed with AM extract significantly reduced liver injury index enzymes and improved liver health (Abdel-Latif et al., 2022). Similar results were obtained in juvenile crucian carp (*Carassius auratus*) fed with AM polysaccharide (Wu, 2020). The protective effect of *Astragalus* polysaccharides on common carp (*Cyprinus carpio*) liver cells was verified in the model of liver injury induced by carbon tetrachloride (CCl_4) (Jia et al., 2012). And the FAM polysaccharides also had antagonistic effects on liver injury and liver fibrosis in rats (Qin, 2012). In the present research, studies have shown that *Astragalus* active substances can prevent the negative effects of internal or external factors on the liver, and then play its liver protection role. In this study, histological examination showed that hepatocytes in group L0 fed only artificial basal diet showed swelling vacuolization and nuclear shift, indicating that the liver integrity of tiger grouper was sensitive to artificial basal diet. Studies have shown that compared with feeding ice fresh feed, feeding artificial basic feed can induce liver lipid accumulation, and liver histological analysis shows more vacuolization, which in turn causes liver inflammation and oxidative stress, and ultimately leads to liver injury (Ma et al., 2020), which is consistent with our research results. In the gradient addition ratio of FAM set in this experiment, the liver histology was gradually improved with the increase of dose, and the liver integrity of the L4 group was the best (cell swelling was significantly improved, and the number of cells in the nucleus was significantly increased). In the L5 group with the highest addition ratio, cell swelling and vacuolization occurred, and severe nuclear deviation occurred. The pathological characteristics showed that liver cell damage occurred in the L5 dose group. The above research results showed that adding appropriate proportion of FAM to the feed can effectively regulate the homeostasis of fat metabolism and is beneficial to liver health. However, high-dose addition may have toxic effects on the liver, destroy the structure and function of the liver, and make the liver lipid metabolism function abnormal, resulting in a certain degree of disorder in the fat transport system, but cell swelling and vacuolization. The possible reasons why FAM is beneficial to liver health are as follows. Astragalus has effective antioxidant components, such as astragaloside, flavonoids and polysaccharides, which can effectively prevent tissue damage through its antioxidant mechanism (Muhammad et al., 2016). In addition, probiotics in FAM may also be beneficial to fish liver health. The application of multiple varieties of probiotics changed the shape of liver nuclei from irregular to regular and reduced the space between liver tissues. Adding *Bacillus subtilis* solid-state fermentation products to zebrafish feed can improve liver lipid metabolism and alleviate lipid deposition (Wang et al., 2022a). The possible mechanism of probiotics improving liver health is to regulate glucose and lipid metabolism, reduce fat accumulation in the liver, regulate intestinal microbiota homeostasis, repair intestinal barrier and relieve inflammation (Yao et al., 2021).

The biological process of fermentation leads to changes in the nutritional composition of the fermentation substrate (Zhang et al., 2012). Among them, the added probiotics have many beneficial effects, such as killing or inhibiting pathogens, affecting the intestinal microflora, and promoting nutrient utilization (Gao et al., 2022), thus affecting the digestion ability of animals. The results of this experiment showed that FAM had different degrees of improvement on the activity of digestive enzymes in tiger grouper. The principle of Chinese herbal medicine as a natural growth promoter may be to induce the secretion of digestive enzymes and stimulate appetite (Zhang et al., 2015), and to cooperate with the probiotics added by fermentation to improve the intestinal microenvironment. The addition of FAM in this experiment increased the activity of digestive enzymes in tiger grouper and reduced the feed coefficient, indicating that the reason why the

addition of FAM in the feed accelerated the growth rate of tiger grouper was not by increasing its food intake, but by enhancing its digestion and absorption capacity.The intestinal morphology of fish is closely related to dietary components (Wang et al., 2017; Fu et al., 2021; Yang et al., 2022). The complete intestinal structure is crucial for the body to prevent pathogens and toxins from entering the systemic circulation, absorb and utilize nutrients, and resist environmental pressure (Yang et al., 2022). It is an important guarantee for the growth rate and health status of aquatic animals. The intestinal mucosa formed by the epithelial cells of the intestinal mucosa is the basic structure of the intestinal tract. The height, quantity, morphological structure and sparse degree of the intestinal mucosa and the development degree of the striated edge on the surface have a great influence on the homeostasis of the intestinal environment and the digestion and absorption of nutrients (Khosravi et al., 2015). Intestinal epithelial cells, tight junction proteins and mucus layer constitute the intestinal barrier function (Yan and Ajuwon, 2017). In this experiment, compared with the control group, the addition of FAM reduced the dissolution and shedding of columnar epithelial cells of intestinal mucosa, improved the morphology of intestinal tissue, significantly increased the height and number of intestinal mucosa and the thickness of muscular layer, the addition ratio of 1% was the best. The reason may be that short-chain fatty acids (Qiao, 2020) or other prebiotics are produced during the fermentation process of AM, which promotes the growth of beneficial bacteria, inhibits harmful bacteria, improves the homeostasis of intestinal environment, and thus facilitates the development of intestinal mucosa (Cheng et al., 2021). The improvement of intestinal morphology enhances the intestinal barrier function, improves the immune and pathogenic microbial defense ability, and may reduce intestinal cell apoptosis (Wu et al., 2022a), and ultimately has a positive impact on the body's nutrient utilization and growth (Yang et al., 2022).

The intestinal microbiota of fish is essential for maintaining the health of aquatic animals (Wang et al., 2021b). Intestinal microbiota is a key factor affecting various functions of the host, including development, digestion, growth, disease resistance and immunity (Burr et al., 2005). Therefore, it is very important to explore how to change the intestinal microbiota when feeding tiger grouper FAM. The results of this experiment showed that the microbial composition of the intestinal tract of tiger grouper changed significantly with the 8-week breeding experiment. Except that the diversity and richness of the L4 group showed an upward trend compared with the other groups, FAM had no significant effect on the Alpha diversity index of intestinal microbiota, but each group in this experiment had a considerable proportion of unique OUT, indicating that FAM changed the composition of intestinal microbial community. At the phylum level, *Proteobacteria* was the absolute dominant phylum in each group, the relative abundance in the control group was 66.78%, and the relative abundance in each treatment group increased by more than 70%. *Bacteroidota* was significantly enriched in the L0 group, *Fusobacteriota* was significantly enriched in the L1 group, and the *Firmicutes* enrichment abundance in the L2-L4 group was lower than that in the L0 and L1 groups. *Proteobacteria*, *Bacteroidota*, *Fusobacteriota* and *Firmicutes* represent more than 80% of intestinal microbes in various marine and freshwater species (He et al., 2018; Li et al., 2022). The similarity of this bacterial taxa indicates that intestinal microbes are involved in important host intestinal functions such as digestion of nutrients and immunity (Xu et al., 2023). The increase of *Proteobacteria* abundance may be beneficial to the absorption and utilization of feed nutrients by fish under the catabolism of feed components by bacteria (Roeselers et al., 2011; Wu et al., 2022b). *Ralstonia* (Bai et al., 2019) and *Vibrio* (Clements et al., 2014) are the main intestinal microbiota of marine fish, but *Vibrio* is also a potential pathogen of marine fish. *Ralstonia* is a potential pathogen for humans and animals (Monica et al., 2019), and no pathogenic cases have been reported in aquatic animals. Some strains of *Photobacterium*, *Pseudomonas*, *Acinetobacter*, and *Escherichia-Shigella* are often considered as potential pathogens (Behera et al., 2018; Shao et al., 2020; Li et al., 2022), and a certain number of these microbiotas exist in fish for a long time, which may cause disease when fish immunity decreases and microbiota imbalance occurs. *Bacillus* can secrete digestive enzymes and has a good inhibitory effect on pathogenic bacteria (Lu, 2021). *Lactobacillus* can metabolize to produce lactic acid and antibacterial bacteriocins, and also has an inhibitory effect on pathogens (Kaktcham et al., 2017). In addition, *Lactobacillus* can also induce the proliferation of short-chain fatty acid-producing strains in intestinal microbiota, increase the content of short-chain fatty acids in intestinal tract, and benefit intestinal health (Liu, 2019). The change trend of *Ralstonia* abundance was consistent with the growth performance. The increase of *Ralstonia* abundance may promote the growth and feed utilization of fish. The possible reason is that the synthesis of short-chain fatty acids in intestinal microbiota is enhanced after intake of FAM, and its activation enhances the energy supply of the genus bacteria. The abundance of *Photobacterium*, *Pseudomonas* and *Escherichia-Shigella* in L0 group was highest. The abundance of *Vibrio* in L4 group was lowest while the abundance of *Acinetobacter* and *Lactobacillus* in L4 group was the highest. The abundance of *Bacillus* in L3 group was lowest. The above changes in the abundance of potential pathogenic bacteria and intestinal colonization probiotics indicated that FAM regulated the complexity of intestinal microbiota structure, which may be related to its dose effect. The L4 group had relatively lower abundance of potential pathogenic bacteria and higher abundance of colonization probiotics, indicating that this dose had the best effect on improving intestinal microbiota. Healthy intestinal microbiota can improve the body's immunity and promote the barrier function of the intestine. It can not only secrete digestive enzymes to promote the conversion and absorption of feed nutrients, but also secrete vitamins to make up for the deficiency of feed nutrients (Wang et al., 2012), which is consistent with the results of FAM improving intestinal morphology and growth performance. Xiao et al. (2023) studies have shown that FAM has a degradation effect on Astragalus polysaccharides. Low molecular weight polysaccharides have high solubility and higher bioavailability, while potential probiotics can ferment polysaccharides or oligosaccharides to produce short-chain fatty acids (Georgina, 2014), thereby promoting fish health. Flavonoids in FAM may inhibit pathogenic bacteria and promote the growth of probiotics in the intestine, thereby regulating the structure of intestinal microbiota (Zhao et al., 2021b). In addition, the probiotics and their metabolites in FAM also affect the composition and structure of fish intestinal microorganisms, and cooperate with the physiologically active

substances in Astragalus to maintain the stability and balance of intestinal microbiota.

5 Conclusion

The fermented *Astragalus* could promote the growth of tiger grouper and improve the feed conversion rate. FAM also could improve intestinal and liver morphology and regulate intestinal microbiota. This study shows that FAM can promote the growth performance and liver and intestinal health of tiger grouper. However, the high addition ratio of FAM may will adversely impact the fish body. Therefore, it is recommended that the addition ratio of FAM in the tiger grouper feed be 1%–2% of the diet weight.

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 SRA under PRJNA1001328.

Ethics statement

The animal study was reviewed and approved by the Animal Care and Use Committee of South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JY: Writing–original draft, visualization, data curation. SZ: Writing–original draft, investigation, conceptualization. ZF: Writing–review and editing, validation, formal analysis. BX: Writing–original draft, methodology. ML: Writing–review and editing, software. GY: Writing–review and editing, resources. ZM: Writing–review and editing, supervision, project administration. HZ: Writing–review and editing, funding acquisition.

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Conflict of interest

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

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Transcriptome analysis and immune gene expression of channel catfish (*Ictalurus punctatus*) fed diets with inclusion of frass from black soldier fly larvae

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The larval waste, exoskeleton shedding, and leftover feed components of the black soldier fly and its larvae make up the by-product known as frass. In this study, we subjected channel catfish (*Ictalurus punctatus*) to a 10-week feeding trial to assess how different dietary amounts of frass inclusion would affect both systemic and mucosal tissue gene expression, especially in regard to growth and immune-related genes. Fish were divided in quadruplicate aquaria, and five experimental diets comprising 0, 50, 100, 200, and 300 g of frass per kilogram of feed were fed twice daily. At the end of the trial, liver, head kidney, gill, and intestine samples were collected for gene expression analyses. First, liver and intestine samples from fish fed with a no frass inclusion diet (control), low-frass (50 g/kg) inclusion diet, or a high-frass (300 g/kg) inclusion diet were subjected to Illumina RNA sequencing to determine global differential gene expression among diet groups. Differentially expressed genes (DEGs) included the upregulation of growth-related genes such as glucose-6-phosphatase and myostatin, as well as innate immune receptors and effector molecules such as toll-like receptor 5, apolipoprotein A1, C-type lectin, and lysozyme. Based on the initial screenings of low/high frass using RNA sequencing, a more thorough evaluation of immune gene expression of all tissues sampled, and all levels of frass inclusion, was further conducted. Using targeted quantitative PCR panels for both innate and adaptive immune genes from channel catfish, differential expression of genes was identified, which included innate receptors (TLR1, TLR5, TLR9, and TLR20A), proinflammatory cytokines (IL-1β type a, IL-1β type b, IL-17, IFN-γ, and TNFα), chemokines (CFC3 and CFD), and hepcidin in both systemic (liver and head kidney) and mucosal (gill and intestine) tissues. Overall, frass from black soldier fly larvae inclusion in formulated diets was

found to alter global gene expression and activate innate and adaptive immunity in channel catfish, which has the potential to support disease resistance in this species in addition to demonstrated growth benefits.

KEYWORDS

channel catfish, frass, alternative diets, feed additives, RNA-seq, innate immunity, adaptive immunity, metabolism

1 Introduction

The aquaculture sector has to become more sustainable and globally competitive through the advancement of knowledge on fish nutritional requirements and through the continuous search for sustainable raw materials that can solve strategic problems in fish nutrition (Yildirim-Aksoy et al., 2020a; Yildirim-Aksoy et al., 2020b; Mohan et al., 2022). During the last few decades, significant efforts have been made toward the development of less expensive and more sustainable alternative protein and lipid sources with respect to fish meal (FM) and fish oil (FO), without compromising fish welfare and the overall nutritional value of its diet (Belghit et al., 2019; Fawole et al., 2021; Weththasinghe et al., 2021; Zarantoniello et al., 2022). Plant-derived ingredients, microalgae and microbial biomass, terrestrial animal by-products, and insects have been utilized as an alternative for FM and FO (Zarantoniello et al., 2019; Mohan et al., 2022). Insects as alternative feed ingredients for aquafeed formulations have been previously investigated in herbivorous/omnivorous fish and are considered one of the most promising cost-effective, sustainable, and alternative ingredients to replace FM and FO in fish diets. In fact, besides their presence in the natural diet of both freshwater and marine fish species, insects are characterized as having a proper nutritional profile (especially in terms of amino acids, vitamins, and minerals) for fish and being environmentally friendly in aquaculture (low energy and water consumption, and no arable lands are required) (Nyakeri et al., 2017; Swinscoe et al., 2019; Xu et al., 2020; Mohan et al., 2022; Yildirim-Aksoy et al., 2022). Furthermore, the majority of insect larvae can grow on low-quality organic wastes so that the reuse of the remaining organic substrate results in efficient bioconversion and the larvae or prepupae can have enriched proteins, lipids, minerals, and vitamins (Rana et al., 2015; Yildirim-Aksoy et al., 2020a; Yildirim-Aksoy et al., 2020b; Xu et al., 2021; Mohan et al., 2022). Additionally, many insect species have shown an inhibitory effect on fungal and bacterial growth such that their dietary inclusion may increase the shelf life of feed and also provide beneficial effects on the gut of fish (Sheppard et al., 2002; Van Huis et al., 2013; Hounmanou et al., 2018; De Silva et al., 2019; Hoc et al., 2019; Koutsos et al., 2022; Hasan et al., 2023).

Over the last decade, interest in incorporation of insects in fish diet formulations has significantly increased. As a result, several studies have been conducted on various fish species, such as carnivores, herbivores, and omnivores, in utilizing feeds containing insects. Among the insects, the black soldier fly (BSF) (*Hermetia illucens*, Linnaeus 1758, Diptera, Stratiomyidae family) and its larvae, which are native to the tropical, subtropical, and warm temperate areas of Southern United States (three generation cycles per year) have been shown to be a good replacement of FM and FO (Hoc et al., 2019). In this regard, feed incorporated with different combinations of BSF led to the best overall response in terms of growth and health in trout (Huyben et al., 2019; Fawole et al., 2021; Bolton et al., 2021; Caimi et al., 2021; Hoc et al., 2021; Hwang et al., 2021; Melenchón et al., 2021; Randazzo et al., 2021; Cho et al., 2022; Liu et al., 2022; Ratti et al., 2023), salmon (Belghit et al., 2019; Li et al., 2019; Weththasinghe et al., 2021), clownfish (Vargas-Abúndez et al., 2019), zebrafish (Zarantoniello et al., 2018; Zarantoniello et al., 2019), seabass (Wang et al., 2019; Moutinho et al., 2021), tilapia (Yildirim-Aksoy et al., 2020b; Tippayadara et al., 2021; Agbohessou et al., 2022), catfish (Xiao et al., 2018; Adeoye et al., 2020; Yildirim-Aksoy et al., 2020a; Sudha et al., 2022), and shrimp (Cummins Jr et al., 2017; Yildirim-Aksoy et al., 2022).

The largest aquaculture sector in the United States is farm-raised channel catfish, *Ictalurus punctatus*. One problem faced by the channel catfish industry is related to FM and FO in aquafeeds, as they are limiting and increasing less economical and sustainable for the future (Yildirim-Aksoy et al., 2020a). Despite the advancement in using BSF as an alternative to FM and FO, as compared to other species, studies related to the immune response, gut microbiome, and transcriptomes in the channel catfish are scarce (Mohan et al., 2022). Immune-related gene expression studies were conducted in several species where BSF was included in formulated feeds. In rainbow trout, significant differential expression of genes such as IL-1β; IL-10, TGF-β, COX-2, and TCR-β was observed but black soldier fly larvae (BSFL) diets did not induce any inflammation (Gaudioso et al., 2021). In addition, myogenesis-related gene expression experiments conducted in zebrafish show that BSFL meal may increase growth without having any negative consequences. Similar to rainbow trout, no evidence of inflammation in the gut intestinal parameters was found, and there were no appreciable alterations in stress and immunological indicators (Zarantoniello et al., 2019; Mohan et al., 2022). Several other studies have convincingly examined the effects of BSL in addition to the diets of other commercially reared fish species. In an attempt to determine the effects of FM replacement with defatted BSF in Jian carp, significant upregulation in the relative gene expression of peroxisome proliferator-activated receptor (PPARα) and heat shock proteins (HSP70) was observed (Li et al., 2017; Zhang et al., 2017; Mohan et al., 2022). Although BSFL meal supplementation in Nile tilapia did not affect the innate immunity and growth (Agbohessou et al., 2022), BSFL-supplemented meals as an alternative to FM in juvenile seabass substantially increased cytokine expression and mucin production in the gut and skin and exhibited no detrimental effects on development (Hender et al., 2021). Furthermore, according to Chaklader et al. (2019), *H. illucens* larval diet coupled with poultry by-product meal increased the growth of seabass and considerably elevated immune gene expression in the head kidney.

The majority of these studies are designed to determine the effects of dietary supplementation with BSL and report various immune-related gene activities. However, tissue- or organ-specific immune gene expression patterns, the global effects of any changes

TABLE 1 Percentage composition of experimental diets.

	Experimental diets (%)[a]				
	1	2	3	4	5
Menhaden fish meal	8	8	8	8	8
Soybean meal	45	44	43	41	39
Frass		5	10	20	30
Wheat short	24	20.4	16.9	9.8	2.5
Corn meal	14	13.8	13.5	13	12.75
Corn oil	4	3.8	3.59	3.18	2.75
Dicalcium phosphate	1	1	1	1	1
CMC	3	3	3	3	3
Vitamin premix[b]	0.5	0.5	0.5	0.5	0.5
Mineral premix[c]	0.5	0.5	0.5	0.5	0.5

CMC, carboxymethyl cellulose. Frass is a by-product of the black soldier fly (*Hermetia illucens*) larva meal industry.
[a]Diets 1, 2, 3, 4, and 5 contained 0, 5, 10, 20, and 30% frass, respectively.
[b]Vitamin premix, diluted in cellulose, provided by following vitamins (mg/kg diet): vitamin A (520,400 IU/g), 7.7; vitamin D3 (108,300 IU/g), 18.5; vitamin E (250 IU/g), 200; vitamin K, 10; thiamin, 10; riboflavin, 12; pyridoxine, 10; calcium pantothenate, 32; nicotinic acid, 80; folic acid, 2; vitamin B12, 0.01; biotin, 0.2; choline chloride, 400; and L-ascorbyl-2-polyphosphate (35% vitamin C activity), 172.
[c]Trace mineral premix provided by following minerals (mg/kg diet): zinc (as $ZnSO_4\_7H_2O$), 150; iron (as $FeSO_4\_7H_2O$), 40; manganese (as $MnSO_4\_7H_2O$), 25; copper (as $CuCl_2$), 3; iodine (as Kl), 5; cobalt (as $CoCl_2\_6H_2O$), 0.05; and selenium (as Na_2SeO_3), 0.09.

in expression levels of these genes, and interactions among adaptive and innate immune factors in response to dietary supplementation with BSL are still not clearly understood in channel catfish. The main goal of using BSFL meal is to find a viable replacement for FM that will allow for sustainable development without impeding fish growth. In addition to benefits to growth and development, the use of BSF as a replacement or additive to fish feed is also used to assess the immune-related gene modulation that supports fish systemic and mucosal immunity during pathogen invasion (Mohan et al., 2022). In channel catfish, multi-organ (especially the liver and intestine)–specific transcriptomic and immune-related gene expression studies in response to the addition of frass from BSFL are lacking. The main goal of this study was to assess the effects of dietary inclusion of frass from BSFL, *H. illucens*, at different concentrations to replace FM on both transcriptomics and immune-related gene expression at the systemic (head kidney and liver) and mucosal (gill and intestine) levels in channel catfish.

2 Materials and methods

2.1 Experimental fish

Fingerlings of the Marion strain channel catfish, *I. punctatus*, were spawned and maintained at the USDA-ARS, Aquatic Animal Health Research Laboratory (Auburn, AL, United States) on commercial fry and fingerling diets and were acclimated to the experimental basal diet for 2 weeks before stocking. At the end of the acclimation period, fish (average weight of 5.24 ± 0.04 g) were randomly stocked into 20, 110 L aquaria at a density of 50 fish per aquarium. The aquaria were supplied with flow-through dechlorinated, heated (28°C) city water with a flow rate of approximately 0.7 L/min. Water was continuously aerated using air stones. Water temperature and dissolved oxygen in three randomly chosen aquaria were measured once every other day in the mornings, using a YSI model Pro DO meter (Yellow Springs Instrument, Yellow Springs, OH, United States). During the trial, the water temperature averaged 26.8°C ± 1.12°C, and the dissolved oxygen averaged 6.35 ± 0.53 mg/L. The photoperiod was maintained at a 12:12 h light/dark schedule.

2.2 Experimental diets and feeding trial

A nutritionally complete, practical basal diet was formulated to contain approximately 31.5% crude protein and 6.2% lipid based on feedstuff values reported in NRC (1993) (Table 1). Five diets containing frass (0, 5, 10, 20, and 30%) as partial replacements of a combination of soybean meal, wheat short, and corn meal on an equal protein basis were prepared. Frass from BSF, *H. illucens*, fed on distillers' dried grain with solubles (DDGS), was donated from EnviroFlight LLC, Yellow Springs, OH, United States. Carboxymethyl cellulose (CMC) was added to all diets as a binding agent. Dry ingredients were thoroughly mixed for 10 min in a Hobart mixer before oil was added. After the oil was diffused, approximately 300 mL of deionized water per kilogram of the diet was added. The moist mixture was extruded through a 3-mm diameter die in a Hobart meat grinder. The resulting moist pellets were air-dried at room temperature to a moisture content of approximately 10%. Pellets were ground into small pieces, sieved to obtain approximate sizes, and stored frozen in plastic bags at −20°C until fed. Fish in four randomly assigned aquaria were fed with one of the five experimental diets twice daily (between 0730 and 0830 h and 1500 and 1600 h) to apparent satiation for 10 weeks (Yildirim-Aksoy et al., 2020a).

2.3 Collection of tissues from channel catfish fed with frass inclusion diets

After the 10-week experiment to evaluate the dietary inclusion of frass in five experimental diets, channel catfish from each treatment (diet) were euthanized by an overdose of buffered MS-222, and the head kidney, liver, intestine, and gills were collected and stored in an RNA stabilization solution (RNA*later*, Thermo Fisher Scientific, Waltham, MA) and stored at −80°C until required. In total, four fish from each aquarium from each of the experimental diets were collected and sampled, and their tissues were pooled together at the end of the trial. Liver and intestine samples were collected for RNA sequencing, while additional samples of the head kidney and gills were collected for quantitative PCR analyses.

2.4 RNA sequencing of channel catfish fed with frass inclusion diets

Intestine (n = 16 in pools of 4) and liver (n = 16 in pools of 4) tissues from fish at the end of the 10-week trial from three experimental diets [control (0 g/kg), low frass (50 g/kg), and high frass (300 g/kg)] were used to make libraries for RNA sequencing. Total RNA was extracted from each sample after homogenizing it in a FastPrep-96™ bead beating grinder (MP Biomedicals, Santa Ana, CA) followed by the Qiagen RNeasy® Plus Mini Kit (Germantown, MD) according to the manufacturer's protocol. Total RNA samples were sent to a service provider (Azenta Life Sciences, South Plainfield, NJ) for library preparation with poly(A) selection and Illumina sequencing. There, RNA samples were quantified using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA), and RNA integrity was checked using an Agilent TapeStation 4200 (Agilent Technologies, Palo Alto, CA). RNA sequencing libraries were prepared using the NEBNext Ultra II RNA Library Prep Kit for Illumina using the manufacturer's instructions (New England Biolabs, Ipswich, MA). The sequencing libraries were validated on the Agilent TapeStation (Agilent Technologies, Palo Alto, CA) and quantified by using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA) as well as by quantitative PCR (KAPA Biosystems, Wilmington, MA, United States). Libraries were then sequenced on an Illumina HiSeq 4000 according to the manufacturer's instructions using a 2 × 150 bp paired end (PE) configuration. A total of 24 samples (n = 12 intestine; n = 12 liver) were sequenced to include no frass control diet (n = 4 each tissue), low-frass inclusion of 50 g/kg (n = 4 each tissue), and high-frass inclusion of 300 g/kg (n = 4 each tissue).

2.5 Transcriptome analysis for the channel catfish

Raw, demultiplexed reads at a minimum of 25M PE reads/sample were delivered by the service provider. Bioinformatics was performed in-house within the OmicsBox software (OmicsBox, 2019) and R-Bioconductor packages. Raw FASTQ files were initially preprocessed for quality control (QC) using FASTQC (Andrews, 2017) and Trimmomatic (Bolger et al., 2014) with the default parameters. This process removed low-quality bases, filtered out short reads, and eliminated any contaminating Illumina sequencing adapters. The genome of channel catfish, *I. punctatus*, was obtained from the NCBI (assembly Coco_2.0; accession #GCA_001660625.3), and QC reads were aligned to it using the STAR aligner (Dobin et al., 2013), with options of 2-pass mapping and the overhang length set to 149. QC of the resultant BAM files was performed using RSeQC modules (Li et al., 2009; Wang et al., 2012; Wang et al., 2016) to assess alignment quality and obtain quality scores, such as transcript integrity numbers (TINs). At this stage, one sample (B1) was removed from further analyses. Sample B1 (a high-frass liver sample) was noted as a technical outlier as the integrity score was quite low (TIN = 18.52). QC BAM files were then used to generate a gene-level count data matrix for all samples via the HTSeq software (Anders et al., 2015). In HTSeq, the quantification level was set to feature type exon and grouped by parent attributes; the strand specificity was set to non–strand specific; and the overlap mode was set to union. The R package DESeq2 (Love et al., 2014) was then used to generate differentially expressed genes (DEGs) in pairwise comparisons of low- or high-frass samples as compared to the control, no frass (baseline) samples. Principal component analyses were performed on the data, and an additional sample (B2) was removed from further analyses. Sample B2 (a low-frass liver sample) was also noted as an outlier since it had a first principal component score >>>1. Finally, histograms of *p*-values were examined and adjusted using the fdrtool (Strimmer, 2008). The resultant DEG lists (Supplementary Table S1) were considered significant if the adjusted *p*-value was less than 5% and the fold change was greater than 2-fold up- or downregulated as compared to the control (*p*-adj <0.05 and FC > 2).

2.6 Quantitation of channel catfish innate and adaptive immune gene expression

Innate and immune gene-specific primers (Table 2) were utilized to independently evaluate channel catfish immunity in response to frass inclusion via reverse transcription quantitative PCR (RT-qPCR). Each total RNA sample was assessed by using spectrophotometry (Bio-Tek Cytation 1, Agilent Technologies, Palo Alto, CA) and Agilent 2100 Bioanalyzer with RNA integrity numbers (RINs) > 8 before reverse transcription. Then, cDNA synthesis was performed using the LunaScript® RT SuperMix Kit (New England Biolabs, Ipswich, MA, United States). Reactions contained 4.0 µL of LunaScript RT SuperMix (5X) and template RNA (200 ng), and the volume was adjusted using nuclease-free water to 20 µL. As a control, to rule out the presence of DNA in the extracted sample, no-RT reactions were prepared for each of the samples along with no template controls (negative control). Reaction conditions for cDNA synthesis included primer annealing at 25°C for 2 min, cDNA synthesis at 55°C for 10 min, and heat inactivation at 95°C for 1 min. cDNA was kept at −20°C after transcription until RT-qPCR.

Using a LightCycler® 480 System (Roche Diagnostics, Indianapolis, IN), RT-qPCR was performed to assess the expression level of systemic (head kidney and liver) and mucosal (gill and intestine) immunity by checking differential regulation of innate and adaptive immune genes. Fourteen innate immune genes

10.3389/fphys.2023.1330368

TABLE 2 List of RT-qPCR primers.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Reference
CD4	CCTCTGCGAACCCATCTTCA	AGGCAGGTCCAGATTCTTGC	Xu et al. (2016)
IL-1β type a	AAAAATGGCCAGCCTGTATG	CAGCCCGGGTATTTAACTGA	
IL-1β type b	GCCTCTTAGTATGCGCCAAG	AACCTTGTCTTGCAGGCTGT	
Complement factor D	AGGCAGAGGACAAAAAGCAA	TGGCTGACTTAGCTGCCAAT	
Complement factor C3	AGTTGAATACCGCTGCCAAC	CTCTCCATGCGCTGAGTACA	
MHC class II	CTGAGGAACGGGAAGGAGAT	CAGATGGGAGTGGACCTGAT	
Interferon γ	CAGCAGTGACTTCAGCCAAA	GCCTCAGAGTACGCCATCAT	
TLR1	AGCCAAAGAAATGCCAACTG	TGAAGTCTCGTTCGTGGTGA	
TLR9	GGAGGAACGGGACTGGATAC	AAGCACAGCCACCCTGATTA	
IgM	TGTGTGTGTGTGTGTGTGTT	CGGATGTCTTGGCTTGTTG	
IL-17	TGGTTGCTCAGGCTGCTCCTT	ACGCCAGCTTGATGTCATGTTCC	Wang et al. (2014)
18S	GAGAAACGGCTACCACATCC	GATACGCTCATTCCGATTACAG	Small et al. (2008)
β2M	AAGGGATGGAAGTTTCATCTGACC	GGAATGAAGCCCAGGAGGTTTAC	
β Actin	CCCATCTATGAGGGTTATGCTCTG	GCTCGGTCAGGATCTTCATCAG	
TLR3	TTGCAGCTGTGAGAGCATTC	AGTGCACCAGGAAGGCTAGA	Pridgeon et al. (2010)
TLR5	TTGGAAGCGCTACAAATCCT	ACCCGGAGGTTGAATAATCC	
TLR20A	CACCTCTCTGGGACTGGTGT	GCTCATCTTTCCCGCAGTAG	
TLR21	TTCCTCTGCAGTGAGTGGTG	TGTGTCCAGAACAGCTCCTG	
LEAP	CCTTTGGAGAATCATGGGTACTAA	GCAGTGTCCTTTCCTGCATA	This study
Hepcidin	GTTGGCGAAGGAGACGAG	ACCCACAGCCTTTATTCTTACA	
TNF α	GCTGCAATCAGAACGACTAGA	GGTCCGTCCACATCCAATAC	

such as six innate receptors (TLR1, TLR3, TLR5, TLR9, TLR20A, and TLR21), five proinflammatory cytokines (TNFα, IFN-γ, IL-17, IL-1β type a, and IL-1β type b), two chemokines (complement factor D and complement factor C3), and one antimicrobial peptide (hepcidin) were used for this study. Four adaptive immune genes were used for this study, that is, an immunoglobulin (IgM), two major histocompatibility class I (CD4 and β2M), and major histocompatibility class II (MHC class 2) genes. The Luna Universal qPCR Master Mix was used for RT-qPCR in 10 μL reactions. Each reaction included 5 μL of Luna Universal qPCR Master Mix (2X), 0.5 μL of forward primer (1 μM), 0.5 μL of reverse primer (1 μM), 2 μL of nuclease free water, and 2 μL of cDNA diluted 1:10 with nuclease-free water. Reactions were carried out in triplicate under the following conditions: 95°C for 15 s, followed by 45 cycles at 95°C for 15 s, 60°C for 30 s, followed by melting curve analysis. Reaction and cycling conditions were prepared following the manufacturer's instructions (NEB). The samples were run in parallel with two reference genes, 18S and EF1α, for normalization.

Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001; Pfaffl, 2001), normalizing with the geometric average of the reference gene relative to the controls. Statistical assessment was performed using the IBM SPSS 20 software (SPSS Inc., Chicago, IL). Data were evaluated by the analysis of variance (ANOVA) to determine differences in means among different groups and then further analyzed *post hoc* using Tukey's multiple comparison test. Differences of means among the groups were considered statistically significant when $p < 0.05$. Data were collected and analyzed, and all graphs of immune genes were plotted using the GraphPad Prism 5 software (Boston, MA).

3 Results

3.1 Global gene expression analyses of channel catfish tissues after being fed either a low-frass (50 g/kg) or high-frass (300 g/kg) experimental diet

The liver and intestine of individual channel catfish fed with different frass diets (50 and 300 g/kg frass) or control diet (0 g/kg frass) were used to build and sequence mRNA libraries. After the raw RNA sequencing data were processed and aligned with the channel catfish genome, sequence libraries from the 50 and 300 g/kg frass for the intestine and liver were compared to the basal-level control intestine or liver (0 g/kg frass) using the cutoff criteria (*p*-adj <0.05, fold change >2). There were 19 downregulated and 10 upregulated genes among the 29 DEGs identified in the low-frass (50 g/kg) intestine, and

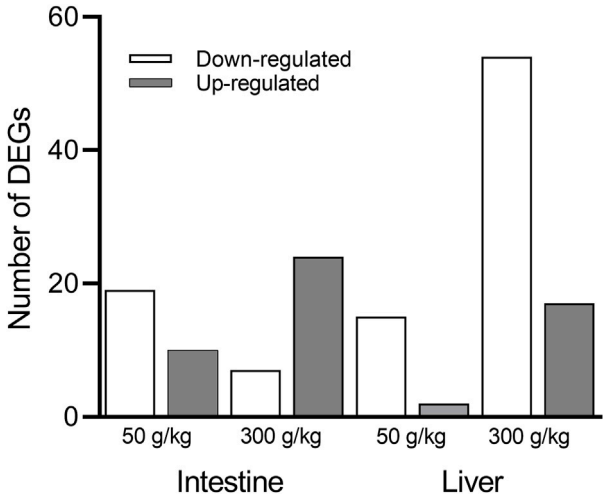

FIGURE 1
Number of significant DEGs identified between the control (0 g/kg) and the low-frass (50 g/kg) and high-frass (300 g/kg) supplemented diets. The downregulated DEGs (white bars) and upregulated DEGs (gray bars) are shown for the intestine and liver (*p*-adj <0.05; fold change >2).

seven downregulated and 24 upregulated genes among the 31 DEGs identified in the high-frass (300 g/kg) intestine (Figure 1) samples. There were 17 DEGs, which included 15 downregulated and two upregulated in the low-frass (50 g/kg) liver samples. There were 54 downregulated and 17 upregulated genes among the 71 DEGs identified in the high-frass (300 g/kg) liver samples (Supplementary Material S1).

We next sought to understand the relationship between the DEGs identified among the different experimental frass diets and their respective tissues. For this analysis, we identified the gene expression interactions through visualizing the DEGs in a Venn diagram (Figure 2 and Supplementary Material S1). There were five DEGs that were co-expressed in the low- and high-frass liver, whereas there was only one co-expressed between the low- and high-frass intestine, and there were no common DEGs among the all frass diets. However, there were three DEGs that were shared among the intestine and liver high-frass diets, two DEGs among the intestine low-frass and liver high-frass diets, and one DEG among the intestine high-frass and liver low-frass diets. Hence, only ~3% of DEGs were common among the liver and intestine in the frass diets and most of the DEGs in each low-frass and high-frass diets in the liver and intestine were unique to the groups when compared with the control diet (Supplementary Material S1).

Several upregulated DEGs that represent different innate immune receptors and effector molecules such as toll-like receptor 5 were identified in the low-frass (FC = 4.6) and high-frass (FC = 6.1) intestine; apolipoprotein A1 (FC = 4.1) in the low-frass intestine; and C-type lectin (FC = 2.4), complement factor H (FC = 4.7) and lysozyme (FC = 13.4) in the high-frass intestine. In the low-frass liver, there was hepcidin (FC = 11.9) that was upregulated in the low-frass liver, and in the high-frass liver, there was lysozyme (FC = 3.0).

Several interferon or interferon-like proteins were downregulated in the liver, as well as genes associated with adaptive immunity such as major histocompatibility complex class I (FC = −35.9) and immunoglobulin-like V-region gene (FC = −9.3) in the low-frass intestine. Alternatively, glucose-6-phosphatase catalytic subunit 1a (FC = 9.0) and myostatin (FC = 4.7) were upregulated in the low-frass

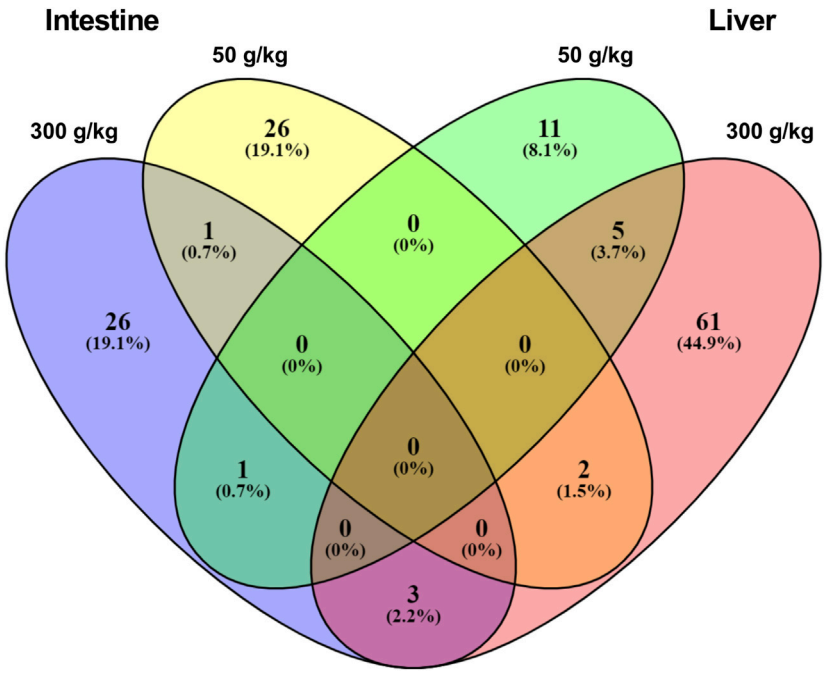

FIGURE 2
Venn diagram showing unique DEGs among the low-frass (50 g/kg) and high-frass (300 g/kg) supplemented diets in the intestine and liver.

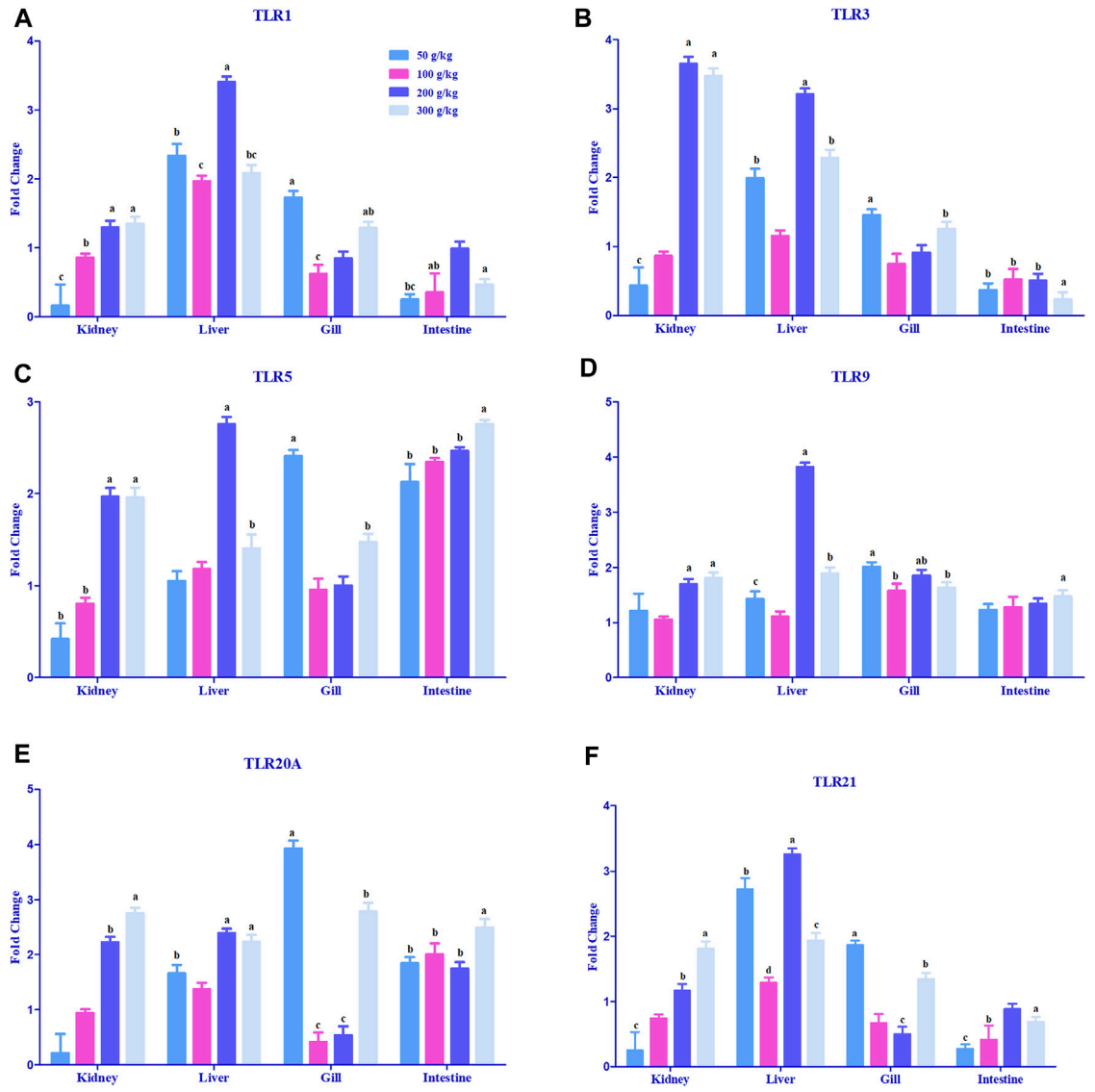

FIGURE 3
Relative expression of toll-like receptors: TLR1 **(A)**, TLR 3 **(B)**, TLR5 **(C)**, TLR 9 **(D)**, TLR20A **(E)**, and TLR 21 **(F)** in the head kidney, liver, gills, and intestine of channel catfish fed with frass-supplemented diets (50–300 g/kg). Data are presented as mean ± SE, and multiple reference genes were used to normalize with the target gene (n = 4). TLR gene expression among the frass diets (50–300 g/kg) was significantly upregulated when compared to the control (0 g/kg). Different alphabets indicate the significant differences between frass-supplemented diets ($p < 0.05$).

intestine, which correspond to the regulation of cell proliferation, apoptosis, and the regulation of skeletal muscle growth in the channel catfish.

3.2 Immune gene expression in channel catfish

Based on the RNA sequencing results of the low-frass (50 g/kg) and high-frass (300 g/kg) diets, we next performed a more thorough analysis of innate and adaptive gene expression using both systemic (head kidney and liver) and mucosal (gill and intestine) tissues from all the frass-supplemented diets (50, 100, 200, and 300 g of frass per kilogram of feed) as compared to the control, no frass inclusion diet (Yildirim-Aksoy et al., 2020a).

3.2.1 Expression of innate immune receptors among different tissues

A significant difference in the expression levels of various toll-like receptor genes was observed in the gill, intestine, head kidney, and liver samples when compared to the control tissues (0 g/kg); however, only some of the tissues demonstrated a threshold of >2-fold change (Figure 3). The expression of TLR1 was mostly upregulated in the liver with a 2-fold increase in expression among the frass diets (Figure 3A). TLR3 expression was largely expressed in the head kidney (3.5-fold) with higher frass concentrations and (2- to 3-fold) across the liver (Figure 3B). TLR5 was the only >2-fold upregulated among the liver and gills in one to two of the frass diets (Figure 3C). The intestine showed >2-fold upregulation among all the frass diets. The TLR9, TLR20A,

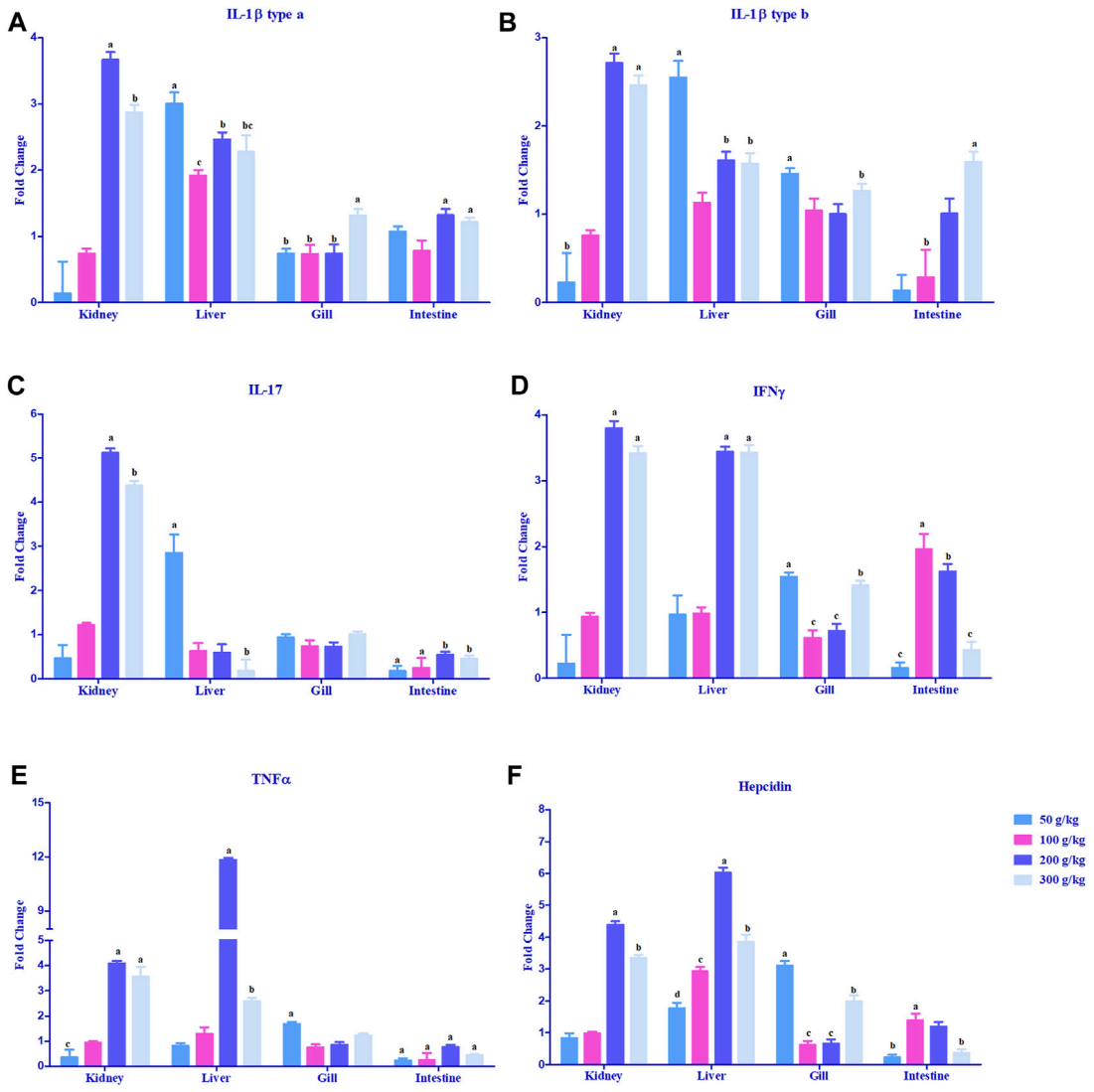

FIGURE 4
Relative expression of proinflammatory cytokines: IL-1β type a **(A)**, IL-1β type b **(B)**, IL-17 **(C)**, TNFα **(D)**, and IFN-γ **(E)**, and antimicrobial peptide and hepcidin **(F)** in the head kidney, liver, gills, and intestine of channel catfish fed with frass-supplemented diets (50–300 g/kg). Data are presented as mean ± SE, and multiple reference genes were used to normalize with the target gene (n = 4). Gene expression among the frass diets (50–300 g/kg) was significantly upregulated when compared to the control (0 g/kg). Different alphabets indicate the significant differences between frass-supplemented diets ($p < 0.05$).

and TLR21 genes were mostly >2-fold upregulated among the high-frass (200 and 300 g/kg) diets in the liver (Figures 3D–F). The TLR20A gene had >2-fold expression among the different frass diets in the head kidney, gills, and intestine.

3.2.2 Expression of proinflammatory cytokines in different tissues

The expression of cytokine genes, IL-1β type a and IL-1β type b, was upregulated >2-fold in the head kidney and liver mostly with the high-frass (200 and 300 g/kg) diets with an exception in the liver where the low-frass (50 g/kg) diet also exceeded the 2-fold change difference (Figures 4A, B). IL-17 and IFN-γ had a similar pattern in the head kidney with >2-fold upregulation mostly among the high-frass (200 and 300 g/kg) diets, whereas in the liver, IL-17 was most abundantly expressed with the low-frass diet and IFN-γ was upregulated among the high-frass (200 and 300 g/kg) diets (Figures 4C, D). The gills and intestine had significant upregulation when compared to the control tissues (0 g/kg), but neither tissue demonstrated a threshold of >2-fold upregulation. TNFα was upregulated >2-fold in the head kidney with high-frass (200 and 300 g/kg) diets, while the liver had greatly overexpressed expression in the 200 g/kg diet and also significant expression in the 300 g/kg diet (Figure 4E).

3.2.3 Expression of hepcidin and complement genes in different tissues

Hepcidin gene expression was upregulated >2-fold in the head kidney with high-frass (200 and 300 g/kg) diets and in the

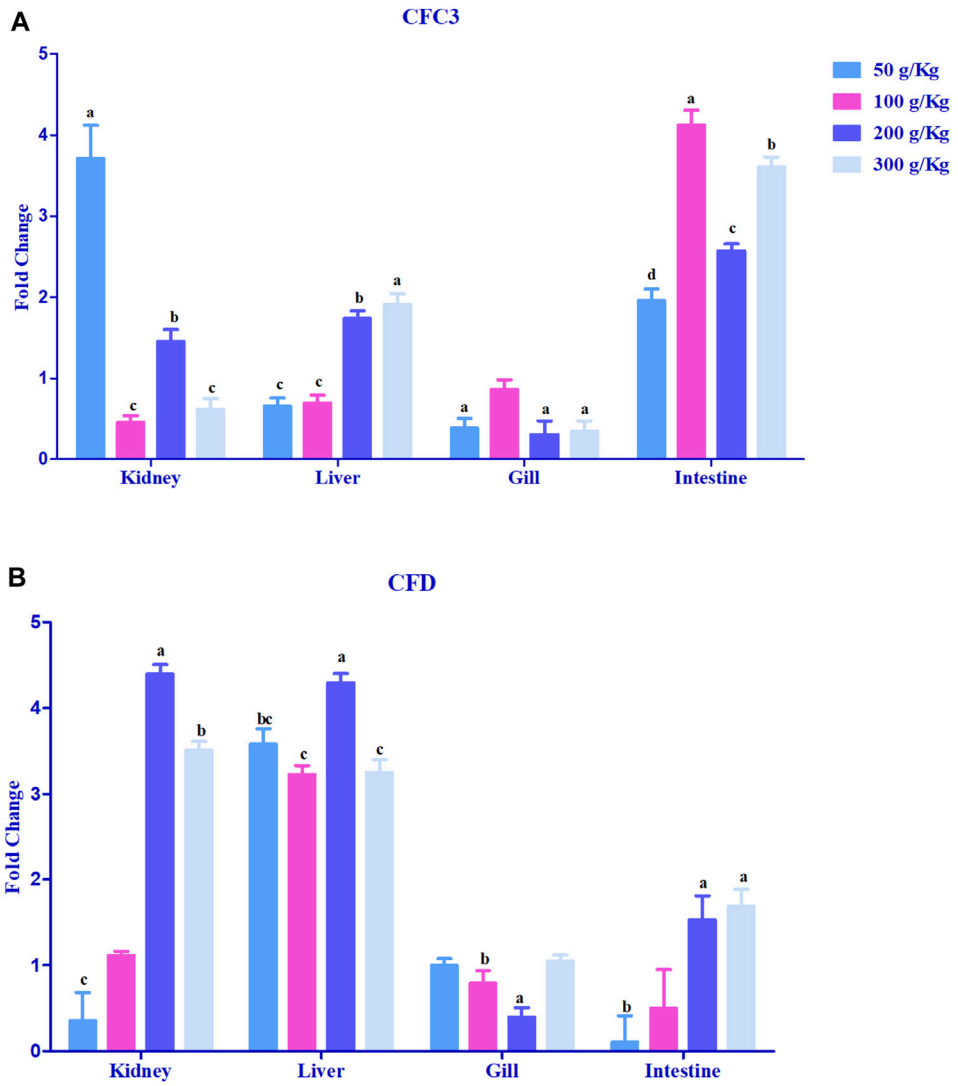

FIGURE 5
Relative expression of complementary factor C3 **(A)** and complement factor D **(B)** in the head kidney, liver, gills, and intestine of channel catfish fed with frass-supplemented diets (50–300 g/kg). Data are presented as mean ± SE, and multiple reference genes were used to normalize with the target gene (n = 4). Different alphabets indicate the significant differences between frass-supplemented diets ($p < 0.05$).

liver among the 100–300 g/kg frass diets (Figure 4F). The gills had >2-fold upregulation with the 50 g/kg frass diet, and the intestine demonstrated no >2-fold upregulation. Complement factor C3 was upregulated >2-fold in the intestine and in the head kidney with >2-fold upregulation with the low-frass diet (Figure 5A). Complement factor D was predominantly upregulated >2-fold in the head kidney with high-frass diets (200–300 g/kg) and in the liver among all frass diets (Figure 5B).

3.2.4 Expression of adaptive immune genes in different tissues

The adaptive immune-related genes (IgM, MHC class 2, β2M, and CD4) showed significant differential expressions in the gills, head kidney, and liver. The IgM gene was mostly >2-fold upregulated with the high-frass (200–300 g/kg) diets in the head kidney and with the 100–200 g/kg frass diets in the liver. The gills had >2-fold upregulation in the 50 g/kg frass diet, and the intestine demonstrated no >2-fold upregulation (Figure 6A). CD4 expression was upregulated >20-fold almost exclusively in the liver tissue among all frass diets, and >2-fold upregulation was identified in the head kidney with the 300 g/kg diet (Figure 6B). In liver, frass diets of 50, 200, and 300 g/kg were the only ones with >2-fold upregulation of the β2M gene (Figure 6C). Again, the liver was the site for >2-fold upregulation of the MHC class 2 gene among the 50, 200 and 300 g/kg frass diets (Figure 6D).

3.3 Validation of RNA-seq with RT-qPCR

RNA-seq and RT-qPCR values were compared for the intestine with the low- and high-frass diets (50 and 300 g/kg). TLR5, complement factor D, and complement factor C3 all had similar fold change values (Table 3).

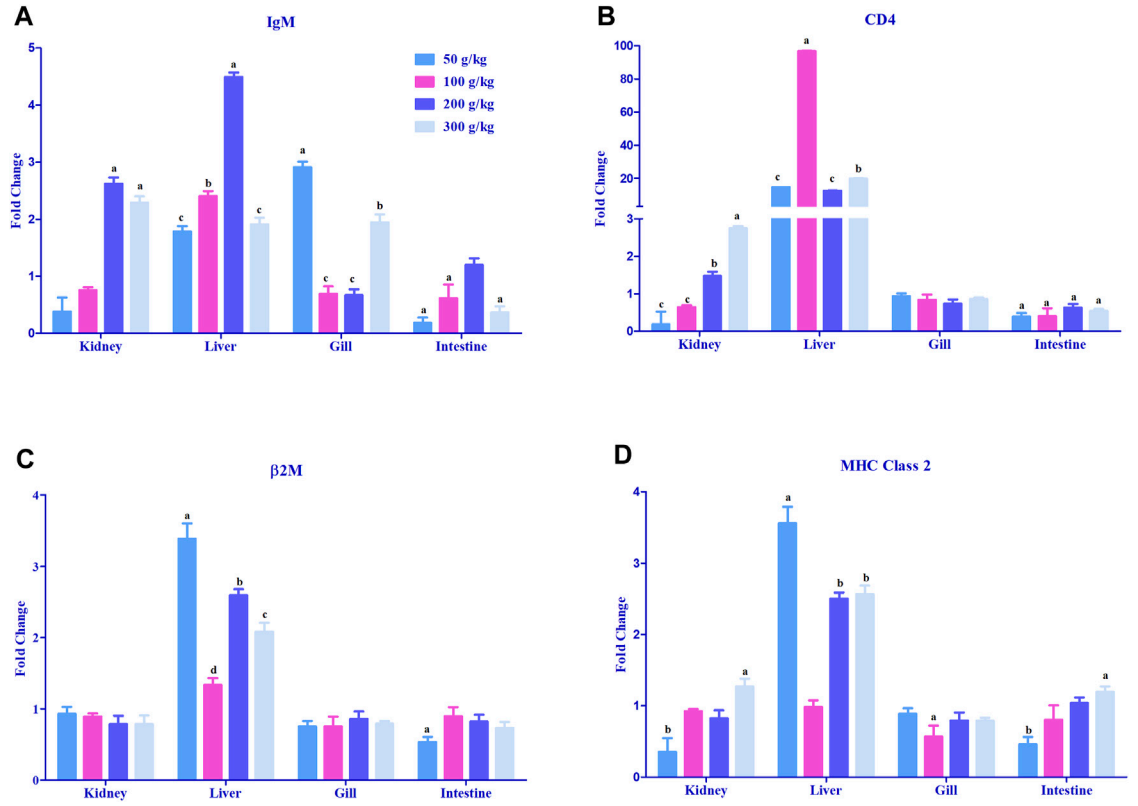

FIGURE 6
Relative expression of adaptive immunity genes: IgM **(A)**, CD4 **(B)**, β2M **(C)**, and MHC class 2 **(D)** in the head kidney, liver, gills, and intestine of channel catfish fed with frass-supplemented diets (50–300 g/kg). Data are presented as mean ± SE, and multiple reference genes were used to normalize with the target gene (n = 4). Different alphabets indicate the significant differences between frass-supplemented diets ($p < 0.05$).

TABLE 3 Comparison of RNA-seq and RT-qPCR results in the intestine with either 50 or 300 g/kg frass in the diet.

Feature	Description	Fold change	
		50 g/kg	300 g/kg
		RT-qPCR (RNAseq)	RT-qPCR (RNAseq)
NM_001200229.1	Toll-like receptor 5	2.1 (4.6)	2.7 (6.1)
NM_001257116.1	Complement factor D	0.1 (ND[a])	1.7 (1.7)
XM_017457539.3	Complement C3	1.9 (ND[a])	3.6 (3.0)

[a]No data.

4 Discussion

Insects are composed of essential amino acids like that in fish meal. BSF (*H. illucens*) larvae are one of the most promising insect species for use in aquaculture feed (Yildirim-Aksoy et al., 2020a; Yildirim-Aksoy et al., 2020b; Yildirim-Aksoy et al., 2022; Mohan et al., 2022). Although substrates for BSFL often vary greatly in their nutritional components, frass generated from larvae fed on distillers' dried grains contain enough protein to serve as an animal feed source. Previous work in our lab has demonstrated the nutritional benefit of frass-supplemented diets in channel catfish with increased growth performance and an increase in overall feed intake and palatability (Yildirim-Aksoy et al., 2020a). In the current study, we investigated the effect of BSFL frass diets on gene expression among different systemic and mucosal tissues in the channel catfish.

A previous work has shown that proinflammatory genes such as toll-like receptor (TLR) signaling pathway enhance disease resistance in teleost fish (Chunyan et al., 2023). An analysis of rainbow trout transcriptome data revealed no activation of the TLRs after 30 days (Chunyan et al., 2023), whereas a previous study in rainbow trout had shown significant upregulation of the TLR genes in the liver and spleen after the use of probiotics (Dang et al., 2022; Chunyan et al., 2023). Different TLRs on the surface of host cells are honed to react quickly to pathogens, and the different probiotic

strains that are species specific likely carry out their "probiotic effect" by modulating host gene expressions, which could lead to a more robust innate immune response (Dawood et al., 2020; Tippayadara et al., 2021).

In the current study, TLR5 was upregulated >2-fold in the intestine in both the 50 and 300 g/kg frass diet RNAseq transcriptome data and in the quantitative PCR analyses of all the frass diets in the intestine. TLR1, TLR3, and TLR21 peaked in the head kidney and liver of channel catfish fed with high-frass diets (200–300 g/kg). To date, there are few studies evaluating the gene expression of innate immune receptors in channel catfish fed with BSFL frass diets. BSFL-supplemented diets consist of chitin and are tightly bound to β-glucan to form chitin–glucan complexes, which are specifically used by probiotic bacteria such as *Lactobacillus* and *Bacillus* for their development in fish intestines (Balcázar et al., 2006; Dawood et al., 2020; Wang et al., 2023). Immunostimulants work by recognizing certain molecular structures that the host cells have discovered. Pathogen-associated molecular patterns (PAMPs) are repetitive structures that are seen in a variety of microorganisms. PAMPs that are present in or on microorganisms during a natural infection, be it on bacteria, viruses, or fungi, are recognized by certain receptors on or in the host cells that are being infected. The receptors, which may be membrane-bound or cytosolic, are collectively referred to as pathogen recognition receptors (PRRs), and they comprise toll-like and RIG-I receptors among others. Following the activation of the innate immune response by these molecules, proinflammatory cytokines may be produced and released (Dawood et al., 2020).

Proinflammatory cytokines and chemokines are essential for cell proliferation, apoptosis, growth, and development where interleukin-10, an anti-inflammatory cytokine, and inflammation mediators NFκB and MYD88 are useful markers of inflammation when evaluating new ingredients in feed formulations (Zarantoniello et al., 2018; Zarantoniello et al., 2020a; Zarantoniello et al., 2020b; Zarantoniello et al., 2022; Ratti et al., 2023). In the current research, channel catfish fed with diets containing BSFL frass had substantially elevated levels of IL-1β type a, IL-1β type b, IL-17, TNFα, and IFN-γ, generally in the head kidney, when fed with higher frass diets and among all frass diets in the liver. The cytokines were much less expressed in the intestine and gills of channel catfish fed with the frass diets, which is comparable with studies with BSFL meal in trout and swamp eel (Fawole et al., 2021; Kumar et al., 2021; Fischer et al., 2022). Among teleost fish, gene expression responses have been shown to be variable, yet appear to be species- and stage-specific as well as connected to the dietary inclusion amounts of BSFL meal. For instance, among the several developmental stages in zebrafish, different immune response markers can be expressed in the gut, for example, in adult zebrafish, these markers (*il1b*, *il6*, and *tnfa*) were not significantly expressed in 25% and 50% full-fat BSF prepupae meal (Zarantoniello et al., 2019). Dietary inclusion of 50, 60, or 100% BSFL meal did not adversely impact Atlantic salmon pre-smolt that had no significant gene expression (*il4*, *tgf1*, *il10*, *ifn*, *il8*, and *myd88*) when compared to the control (Li et al., 2019) and seawater phase (*il1b*, *il17a*, *myd88*, *il8*, *il4*, *mhcl*, *il10*, *ifn*, *tgf*, *cd8*, and *foxp3*) with no significant expression of cytokines (Li et al., 2020). Immune gene expression was activated in the intestines of both larval and juvenile zebrafish (*IL1b*, *IL10*, *IL6*, and *tnfa*) (Zarantoniello et al., 2018; Zarantoniello et al., 2019; Zarantoniello et al., 2020a; Zarantoniello et al., 2020b; Zarantoniello et al., 2021; Zarantoniello et al., 2022), juvenile rainbow trout (*IL10*, *tnf*, and *tlr5*) (Cardinaletti et al., 2019), both the proximal and distal intestines of Atlantic salmon (*cd3* and *foxp3*) (Li et al., 2019), and in the distal intestine of rainbow trout (*il1b* and *tlr1*) (Mohan et al., 2022; Ratti et al., 2023), which supports our research findings in the channel catfish. As an alternative to fish meals, BSFL-supplemented meals had no detrimental effects on growth and dramatically increased the cytokine expression in the gut of seabass (Hender et al., 2021). In fish, TNFs regulate apoptosis, lipid metabolism, inflammation, and organ regeneration, among other well-documented physiologically significant roles (Yaoguo et al., 2021). The 25% inclusion of BSFL meal diets showed a 9.2-fold increase in IFN-γ relative to control in the Atlantic salmon (Weththasinghe et al., 2021). By producing a variety of iNOS, superoxide anions, and oxygen and nitrogen radicals, TNFα and IFN-γ were upregulated, which in turn activated macrophages (Muñoz-Carrillo et al., 2018). In this study, TNFα and IFN-γ were greatly induced in channel catfish head kidney and liver when fed with increasing amounts of BSFL frass.

In the present study, adaptive immune genes especially IgM have resulted in decreased expression in the intestine, while the head kidney, liver, and gills show varied upregulation among frass diets. The results of another study using BSFL diets identified decreased IgM expression in the distal intestine of salmon as well (Weththasinghe et al., 2021). β2M and MHC class 2 were also significantly upregulated in all BSFL frass diets in liver tissues. The increased β2M expression in BSFL frass diets lead to the activation of cytotoxic lymphocytes as a preventive defense mechanism against pathogens. β2M and CD4 are an essential part of the adaptive immune response from MHC class I molecules, which are responsible for their structure, correct folding, and cell surface expression (Pikulkaew et al., 2020).

Metabolically, channel catfish fed with frass diets developed better than the control (Yildirim-Aksoy et al., 2020a). This could be associated with upregulated genes in the intestine such as glucose-6-phosphatase, which corresponds to the regulation of cell proliferation, and myostatin in the intestine, which regulates skeletal muscle growth. Previous work have demonstrated that increased myostatin leads to muscle growth in catfish (Gregory et al., 2004; Weber et al., 2005; Khalil et al., 2017; Coogan et al., 2022).

Based on these results, meal composition may have an impact on the activity of digestive enzymes in the intestine, which could also be related to mucosal immunity in channel catfish. The upregulated genes associated with cell proliferation and downregulation of several chemokines in the intestine could be involved in nutrient absorption. Previous studies analyzing the transcriptome of probiotic-fed fish have shown a shift in the functional effect of signaling pathways, immune-related pathways, protein digestion and absorption, and starch and sucrose metabolism (Tacchi et al., 2011; Tacchi et al., 2012; Gonçalves et al., 2017; Dawood et al., 2020; Chunyan et al., 2023). Additionally, we observed the upregulation of metabolic pathways that include glucose-6-phosphatase in the intestine of frass-fed fish, which is on par with the studies related to probiotic-incorporated feed (Chunyan et al., 2023; Wang et al., 2023).

5 Conclusion

Transcriptome analyses of channel catfish fed with diets formulated with the inclusion of frass from BSFL showed that a series of metabolic and immune-related genes were differentially regulated after being fed either a low- or high-frass diet for 10 weeks. Further analysis of both systemic and mucosal tissues using quantitative PCR revealed an upregulation of multiple innate and adaptive immune genes with various levels of frass supplementation. Overall, these data suggest that the activation of multiple immune-related genes after being fed BSFL frass may improve pathogen resistance in channel catfish, of which further study is warranted.

Data availability statement

The RNA sequencing datasets generated for this study can be found in the NCBI Gene Expression Omnibus (GEO) repository and can be accessed under the accession number GSE231874. All other data that support the findings of this study have been included in the manuscript and Supplementary Materials.

Ethics statement

The animal study was approved by the USDA-ARS Aquatic Animal Health Research Unit (AAHRU) Institutional Animal Care and Use Committee (IACUC) and conformed to USDA-ARS Policies and Procedures 130.4.v4 and 635.1. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

NS: Formal analysis, investigation, validation, and manuscript writing–original draft. ML: Conceptualization, investigation, manuscript writing–original draft, review, and editing, methodology, resources, and validation. MY-A: Conceptualization, funding acquisition, investigation, supervision, validation, and manuscript writing–review and editing. RE: Investigation and manuscript writing–review and editing. HK: Investigation and manuscript writing–review and editing. BB: Manuscript writing–review and editing, funding acquisition, project administration, resources, and supervision. JA: Project administration, resources, supervision, manuscript writing–review and editing, conceptualization, data curation, formal analysis, investigation, methodology, validation, and manuscript writing–original draft.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2023.1330368/full#supplementary-material

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Effects of yeast extract supplemented in diet on growth performance, digestibility, intestinal histology, and the antioxidant capacity of the juvenile turbot (*Scophthalmus maximus*)

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An 8-week feeding experiment was conducted on the juvenile turbot (*Scophthalmus maximus*) to evaluate the influence of yeast extract (YE) supplementation in the diet on growth performance, feed utilization, body composition, nutrient digestibility, intestinal histology, and antioxidant capacity. Four experimental diets were formulated with graded levels of yeast extract 0 (YE0), 1% (YE1), 3% (YE3), and 5% (YE5) and fed to turbots (initial body weight: 4.2 ± 0.1 g) with three replicates per diet and 200 fish in each replicate, respectively. The results showed that turbots fed with diets YE1 and YE3 displayed a significantly higher specific growth rate and protein efficiency rate than those fed with diets YE0 and YE5, while the feed conversion ratios in YE1 and YE3 groups were lower than those in YE0 and YE5. Fish fed with diets YE3 and YE5 showed higher body crude protein contents than those in groups YE0 and YE1. The highest apparent digestibility coefficients for dry matter and crude protein, digestive enzyme activities (trypsin, lipase, and amylase), and the height of the intestinal fold were observed in the YE3 group. YE3 treatment displayed a significantly higher superoxide dismutase (SOD) activity than the YE0 group, while the malondialdehyde (MDA) content in YE1 was significantly lower than those in YE0 and YE5. No significant difference was observed in serum physiological and biochemical parameters among all treatments. Overall, appropriate dietary supplementation of the yeast extract could improve the growth performance, digestibility, and antioxidant capacity of the juvenile turbot, and the recommended yeast extract level in the feed is 2.47%.

KEYWORDS

yeast extract, growth, feed utilization, digestibility, intestinal histology, antioxidant capacity

1 Introduction

Global aquaculture production has currently achieved a record high level and is expected to become more significant in the future as a source of food and nutrition for humans (FAO, 2022). However, the limited natural resources and environmental issues have become the bottleneck for the further development of aquaculture. The supply of fish meal for aquafeed is continuing to decrease, and the aquatic environment is deteriorating with the rapid rise of aquaculture (Han et al., 2018). Generally, aquatic feed accounts for 50%–60% of the total cost in aquaculture (Hardy, 2010), and its quality is closely related to the production efficiency of aquaculture and the output of waste discharged into surroundings (Hossain et al., 2023). Hence, successful sustainable aquaculture in the future heavily depends on the efficiency of the artificial feed. Yeast products are considered to be potential feed ingredients or additives that can boost fish growth performance and enhance the overall quality of aquatic feeds in an effective and sustainable way (Tao et al., 2023).

Yeast products, including yeast spent and yeast extract, are the by-products of the brewing and baking industries. Brewing spent yeast, as the second by-product of the brewing industry, has been reused as a functional ingredient to save fish meal in diets because of its higher contents in protein, polysaccharides, nucleotides, and other bioactive components. This is beneficial to both the brewing industry and aquaculture industry with regard to sustainability and environmental impact (Baiano, 2014). Many studies have reported that intact spent yeast was reused in aquafeed as an alternative protein ingredient to replace a fish meal (Tao et al., 2023). However, nutrients in spent yeast were not sufficiently utilized, owing to the thick cell wall of yeast, which blocks many bioactive components from being released for use (Baiano, 2014). Nevertheless, yeast extract is produced from spent yeast by disrupting the cell membrane with various methods (Demirgul et al., 2022). Indeed, yeast extract exhibits a notable release of polysaccharides, nucleotides, and other bioactive components compared to the intact spent yeast. Yeast extract is a water-soluble extract and is rich in peptides, free amino acids, nucleotides, ß-glucans, mannan oligosaccharides, B-complex vitamin, etc. (Tao et al., 2023). Peptides, amino acids, and vitamins are essential nutrients and play vital roles in the development of fish; nucleotides have been considered conditionally essential nutrients for fish under stress conditions or during rapid growth periods (Hossain et al., 2020); polysaccharides (e.g., ß-glucan and mannan oligosaccharides) are used as immunostimulants to better the intestinal health, immunity, and growth performance of fish and shrimp (Guzmán-Villanueva et al., 2013; Rajan et al., 2023). The beneficial bioactive components found in the yeast extract aid in enhancing the wellbeing of aquatic creatures and optimizing the effectiveness of aquafeed.

Earlier research studies have suggested that the addition of yeast extract to the diet resulted in an elevated specific growth rate and a reduced feed conversion ratio in some freshwater fish species, including snakehead fish (*Ophiocephalus argus*×*Channa maculata*) (Zhou et al., 2012), Gibel carp (*Carassius auratus gibelio*) (Chen et al., 2009), and Nile tilapia (*Oreochromis niloticus*) (Berto et al., 2016; Han et al., 2018). On the other hand, yeast extract did not show any notable effects on the growth of Chinese mitten crab (*Eriocheir sinensis*) and Pacific white shrimp (*Litopenaeus vannamei*), while the antioxidant capacities of shrimp and crab were improved by adding yeast extract in the feed (Zhang et al., 2019; Zheng et al., 2021). Moreover, yeast extract has been shown to be a superior alternative protein resource to intact spent yeast in shrimp (Zhao et al., 2017). Therefore, yeast extract is now more accurately defined as a functional additive that can enhance the growth performance, immunity, and antioxidant ability of aquatic animals (Podpora et al., 2015; Zheng et al., 2021). Nonetheless, few studies have been reported about the application of yeast extract in the feed of marine cultured fish.

The turbot is native to Europe and has been introduced to China for over 30 years due to its fast growth gate, good adaptability to intensive industrial aquaculture, and high market acceptance (Zhang et al., 2023). To date, the turbot has become an important mariculture flatfish in China, yielding more than 100,000 tonnes per year (Ministry of Agriculture and Rural Affairs of the People's Republic of China, National Aquatic Technology Extension Station, China Society of Fisheries, 2023). Simultaneously, the culture of turbot in China is also facing the challenge of sustainable development because of the shortage of dietary protein sources and a high disease outbreak rate under intensive conditions. The effects of single components extracted from spent yeast, such as ß-glucans (Jiang et al., 2019; Gu et al., 2021), mannan oligosaccharides, and nucleotides (Fuchs et al., 2015; Bai et al., 2017), on the turbot have been assessed in several studies. Furthermore, the recent studies by Yang et al. (2020) and Wang et al. (2021) have illustrated the effectiveness of yeast cell wall extract in reducing toxins in turbot feed. Nevertheless, the influence of yeast extract, as a practical feed additive with several bioactive components, on the physiological status of turbot is still unknown. Hence, the objective of this investigation was to evaluate the effects of dietary yeast extract on growth, feed utilization, intestinal health, digestive ability, serum biochemical indexes, and antioxidant potential in turbot. The findings from this research will offer a valuable proof for the application of yeast extract in practical diets of turbot to improve the feed efficiency.

2 Materials and methods

2.1 Experimental diets

Yeast extract (YE) was obtained from Guangzhou Xintun Aquatech Co., Ltd. (Guangzhou, China); it contained moisture 57.30%, crude proteins 20.18%, small peptides 7.99%, ß-glucans 8.5%, mannan oligosaccharides 5.2%, nucleotides 3.2%, and amino nitrogen 1.1% (Table 1). Four isonitrogenous and isocaloric diets were formulated with graded levels of YE in 0 (YE0), 1.00% (YE1), 3.00% (YE3), and 5.00% (YE5). The formulation and proximate composition of experimental diets are listed in Table 2. To assess the apparent digestibility of nutrients, an inert marker consisting of 0.1% Y_2O_3 was incorporated into every diet. All feedstuffs were carefully weighed, mixed together, and ground before passing through a mesh sieve with a diameter of 178 μm. Next, the oil was added to the powder and thoroughly mixed together. The 2-mm soft pellets were produced using a pelletizer (EL-260, Weihai Youyi Factory, Shandon, China) and stored at −20°C until use.

TABLE 1 Composition of yeast extract used in this experiment (%, wet weight basis).

Component	Content (%)	Component	Content (%)
Moisture	57.30	ß-Glucan	8.50
Crude protein	20.18	Mannan oligosaccharide	5.20
Small peptide	7.99	Nucleotides	3.20
Amino nitrogen	1.10		

Yeast extract was purchased from Guangzhou Xintun Aquatech Co., Ltd. (Guangzhou, China).

TABLE 2 Ingredients and proximate composition of experimental diets of the turbot (%, dry matter basis).

Ingredient	YE0	YE1	YE3	YE5
Fish meal[a]	45.00	45.00	45.00	45.00
Meat and bone meal[a]	10.00	10.00	10.00	10.00
Squid meal powder[a]	4.00	4.00	4.00	4.00
Extruded soybean[a]	6.00	6.00	6.00	6.00
Corn gluten meal[a]	2.00	2.00	2.00	2.00
Cotton seed meal[a]	2.75	2.19	1.11	0
α-Starch[a]	14.00	14.00	14.00	14.00
Soybean oil[a]	2.50	2.50	2.50	2.50
Fish oil[a]	2.50	2.50	2.50	2.50
Calcium monophosphate[a]	4.50	4.50	4.50	4.50
Limestone powder[a]	0.50	0.50	0.50	0.50
Yeast extract[b]	0	1.00	3.00	5.00
Zeolite meal[a]	4.15	3.71	2.79	1.90
Mineral–vitamin premix[c]	2.00	2.00	2.00	2.00
Yttrium oxide (Y_2O_3)	0.10	0.10	0.10	0.10
Proximate composition				
Crude protein	45.73	45.80	45.02	45.03
Crude lipid	7.48	7.68	7.83	7.84
Crude ash	18.60	17.95	18.20	18.49
Gross energy/(MJ/kg)	18.12	18.45	18.19	18.56

[a]These ingredients were provided from Hebei Haitai Tech. Ltd. (Shijiazhuang, China).
[b]Yeast extract was purchased from Guangzhou Xintun Aquatech Co., Ltd. (Guangzhou, China).
[c]Mineral–vitamin premix: according to Zhang et al. (2023).

2.2 Fish and feeding trial

Juvenile turbots were acquired from Minfeng Aquafarm (Tianjin, China) and adapted to experimental conditions for 14 d. During this time, they were fed with diet YE0. Subsequently, 2400 healthy turbots (average initial body weight of 4.2 ± 0.1 g) were randomly placed into 12 rectangular fiberglass tanks (200 cm × 100 cm × 100 cm), with 200 fish per tank. The tanks were provided with constant aeration. Before a formal meal, the feces and other waste in each tank were cleaned out. The fish were fed by hand with their specific diets twice a day (at 8:30 a.m. and 17:30 p.m.) to apparent satiation. In order to accurately measure the feed intake, the remaining feeds were siphoned out to collect, dry, and weigh 0.5 h post-feeding. Feces were collected from the third week, and the fresh feces were suctioned out after 1 h of feeding. Feces in one tank were pooled together and preserved at −20°C for the purpose of analyzing nutrient digestibility. The feeding trial was conducted under controlled environmental conditions, with the water temperature maintained between 15°C and 18.5°C, salinity levels ranging from 20‰ to 25‰, dissolved oxygen levels maintained above 6.0 mg/L, and ammonia–nitrogen levels kept below 0.5 mg/L. The duration of light was adjusted to 14 h, and the period of

darkness was set to 10 h. The duration of the feeding trial was 8 weeks.

2.3 Sample collection

After an 8-week feeding trial, all fish were subjected to 24-h starvation and then were anesthetized using a solution of MS-222 (100 mg/L, Sigma-Aldrich, MO, United States). They were carefully weighed and counted. Afterward, four fish from each tank were randomly chosen and stored at −20°C in a refrigerator for the purpose of body composition analysis. Two fish per tank were selected to measure the body weight and then dissected on ice to obtain samples from the liver and viscera. The liver and viscera were weighed accurately to calculate the hepatosomatic index (HSI) and viscerosomatic index (VSI). Subsequently, the liver was stored at −20°C in a refrigerator for the analysis of antioxidant parameters. Moreover, two more fish in each tank were taken for blood samples using 1-mml syringes via the caudal vein. Serum was collected by centrifugation at 3000 *g* for 10 min and stored under −20°C for measuring physiological and biochemical parameters. The phlebotomized turbots were dissected to separate the anterior part of the gut for measuring the activities of digestive enzymes. Simultaneously, sections of the mid-intestine of approximately 1 cm were excised and placed in a solution of 4% paraformaldehyde for the purpose of histology examination. The intestinal segments (4-μm slides) were stained with hematoxylin and eosin (H&E) and used to observe the histological structures with a Zeiss microscope (Imager A1m, Oberkochen, GER).

2.4 Chemical analysis

All diets, fish bodies, and feces were ground into powder, and the powder was then examined for its proximate composition, including moisture, crude protein, crude lipid, crude ash, and gross energy, according to AOAC (2005) and Shi et al. (2022). The moisture content was determined using an oven at 105°C. Crude lipids were extracted using the Soxhlet method with petroleum ether. The Kjeldahl method was employed to measure the crude protein using a unit (4800 Kjeltec Analyzer, Hoganas, Sweden). The measurement of gross energy was conducted using a 6300 oxygen calorimeter (Parr instrument Company Moline, United States). The samples were incinerated in a muffle furnace at 550°C for a duration of 12 h to analyze the crude ash. An inductively coupled plasma source mass spectrophotometer (X Series 2 ICP-MS) (Thermo Fisher Scientific, United States) was used to measure the Y_2O_3 levels in both the diets and feces.

2.5 Physiological and biochemical index analysis

Serum levels of triglycerides (TGs), total cholesterol (TCHO), glucose (GLU), total protein (TP), blood urea nitrogen (BUN), alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) were analyzed using commercial kits (Leadman Biochemistry Company, Beijing, China) through an automatic biochemical analyzer (Mindray BS-180, Shenzhen, China). The activities of superoxide dismutase (SOD) and contents of malondialdehyde (MDA) in the liver were evaluated using the hydroxylamine method and thiobarbituric acid method, respectively, according to the guidelines of commercial kits (A001, A003; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The activities of intestinal trypsin, lipase, and amylase were measured using the colorimetric method with commercial reagent kits (A080, A054, C016, Jiangcheng Corp. Nanjing, China) using a microplate reader (BioTek Instruments, Inc., Winooski, VT, United States).

2.6 Statistical analysis

The data were present as means ± SE and analyzed with STATISTICA 10.0 software (StatSoft Inc., Tulsa, OK, United States). Confirmation of normality and variance homogeneity was conducted prior to performing statistical analysis. One-way ANOVA was conducted to assess the impact of yeast extract on all response variables, and in cases where a significant effect was detected in the ANOVA analysis, Duncan multiple comparisons were employed. The significance level was set at $p < 0.05$. In order to estimate the optimal level of yeast extract in the diets, a quadratic regression analysis based on the specific growth rate and graded levels of dietary yeast extract was employed.

3 Results

3.1 Growth performance

The growth, feed utilization, and body condition indexes of the turbot fed with graded levels of yeast extract are presented in Table 3. The survival rate of turbot in each group was 100%, and no dead fish were observed during the whole experimental period. Dietary supplementation with yeast extract significantly influenced the final body weight (FBW), weight gain rate (WGR), specific growth rate (SGR), feed conversion ratio (FCR), feeding rate (FR), and protein efficiency rate (PER) ($p < 0.05$). The values of FBW, WGR, and SGR in YE3 group were higher than those in other groups, and these parameters were distinctively higher in the YE1 group than those in the YE0 and YE5 groups ($p < 0.05$). The regression formula between SGR and dietary YE levels was determined as SGR $=-0.0388YE^2 + 0.1913\,YE + 2.6086$ ($R^2 = 0.979$, $p < 0.05$.), and the optimal supplemental level of YE in the diet was calculated as 2.47% based on this quadratic regression relationship (Figure 1). The YE3 group showed the lowest FCR among all groups, and the FCR of the turbot fed with diet YE1 was significantly lower than that of the turbot fed with the YE0 and YE5 diets ($p < 0.05$). FR in YE3 was higher than that in YE0 and YE5 groups, and group YE1 showed a lower FR than group YE5 ($p < 0.05$). Moreover, YE0 and YE5 displayed significantly inferior PER to YE1 and YE3 ($p < 0.05$). No significant differences in HSI and VSI parameters

TABLE 3 Effects of yeast extract supplemented in the diet on the growth performances of the turbot.

Parameter	YE0	YE1	YE3	YE5
IBW (g)	4.20 ± 0.00	4.24 ± 0.04	4.22 ± 0.04	4.25 ± 0.02
FBW (g)	18.09 ± 0.16^{a}	19.93 ± 0.17^{b}	20.64 ± 0.18^{c}	18.15 ± 0.28^{a}
WGR (%)	330.81 ± 2.87^{a}	369.67 ± 0.69^{b}	388.49 ± 0.70^{c}	327.75 ± 4.00^{a}
SGR (%/d)	2.61 ± 0.01^{a}	2.76 ± 0.00^{b}	2.83 ± 0.00^{c}	2.60 ± 0.02^{a}
FR (%/d)	1.85 ± 0.01^{bc}	1.82 ± 0.01^{ab}	1.79 ± 0.02^{a}	1.88 ± 0.01^{c}
FCR	0.83 ± 0.01^{c}	0.79 ± 0.00^{b}	0.76 ± 0.01^{a}	0.84 ± 0.01^{c}
PER (%)	257.89 ± 1.58^{b}	272.19 ± 1.48^{c}	279.66 ± 3.43^{c}	250.30 ± 2.23^{a}
CF	3.15 ± 0.65^{a}	3.32 ± 0.77^{b}	3.35 ± 0.73^{b}	3.07 ± 0.3^{a}
HSI (%)	1.52 ± 0.23	1.49 ± 0.06	1.46 ± 0.23	1.58 ± 0.10
VSI (%)	6.19 ± 0.65	6.23 ± 0.77	6.00 ± 0.73	6.49 ± 0.30
SR (%)	100	100	100	100

IBW, initial body weight; FBW, final body weight; weight gain rate (WGR, %) = [(final body weight–initial body weight)/initial body weight] × 100; specific growth rate (SGR, %/d) = [(Ln final body weight–Ln initial body weight)/days] × 100; feeding rate (FR, %/d) = 100 × feed intake/[days × (final body weight + initial body weight)/2]; feed conversion ratio (FCR) = [total dry feed intake (g)/wet weight gain (g)]; protein efficiency rate (PER, %) = [fresh body weight gain (g)/protein intake (g)] × 100; Fulton's condition factor (CF) = 100 × [body weight (g)/body length (cm^3)]; hepatosomatic index (HSI, %) = liver weight (g)/body weight (g) × 100; viscerosomatic index (VSI, %) = visceral weight (g)/body weight (g) × 100; survival rate (%) = final fish number/initial fish number. Data are presented as mean ± SE (N = 3). Means with different letters in a row are significantly different ($p < 0.05$).

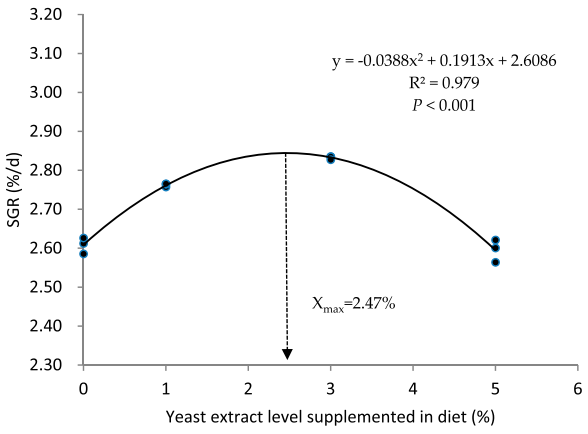

FIGURE 1
Quadratic regression relationship between the specific growth rate (SGR) of the turbot and yeast extract levels supplemented in experimental diets.

were observed among all treatments ($p > 0.05$). However, the values of CF in YE1 and YE3 treatments were significantly higher than those in YE1 and YE3 groups ($p < 0.05$).

3.2 Whole-body proximate composition

The moisture, crude lipid, and crude ash contents of whole body fish were not significantly different among all diets ($p > 0.05$) (Table 4). However, crude protein contents were significantly higher in the YE3 and YE5 groups than those in the YE0 and YE1 groups ($p < 0.05$).

3.3 Serum physiological and biochemical parameters

Dietary yeast extract supplementation has not markedly influenced the serum physiological and biochemical indexes (Table 5), and there were no significant differences observed in serum ALT, AST, TP, BUN, GLU, TG, and TCHO among all groups ($p > 0.05$).

3.4 Digestion-related parameters

The digestion-related parameters of the turbot fed with four experimental diets are shown in Table 6. Yeast extract supplementations in diets led to a notable increase in the activities in contrast to the control group (YE0). Additionally, turbots in YE3 treatment exhibited higher levels of trypsin and amylase than those in YE0, YE1, and YE5 treatments ($p < 0.05$). Moreover, the apparent digestibility coefficient of dry matter (ADC_{DM}) in the YE3 group was significantly higher than that in YE0 and YE5, and ADC_{DM} of YE5 was found to be the lowest among all treatments ($p < 0.05$). YE3 exhibits the greatest apparent digestibility coefficient of crude protein (ADC_{CP}) among all treatments with a statistical difference, and the value of ADC_{CP} in the YE1 group was higher than that of YE5 significantly ($p < 0.05$).

3.5 Intestinal histology

Figure 2; Table 7 display the mid-intestinal morphologies of the turbot fed with various experimental diets. Yeast extract had no different impact on the thickness of the intestinal wall ($p > 0.05$), while it significantly increased the height of mid-intestinal folds in

TABLE 4 Effects of yeast extract supplemented in the diet on the whole body proximate composition of turbot.

Parameter (%)	YE0	YE1	YE3	YE5
Moisture	78.99 ± 0.22	78.53 ± 0.55	78.67 ± 0.68	78.70 ± 0.65
Crude protein	12.89 ± 0.27^{a}	13.17 ± 0.26^{a}	14.36 ± 0.28^{b}	14.07 ± 0.37^{b}
Crude lipid	2.96 ± 0.31	2.61 ± 0.25	2.46 ± 0.16	2.63 ± 0.07
Crude ash	3.50 ± 0.06	3.75 ± 0.19	3.47 ± 0.16	3.52 ± 0.21

Data are presented as mean ± SE (N = 3). Means with different letters are significantly different ($p < 0.05$).

TABLE 5 Effects of yeast extract supplemented in the diet on serum physiological and biochemical indexes of the turbot.

Parameter	YE0	YE1	YE3	YE5
ALT(U/L)	13.5 ± 2.81	14 ± 2.31	12.5 ± 3.91	13 ± 2.31
AST(U/L)	81.33 ± 10.7	96.50 ± 8.62	76.67 ± 15.53	85.50 ± 12.83
TP(g/L)	29.1 ± 1.11	29.4 ± 1.75	29.2 ± 2.75	29.8 ± 1.28
ALP(U/L)	8.17 ± 3.48	9.17 ± 4.98	8.50 ± 3.86	9.33 ± 3.35
BUN(mmol/L)	3.1 ± 0.45	3.3 ± 0.64	3.2 ± 0.59	3.6 ± 0.82
GLU(mmol/L)	2.04 ± 0.61	2.28 ± 0.38	1.93 ± 0.57	1.92 ± 0.26
TG(mmol/L)	1.55 ± 0.25	1.63 ± 0.18	1.35 ± 0.29	1.69 ± 0.33
TCHO(mmol/L)	1.92 ± 0.14	2.02 ± 0.15	1.82 ± 0.19	2.10 ± 0.19

ALT, alanine transaminase; AST, aspartate transaminase; TP, total protein; ALP, alkaline phosphatase; BUN, blood urea nitrogen; GLU, glucose; TG, triglyceride; TCHO, total cholesterol. Data are presented as mean ± SE (N = 6).

TABLE 6 Effects of yeast extract supplemented in the diet on digestive enzymes activities and apparent digestibility coefficients of the turbot.

Parameter	YE0	YE1	YE3	YE5
Digestive enzyme activities				
Trypsin (U/mg prot)	486.10 ± 31.18^{a}	508.08 ± 27.79^{a}	613.93 ± 3.98^{b}	546.32 ± 19.13^{a}
Lipase (U/g prot)	21.12 ± 2.51^{a}	36.13 ± 3.55^{b}	43.36 ± 6.61^{b}	32.84 ± 2.94^{b}
Amylase (U/mg prot)	0.18 ± 0.02^{a}	0.17 ± 0.01^{a}	0.24 ± 0.02^{b}	0.19 ± 0.01^{a}
Apparent digestibility coefficients				
ADC_{DM} (%)	60.56 ± 0.80^{b}	61.93 ± 0.08^{bc}	63.01 ± 0.09^{c}	58.96 ± 0.49^{a}
ADC_{CP} (%)	84.50 ± 0.15^{ab}	85.24 ± 0.58^{b}	86.37 ± 0.20^{c}	83.83 ± 0.44^{a}

Data are presented as mean ± SE (N = 6 for enzyme activity; N = 3 for digestibility parameters). Apparent digestibility coefficient of dry matter (ADC_{DM}, %) = (1 - Y_2O_3 content in diet/Y_2O_3 content in feces) × 100; apparent digestibility coefficient of crude protein (ADC_{CP}, %) = 1–(crude protein content in feces/crude protein content in diet) × (Y_2O_3 content in diet/Y_2O_3 content in feces) × 100; means with different letters in a row are significantly different ($p < 0.05$).

YE1 and YE3 when compared with those in YE0 and YE5, and the fold height in YE3 was higher than that in YE1 treatment ($p < 0.05$).

3.6 Antioxidant-related parameters

The SOD activities and MDA contents in the liver of turbot are exhibited in Figure 3. YE3 treatment displayed a significantly higher SOD activity than the YE0 group ($p < 0.05$). Additionally, YE1 exhibited a notable lower MDA content than YE0 and YE5, and YE3 had a remarkable lower MDA level than YE5 ($p < 0.05$).

4 Discussion

In this study, yeast extract has improved the growth and feed utilization of the turbot, which aligns with previous studies in Gibel carp, snakehead fish, and Nile tilapia, while differing from those observed in Pacific white shrimp and Chinese mitten crab (Chen et al., 2009; Zhou et al., 2012; Berto et al., 2016; Hassaan et al., 2018; Zhang et al., 2019; Zheng et al., 2021). The positive influence of yeast extract on the growth of turbot might be implicated with rich contents of bioactive components present in the yeast extract. The yeast extract utilized in this investigation was a product of crude extract paste from the brewing industry and comprised small

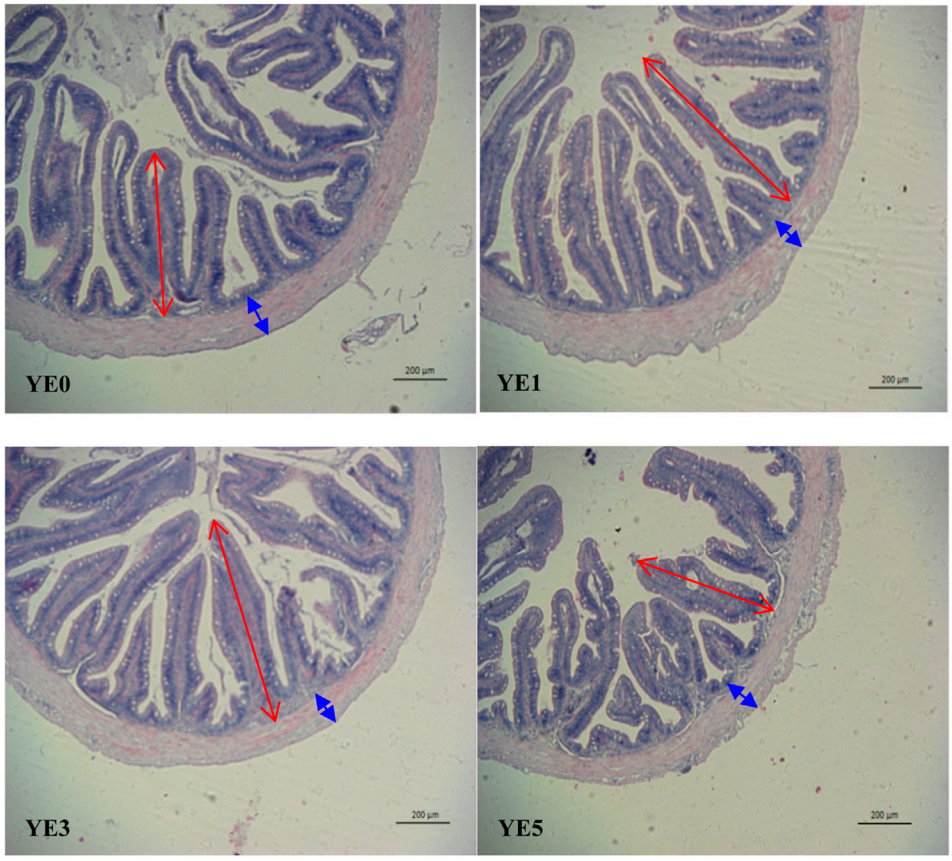

FIGURE 2
Mid-intestinal histology of the turbot fed four experimental diets with graded levels of yeast extract. The red arrow indicates the height of the intestinal fold, and the blue arrow indicates the thickness of the intestinal wall.

TABLE 7 Effects of yeast extract supplemented in the diet on the intestinal histological structure of the turbot.

Parameter	YE0	YE1	YE3	YE5
Height of intestinal folds (μm)	484.82 ± 23.91[a]	546.13 ± 15.63[b]	616.86 ± 18.40[c]	439.09 ± 13.99[a]
Thickness of the intestinal wall (μm)	139.90 ± 5.04	122.62 ± 7.07	126.99 ± 6.65	118.78 ± 6.16

Data are presented as mean ± SE (N = 3). Means with different letters in a row are significantly different ($p < 0.05$).

peptides, ß-glucans, mannan oligosaccharides, nucleotides, and amino acids. Protein synthesis requires amino acids, which are also regarded as feed attractants in fish (Dabrowski et al., 2010), and the turbot was found to utilize small peptides more effectively than free amino acids (Wei et al., 2020). Nucleotides are intracellular compounds that play important roles in almost all biochemical processes. Although nucleotides can be produced from amino acids, they are insufficient during stress conditions and stages of rapid growth and development. Positive effects of nucleotides on growth and feed utilization have been demonstrated in the turbot (Li et al., 2018), beluga sturgeon (*Huso huso*) (Abtahi et al., 2013), rainbow trout (*Oncorhynchus mykiss*) (Tahmasebi-Kohyani et al., 2011), red drum (*Sciaenops ocellatus*) (Li et al., 2007), grouper (*Epinephelus malabaricus*) (Lin et al., 2009), and European sea bass (*Dicentrarchus labrax*) (Pelusio et al., 2023). The addition of nucleotides to the diet was discovered to enhance the expression of specific proteins involved in muscle metabolism and the structure in rainbow trout, along with insulin-like growth factor 1 in red sea bream (Keyvanshokooh and Tahmasebi-kohyani, 2012; Hossain et al., 2016). Moreover, polysaccharides are regarded as substances that enhance the growth and stimulate the immune system of fish and crustaceans (Mohan et al., 2019; Rajan et al., 2023). It is reported that dietary supplementation of mannan oligosaccharides has enhanced the growth and feed efficiency of turbots (Bai et al., 2017). Hence, these findings are helpful to understand the possible mechanisms of yeast extract in the aspect of contributing to good growth in turbots. However, an overabundance of nucleotides and polysaccharides in diets may result in a heightened immune response and hindered growth performance (Burrells et al., 2001; Hossain et al., 2020). Song et al. (2012) found that the growth of olive flounder (*Paralichthys olivaceus*) was hindered when fed a diet containing

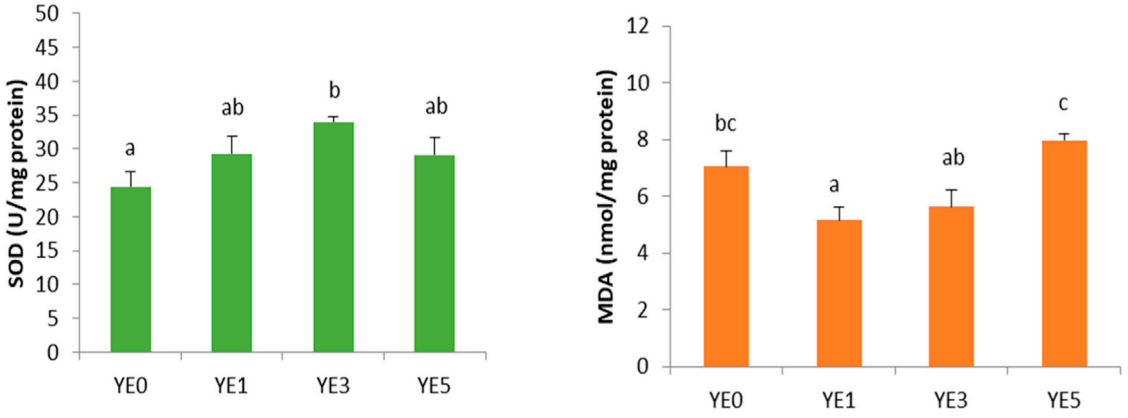

FIGURE 3
Effects of four experimental diets supplemented with graded levels of yeast extract on the anti-oxidant-related parameters in the liver of the turbot. SOD, superoxide dismutase; MDA, malondialdehyde. Data are presented as mean ± SE (N = 6). Means with different letters are significantly different ($p < 0.05$).

1.0% IMP, in comparison to fish fed with 0.1%–0.2% IMP; dietary nucleotides at a high concentration (more than 1.5 g kg^{-1}) inhibited the growth of red sea bream (Hossain et al., 2016). In the YE5 group of this study, the contents of nucleotides, ß-glucans, and mannan oligosaccharides in diet YE5 were calculated at 1.5 g kg^{-1}, 4.25 g kg^{-1}, and 2.6 g kg^{-1} in diets, respectively. They are at relatively higher levels according to previous results. Therefore, the decrease in turbot's growth performance in YE5 treatment compared with the YE3 group may be due to the overdosing of these components in diet YE5. Turbot's growth in this study showed a classic dose-dependent response to dietary yeast extract levels, and the appropriate dietary yeast extract content for improving the growth of turbot is 2.47% based on the quadratic regression relationship between SGR and dietary yeast extract levels.

As for the body compositions of turbot in this research, solely the amount of crude protein notably increased with the increasing levels of dietary yeast extract. This could be attributed to the yeast extract's abundance of amino acid and nucleotide components. Nucleotides are served as the fundamental units for DNA to produce protein. Dietary supplementation of yeast extract would enhance protein biosynthesis by controlling the levels of nucleotides and amino acids in the cells (Hossain et al., 2020). On the other hand, appropriate supplementations of nucleotides and amino acids are helpful in improving the utilization of protein in the diet, and the increased PER observed in YE1 and YE3 groups of this study has shed light on this issue. Consequently, the nucleotide-rich yeast extract in this study promoted the protein synthesis of the turbot and enhanced its body protein content.

The beneficial impact of yeast products at appropriate levels on the intestinal health of fish and crustaceans was reported in many literature works; in general, 1%–2% yeast extract supplementation has increased the fold height of the anterior intestine in snakehead fish (Zhou et al., 2012), and the addition of additives extracted from yeast exhibited modulatory effects on intestinal morphology, particularly enhanced the intestinal mid-intestinal villi length and lamina propria width in gilthead sea bream (*Sparus aurata*) (Mallioris et al., 2022). Furthermore, the utilization of yeast and yeast extract led to a notable enhancement in the Shannon indexes of shrimp's intestinal microbiota (Zheng et al., 2021). The substitution of fish meal with the yeast extract increased the nutrient digestibility of shrimp, as well as the hepatic trypsin activities (Zhao et al., 2017). Similar findings were found in our study, where 3% yeast extract enhanced the height of mid-intestinal folds, improved the digestibility of crude protein and dry matter, and increased the activities of trypsin, lipase, and amylase in the anterior intestine. Eventually, the intestinal health and feed utilization capacity of turbot were improved by dietary yeast extract addition. This may be attributed to the presence of mannan oligosaccharides and nucleotides in yeast extract. It was found that mannan oligosaccharides enhanced the growth and feed effectiveness while counteracting the negative impacts of soybean meal on intestinal wellbeing, which was achieved by boosting the performance of digestive enzymes and protecting against alterations in mucosal folds (Fuchs et al., 2015; Bai et al., 2017). Additionally, mannan oligosaccharides positively influenced the structure and microbial population of the gastrointestinal tract in rainbow trout and *Sparus aurata* (Dimitroglou et al., 2009; 2010). Regarding nucleotides, Hossain et al. have reviewed and summarized that nucleotides could enhance the intestinal health of fish by regulating the physiological and microbiological parameters in the gut (Hossain et al., 2020). It has also been found that nucleotides have upregulated the tight junction gene claudin 3 (*cldn3*) and induced the expression of interleukin 1β (*il1b*) and interleukin 8 (*il8*) in the intestinal epithelial cell line of rainbow trout (Wang et al., 2019). Therefore, the addition of yeast extract also demonstrates a beneficial impact on the intestinal wellbeing of the turbot, similar to the majority of fish and shrimp. Dietary yeast extract improved the apparent digestibility of turbot by enhancing the histological structure of the intestines and increasing the secretion of various digestive enzymes.

The cells of the body can be harmed by highly reactive molecules known as superoxide anions, which are produced *in*

vivo as a result of oxidative stress (Halliwell and Gutteridge, 1984). SOD is an important antioxidant enzyme that helps neutralize these harmful molecules by converting them into less-damaging hydrogen peroxide, which is helpful in preventing the accumulation of MDA and other detrimental by-products caused by oxidative stress in the body (Vlahogianni et al., 2007). In this study, the hepatic SOD activities in the YE3 group were significantly higher than those in control, and the MDA contents in YE1 and YE3 were relatively lower than those in the control group, which indicated that the yeast extract has increased the antioxidant capacity of turbots. The positive effects of yeast production on the antioxidant capacity were also observed in the studies of tilapia (Xu et al., 2015; Reda et al., 2018), shrimp (Xiong et al., 2018; Zheng et al., 2021), and crab (Zhang et al., 2019). Vieira et al. (2016) conducted a study to assess the antioxidant characteristics of brewer's spent yeast extract using various assays, such as the DPPH (1,1-diphenyl-1-picrylhydrazyl) radical-scavenging capacity assay, ferricyanide-reducing power assay, and ferric-reducing antioxidant potential assay. They found that yeast extract displayed a favorable ability to counteract oxidation, despite the fact that the precise mechanism behind it remains unidentified. Furthermore, the healthy condition of fish was reflected by serum biochemical indexes. It is shown that no significant variations were observed in these parameters among all treatments in this study, which implies that yeast extract did not adversely impact the turbot's wellbeing. Therefore, yeast extract has shown potential as a feed additive for the turbot, with promising results in terms of growth and digestibility promotion, and antioxidant properties.

5 Conclusion

In this study, the growth performance, feed conversion efficiency, body crude protein contents, apparent digestibility of nutrients, digestive enzyme activities, height of the intestinal fold, and the antioxidant capacity of juvenile turbots were significantly enhanced by the addition of yeast extract in the diet. The optimal growth and intestinal health of the turbot can be achieved by supplementing 2.47% yeast extract in the diet.

Data availability statement

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

Ethics statement

The animal study was approved by the Biomedical Ethics Committee of Hebei Normal University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JS: data curation, formal analysis, investigation, methodology, software, and writing–original draft. YaL: investigation, methodology, and writing–original draft. TR: data curation, software, validation, visualization, and writing–review and editing. QG: data curation, software, validation, visualization, and writing–review and editing. LY: validation, visualization, and writing–review and editing. YuL: validation, visualization, and writing–review and editing. HL: conceptualization, funding acquisition, project administration, supervision, and writing–review and editing.

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Conflict of interest

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

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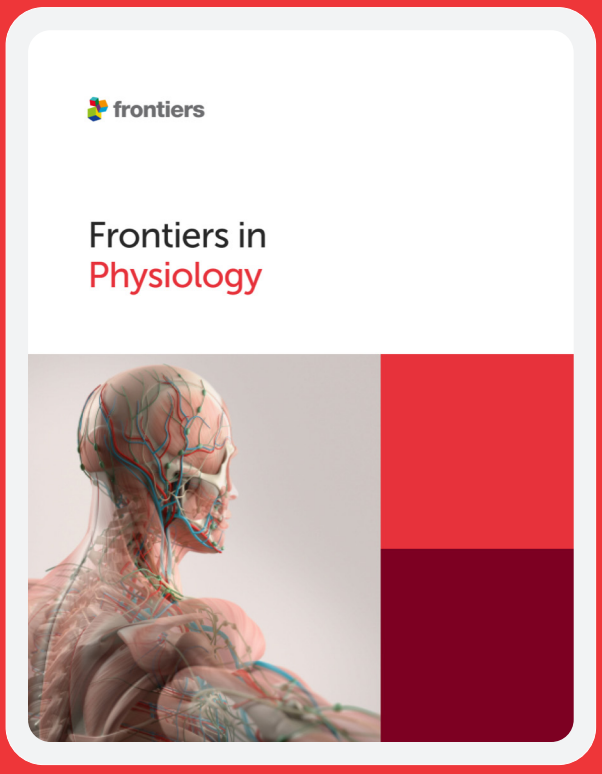

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