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Aquatic nutrition and intestine immunity

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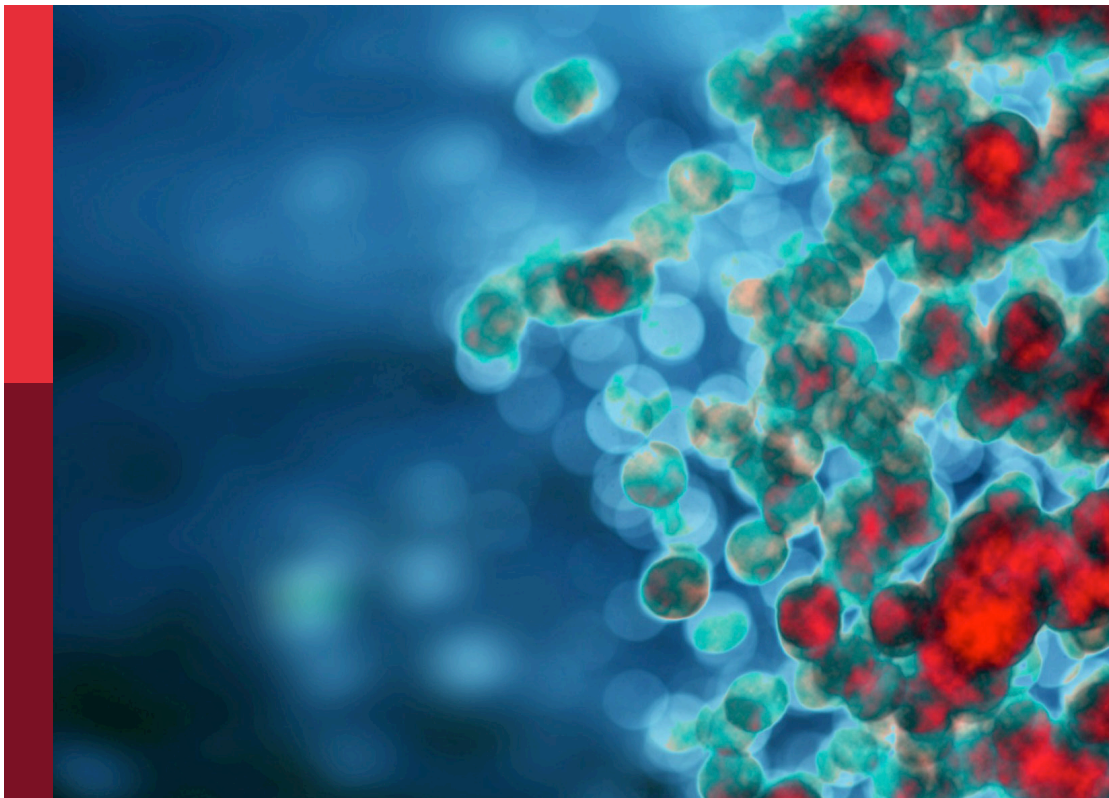
Published in
Frontiers in Immunology



ISSN 1664-8714
ISBN 978-2-8325-3571-4
DOI 10.3389/978-2-8325-3571-4

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Aquatic nutrition and intestine immunity

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Citation

Xu, Q., Ren, M., Wang, L., Yamamoto, F. Y., Qi, C., eds. (2023). *Aquatic nutrition and intestine immunity*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-3571-4

Table of contents

frontiers | Frontiers in Immunology

TYPE Editorial
PUBLISHED 12 September 2023
DOI 10.3389/fimmu.2023.1274213

OPEN ACCESS

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RECEIVED 08 August 2023
ACCEPTED 04 September 2023
PUBLISHED 12 September 2023

CITATION
Xu Q, Ren M, Wang L, Yamamoto FY and Qi C (2023) Editorial: Aquatic nutrition and intestine immunity.
Front. Immunol. 14:1274213.
doi: 10.3389/fimmu.2023.1274213

Editorial: Aquatic nutrition and intestine immunity

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KEYWORDS

nutrition, feed additive, intestine, immunity, aquatic animal

Editorial on the Research Topic
Aquatic nutrition and intestine immunity

Aquaculture supplies large quantities of high-value proteins for human consumption (1). Due to the limited supply of fishmeal and the increasing demand of aquaculture worldwide, novel protein ingredients are being widely adopted for their lower cost and sustainability as a fish meal replacement in the feed industry (2). Previous studies have shown that these alternative proteins can partially replace fishmeal in the diets of fish. However, excessive inclusion levels of these novel proteins may result in impair growth performance, induce liver inflammation, and compromise the intestinal structure of farmed fish (3). It is of great importance to explore functional nutrients to improve immunity and maintain health of different aquatic animals. The intestinal tract is the main site for digestion and absorption of nutrients, and it is crucial for fish growth and physiological functions. Therefore, it is of utmost importance to investigate the fish intestinal health when fed low fish meal diets and develop protocols to mitigate potential side effects caused by the inclusion of alternative protein ingredients.

The goal of this Research Topic is to provide literature on advanced research and improving our understanding of the nutritional immunity regulation in multiple aspects of aquatic animals on intestine. Areas covered include: 1) Effects of replacing fishmeal with novel proteins on growth performance and intestinal health; 2) Effects of supplements on growth, intestine immunity of aquatic animal fed with a low-fishmeal diet; 3) Dietary supplementation of additives to improve intestinal health.

The global fishmeal production has been stagnated for decades and cannot keep up with the increased demand by aquaculture; Thus, fishmeal price has significantly increased over the years (4). It is important to search for alternative protein sources to reduce fishmeal levels in the diet. Soybean meal is the most common fishmeal substitute in aquafeed due to its steady supply and relatively balanced amino acid profile (5). But long-term feeding of soybean meal may result in structural and functional changes in the distal

intestine (6). The negative influences may be correlated with antinutritional factors in soybean meal (7). Wang et al. reported that diet supplementation with 2.46% soybean saponin not only hindered the growth performance by negatively affecting the macronutrients absorption in the intestine, but also induced an inflammatory response in the large intestine possibly by damaging the intestinal morphology, disrupting the intestinal microbiota and decreasing intestinal epithelial cell membrane permeability. Luo et al. reported that dietary soybean meal compromised the intestinal health, and the adverse effects were related to the presence of β-conglycinin and glycinin, especially glycinin. Cottonseed protein concentrate (CPC) is a novel non-food protein derived from cottonseed. Wang et al. replaced fishmeal with 15% CPC and largemouth bass even exhibited better growth potential during the last three weeks of whole feeding trial, which was accompanied with higher phosphorylation level of TOR signaling and higher mRNA expression level of myogenin (myog). However, largemouth bass fed with CPC presented higher inflammation in both liver and gills during *N. seriolae* infection challenge. Liu et al. showed no adverse effects on growth performance of juvenile rainbow trout (*Oncorhynchus mykiss*) when 75% dietary fishmeal was replaced by CPC. Nevertheless, the CPC-based diet resulted in reduced the activity of intestinal trypsin, decreased villus height and width in the distal intestine, upregulated mRNA expression levels of inflammatory cytokines in the intestine, and impaired gut microbiota with reduced bacterial diversity and decreased the relative abundance of *Bacillaceae*. *Clostridium autoethanogenum* protein (CAP) is a byproduct of *Clostridium autoethanogenum* fermentation to produce ethanol. Chen et al. reported that dietary CAP could improve the growth, disease resistance, digestive capacity, and modulate the intestinal microbiota of *L. vannamei* with a higher immune response and enhanced the ability of shrimp to cope with salinity stress.

Previous studies have shown that appropriate inclusion of functional supplements in aquatic diets can effectively mitigate the adverse effects of low-fishmeal diets on farmed fish (8). Zheng et al. reported that CPC substitution induced a significant decrease in digestive enzyme activities and gut barrier protein PT-1 expression and a significant increase in g-GT enzyme activity and inflammatory related factors (Relish and Toll) expression of *Macrobrachium rosenbergii*, and B. coagulans could mediate specific gut microbes and the combined action of multiple functional secondary metabolites to affect intestinal barrier function, digestion, and inflammation. Liu et al. reported that 1.2% taurine supplementation in diets greatly enhanced the weight gain of juvenile golden pompano fed a low fishmeal diet. Moderate exogenous taurine increased the muscular thickness and villus length within the intestine, maintained intestinal physical barrier stability, activated the Nrf2/Keap-1/HO-1 signaling pathway, suppressed NF-kB signaling and intestinal pro-inflammatory cytokine gene expression, and increased anti-inflammatory cytokine gene expression. Liu et al. also reported that dietary cysteine (the precursor of taurine) greatly increased the SGR of golden pompano, upregulated the Nrf2/Keap1/HO-1 signaling pathway, improved muscle thickness and villi length, increased the diversity and relative abundance of the intestinal flora of golden pompano. Supplementation of cysteine also suppressed intestinal NF-kB/IKK/IkB signaling and pro-inflammatory cytokine mRNA levels. Conversely, intestinal anti-inflammatory cytokine gene expression and serum immune parameters were upregulated. Zhao et al. reported 1%-2% alanyl-glutamine and 0.1%-0.2% tributyrin can alleviate enteritis caused by high inclusion levels of soybean meal. The activities of intestinal trypsin, lipase and amylase in tributyrin and alanyl-glutamine groups increased significantly, and the gene expression levels of acetyl-CoA carboxylase (ACC), caspase-3, caspase-8, caspase-9, tumor necrosis factor alpha (TNF-α), and interleukin-1 beta (IL-1b) were down-regulated, while the gene expression level of target of rapamycin (TOR) and eIF4E-binding protein (4E-BP) were up-regulated.

Another aspect of interest, is the functional feed additives as an alternative prophylactic approach to improve animal health and performance (Rawling et al.). Porter et al. established rainbow trout cell lines as potential alternative method to test functional feed ingredients, GALT leucocytes were deemed most effective to act as a health screen over the 4 hr time point demonstrating responses to Poly I:C, PHA, and rIL-1b. RTS11 and RTgutGC also responded to the stimulants but did not give a strong T-cell response, most likely reflecting the nature of the cell type as opposed to the mixed cell populations from the primary GALT cell cultures. When stimulated with both forms of β-glucan, GALT leucocytes of rainbow trout demonstrated a strong proinflammatory and T-cell response. Zhang et al. reported that fish fed the diet with 300 mg kg^{-1}β-glucan significantly increased the activity of lysozyme. Transcriptome analysis showed that 109 immune-related genes were differentially expressed. 300 mg kg^{-1} β-glucan significantly increased the relative abundance of *Mycoplasma* and decreased *Proteobacteria* (mainly *Escherichia-Shigella* and *Escherichia coli*) and *Bacillus anthracis* in largemouth bass intestinal microflora. Shen et al. suggested that β-1,3-glucan supplementation improved the intestinal health of white shrimp (*Litopenaeus vannamei*) through the modulation of intestinal microbiota, the suppression of intestinal inflammatory responses, the elevation of immune and antioxidant capacity, and promoted growth performance of white shrimp. The yeast cell wall (YCW) is an established prebiotic. Rawling et al. identified α-mannan content as a potent driver of GCD and IEL hyperplasia, suggestimg the fortification of intestinal barrier integrity and immune competence. Further the structural molecular differences of the yeast cell wall polysaccharides, in terms of and β-1,3-glucans, were shown to modify the expression pattern of PRR responses. Zhou et al. reported that oral administration of recombinant hepcidin improved the growth performance and regulated the iron metabolism. The immunity and survival of grass carp were improved, and hepcidin in combination with chitosan was better than that of hepcidin alone. Yang et al. suggested that tea polysaccharides promoted immunity, antioxidant capacity and intestinal barrier function and reduced lipogenesis and glucose transporter of common carp. Lu et al. reported mannose

oligosaccharide (MOS) enhances immunity partly related to increasing antibacterial substances content and antimicrobial peptides expression. MOS attenuates inflammatory response partly related to regulating the dynamic balance of intestinal inflammatory cytokines. MOS regulates immune barrier function may partly be related to modulating TLRs/MyD88/NFkB and TOR/S6K1/4EBP signaling pathways. Luo et al. reported a-ketoglutarate may regulate intestinal energy via tricarboxylic acid cycle, thereby alleviating the damage intestinal morphology induced by the dietary soybean antigen proteins.

The underlying regulatory mechanism in aquatic animal nutrition and intestine immunity remains largely unresolved. This Research Topic showcases a collection of original research that highlights the latest discoveries and advances in the field of aquatic animal nutrition and intestinal immunity. By improving our understanding of the nutritional immunity regulation in multiple aspects of aquatic animals, there is potential to develop functional feeds and assist to the infinite growth potential of the aquaculture industry.

Author contributions

QX: Writing – original draft, Writing – review & editing. MR: Conceptualization, Writing – review & editing. LW: Conceptualization, Writing – review & editing. FY: Writing – review & editing. CQ: Writing – review & editing.

Funding

This work was supported by the Natural Science Foundation of China, NSFC (31972800), and the Zhejiang Province R&D Plan 2023C02024.

Acknowledgments

We would like to thank all the authors and reviewers who assisted in our Special Research Topic.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 13 September 2022
DOI 10.3389/fimmu.2022.1010221

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Comparative Immunology, a section of the journal Frontiers in Immunology

RECEIVED 02 August 2022
ACCEPTED 23 August 2022
PUBLISHED 13 September 2022

CITATION
Lu Z-Y, Feng L, Jiang W-D, Wu P, Liu Y, Jiang J, Kuang S-Y, Tang L, Li S-W, Zhong C-B and Zhou X-Q (2022) Dietary mannan oligosaccharides strengthens intestinal immune barrier function *via* multipath cooperation during *Aeromonas Hydrophila* infection in grass carp (*Ctenopharyngodon Idella*). *Front. Immunol.* 13:1010221. doi: 10.3389/fimmu.2022.1010221

Dietary mannan oligosaccharides strengthens intestinal immune barrier function *via* multipath cooperation during *Aeromonas Hydrophila* infection in grass carp (*Ctenopharyngodon Idella*)

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In recent years, mannose oligosaccharide (MOS) as a functional additive is widely used in aquaculture, to enhance fish immunity. An evaluation of the effect of dietary MOS supplementation on the immune barrier function and related signaling molecules mechanism of grass carp (*Ctenopharyngodon idella*) was undertaken in the present study. Six diets with graded amounts of MOS supplementation (0, 200, 400, 600, 800, and 1000 mg/kg) were fed to 540 grass carp over 60 days. To examine the immune response and potential mechanisms of MOS supplementation on the intestine, a challenge test was conducted using injections of *Aeromonas hydrophila* for 14 days. Results of the study on the optimal supplementation with MOS were found as follows (1) MOS enhances immunity partly related to increasing antibacterial substances content and antimicrobial peptides expression; (2) MOS attenuates inflammatory response partly related to regulating the dynamic balance of intestinal inflammatory cytokines; (3) MOS regulates immune barrier function may partly be related to modulating TLRs/MyD88/NFκB and TOR/S6K1/4EBP signalling pathways. Finally, the current study concluded that MOS supplementation could improve fish intestinal immune barrier function under *Aeromonas hydrophila* infected conditions.

KEYWORDS

mannan oligosaccharides, immune barrier, NFκB, TOR, Grass carp (*Ctenopharyngodon idella*)

Introduction

A growing global fish demand has prompted intensive aquaculture research. However, the intensive expansion of aquaculture is often accompanied by fish health/disease issues. Fish enteritis is one of the common diseases in intensive aquaculture, which leads to high mortality in farmed fish and causes huge annual economic losses in the world (1). Mannan oligosaccharides (MOS) is functional oligosaccharides, which has received considerable attention in recent years for its promotion of fish health (2). According to existing studies, we know that MOS has a beneficial effect on the immune function of multiple functional organs (such as head kidney, spleen, skin, etc.) in fish (3–6). Different from other functional organs, the intestinal tract, as a bridge connecting the internal and external environment of the body, can directly contact with foreign substances (7, 8). Hence, fish intestine is very susceptible to direct effects of feed composition. Studies have shown that mannose oligosaccharides contain mannose residues that can specifically bind to intestinal epithelial pattern recognition receptors (PRR) to induce immune responses (8, 9). Therefore, the appropriate level of MOS supplementation maybe involves the interaction of the intestinal epithelium to maintain intestinal health. Generally, intestinal health is closely related to the intestinal immune barrier. Several fragmentary reports have been published about the influence of MOS addition on the teleost intestinal immune barrier. According to these limited studies, in the intestine, MOS addition could up-regulate the immunoglobulin (Ig) and tumor necrosis factor (TNF)-α expression in European sea bass (*Dicentrarchus labrax*) (10), IL-10 in rockfish (*Sebastes schlegelii*) (11), MUC-2 in tropical gar (*Atractosteus tropicus*), TGF-β in turbot *Scophthalmus maximus* (12) and down-regulate IL-1β, IL-6 and IL-8 in European sea bass (13, 14). Although many intestinal inflammatory cytokines have been affected by MOS supplementation, the specific mode of action and related mechanisms in the intestine underlying the beneficial effects of MOS remains unclear in fish. Thus, a more comprehensive and in-depth exploration of the molecular mechanisms will be necessary to understand this relationship.

As is well-known, the intestinal immune barrier is related to its immune components such as antimicrobial compounds (e.g., acid phosphatase (ACP), lysozyme (LZ), complement 3 (C3) and C4), antimicrobial peptides (e.g., hepcidin, liver-expressed antimicrobial peptide (LEAP)-2A, and β-defensin), Ig production and T lymphocytes (4, 15). Apart from study looking into Ig, no evidence has been found that MOS supplementation affects the innate immune components of fish. A study on growing pig intestines indicated that MOS supplementation could increase Zn retention (16). A previous study from our laboratory confirmed that Zn could increase the content of IgM in the intestine of grass carp (17). It was also reported that MOS supplementation could increase phosphorus digestibility in the ileum of piglets (18). We have demonstrated in previous studies that phosphorus regulates hepcidin and LEAP2B mRNA levels in the head kidney and spleen of grass carps (19). To further build on these findings, further investigations into non-cellular immune components are needed to determine whether MOS influences fish immune barrier function in the intestinal tract.

In addition, the fish immune barrier is also strongly linked to intestinal cells (e.g., epithelial cells, mesoderm cells and immune cells) and multiple cytokines (19). Studies on human PBMCs indicated that cytokines are regulated by mammalian target of rapamycin (mTOR) signaling (20) and nuclear factor-κB (NFκB) signaling (21). There is, however, little information available regarding the effect of MOS supplementation on intestinal cytokines and the underlying mechanisms (NFκB and TOR pathway). It has been reported that the addition of MOS to turkey intestines increased butyrate concentrations (22). Butyrate has been found to down-regulate the mRNA levels of TNFα, IL-15, and the signaling molecule NFκBp65 in our lab in a prior study (23). The presence of prostaglandins is increased in the intestine of European sea bass with MOS addition (24). Another study showed that prostaglandin could increase mTOR protein levels in mouse hepatocytes (25). Furthermore, the Toll-like receptors (TLRs) family of proteins has emerged as one of the most important determinants of immune activation in immune response research (26). As of now, only one study has been published about the influence of MOS on fish intestinal TLR family proteins. The study showed that the gene expression of TLR3 was up-regulated with MOS supplementation in the intestine of juvenile hybrid grouper (*Epinephelus lanceolatus* ♂ × *Epinephelus fuscoguttatus* ♀) (27). Based on these data, a relationship may exist between dietary MOS supplementation and the inflammatory cytokines and related signalling molecules in fish, which requires further investigation.

Based on our previous work on the intestinal tract health and growth performance of grass carp with MOS supplementation (6, 28), the present study examined the effects of dietary MOS supplementation on intestinal immune barrier function in grass carp under *Aeromonas hydrophila* (*A. hydrophila*) challenged conditions. Specifically, we explored the impact of MOS addition on antimicrobial substances, multiple cytokines, and possible pathways in the fish intestine, which might shed light on the underlying mechanisms in the intestines as well as the effect of MOS on the immune response. Currently, grass carp ranks as one of the most popular aquaculture species (29). According to the FAO, grass carp production reached 5.7 million tons in 2018 (29). Consequently, this present study not only provides a scientific reference for grass carp commercial feed formulations, which is of great importance for grass carp cultivation but also offers a reliable theoretical foundation for the development of an oral vaccine.

Materials and methods

Experimental design

Preparation and storage methods of the MOS (Sciphar Hi-Tech Industry, Xi'an, purity: 99.12%) diet are based on our previous research (28). As shown in Supplementary Table 1, the formulation of the experimental diet and proximate composition analyses were conducted. In place of cornstarch, different levels of MOS (0, 200, 400, 600, 800, and 1000 mg/kg) were added to the basal diet. A completed diet was stored at 4°C until it was given as feed.

Animals and experimental management

The procedures used in this research were allowed by the Animal Care Advisory Committee of Sichuan Agricultural University under permit No. LZY-2018114005 throughout the feeding trial. Tong Wei fisheries (Sichuan, China) provided the grass carp, which were adapted to fishpond culture conditions for a month before the experiment. 540 fish are randomly assigned to eighteen nylon cages [1.4 L × 1.4 W × 1.4 H (m)], resulting in 30 fish per cage, with the same feeding frequency and experimental period as previously described (28). The typical management parameters for a test are that the level of dissolved oxygen > 6.0 mg/L, the temperature of the water is 28.55°C ± 2.0°C, the pH value is 7.5 ± 0.3, and the experiment is conducted with a natural light cycle.

Challenge trial

In accordance with our published work (6, 28), we conducted a 14-day challenge trial to assess the effects of dietary MOS on intestinal immune function after the growth trial. In brief, 1.0 ml of *A. hydrophila* (FDL20120711) was intraperitoneally injected into five randomly selected fish per replicate from each MOS group. Other conditions of the challenge trial were same as that of the feeding trial. In previous studies, we have successfully established *A. hydrophila* challenged models (28).

Sample preparation and biochemical analysis

At the end of the challenge trial, all grass carp were anesthetized in a benzocaine bath. Then, the abdominal cavity was carefully opened and the intestine was removed immediately. After quickly removing the intestines of each fish, the proximal intestine (PI), middle intestine (MI) and distal intestine (DI) were separated, then fish intestine segments were rapidly collected and temporarily stored in liquid nitrogen. To facilitate later biochemical analysis, the samples were stored at -80°C. An intestine tissue homogenate containing 10% (w/v) saline (4°C) was centrifuged (6000 g, 20 minutes) to determine immune-related parameters. After that, the supernatant was collected. ACP, C3, C4, LZ, and IgM biochemical analysis methods are shown in Supplementary Table 2.

Real-time PCR

The *q*RT-PCR was conducted in reference to our previous work (28). In brief, total RNA was isolated from intestinal segments by using Takara's RNAisoPlus Kit (Dalian, China). The quality of the RNA was determined by electrophoresis on 1% agarose gels and then quantified by spectrophotometry at 260/280 nm using a Nanodrop 2000 (Thermo Scientific, USA). After RNA extraction, the PrimeScript™ RT reagent kit was used (Takara, Dalian, China) to reverse-transcribe it into cDNA. Supplementary Table 3 shows the primers we designed for *q*RT-PCR based on the sequences we previously cloned. As described previously, we screened four internal reference genes and selected β-Actin and GAPDH as our final two candidates (28). Calculation of target gene amplification efficiency was done according to manufacturer instructions after preparing melting curves. The transcription level of genes was calculated according to Livak and Schmittgen's method ($2^{-\Delta\Delta CT}$) (30).

Immunohistochemistry

To prepare the intestinal samples for immunohistochemical staining, fresh intestinal samples were fixed in 4% paraformaldehyde solution. Intestinal samples were prepared by Lilai Biotechnology (Sichuan, China) as paraffin sections. We purchased an IHC kit from BOSTER Biological Technology (SA1028, Wuhan, China). The process was as follows: in short, paraffin sections were dewaxed with xylene and rehydrated in a graded ethanol series before removing endogenous peroxidase activity with hydrogen peroxide 3%. Then, a microwave-repaired antigen was then incubated with EDTA-repair solution, followed by 5% BSA blocking solution, before being incubated overnight with a primary antibody. Following SABC incubation and biotin labeling, slices were created for DAB coloring and hematoxylin re-staining. As a final step, dehydration of the sections was accomplished with graded ethanol solutions, followed by xylene transparency, and finally sealing with neutral gum. A light microscope (TS100, Nikon, Tokyo, Japan) was used to acquire and visualize images of the prepared IHC slices after drying overnight at 60°C. The expression of p-IRAK1, MyD88, TRAF6, and NFκBp65 was quantified using Image Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). For IHC measuring, each antibody is

described in Supplementary Table 4 is described in detail. The reagent instructions were followed and adjustments were made as necessary.

Western blot analysis

Laboratory analysis and the operational parameters of the blotting of intestinal homogenates and antibodies were performed as previously described (4, 5). Extraction and determination of tissue proteins was done using the RIPA and BCA assay kit (Beyotime). The prepared samples (40 μg/lane) were transferred to a PVDF membrane after separation by 10% SDS-PAGE. The membranes were then incubated with primary antibodies overnight (4°C, 14h). Following membrane washing, incubation with secondary antibodies was done incubated at room temperature for 90 minutes. Based on the previous description, protein signals were then visualized and quantified using NIH Image J, 1.42q (4, 5, 28). The detailed description of all antibodies is listed in Supplementary Table 5 of the current study.

Statistical analysis

By using Levene's test for homogeneity and the Shapiro-Wilk test for normal distribution, we assessed data homogeneity and normality. One-way ANOVA with Tukey's multiple comparisons test was used to analyze biochemical, gene expression, and protein level data, while the Student t-test was used to analyze immunohistochemical data (IOD), $P < 0.05$ was considered significant. As previously described, data analyses were performed using PROC MIXED of SAS software version 9.4 (SAS Institute Inc. 2004) (28). The linear and quadratic effects of MOS supplementation were assessed using orthogonal polynomial contrasts. The visualization of antibiotic substance, cytokines, immunohistochemical, and correlation data was done with *R* Studio v4.0.2 and Hiplot (https://hiplot.com.com) (5).

Result

Growth and disease resistance phenotypes

In this study, we used the same growth trials as we used in our previous grass carp research (28). The effectiveness of MOS supplementation on fish growth performance was found to be a quadratic effect ($P < 0.01$) after the growth experimental period. In comparison with the control group, final body weight (21.59%), specific growth rate (16.24%) and percent weight gain (31.34%) of the optimum group (400 mg/kg MOS) were significantly elevated along with increased intestine length (14.82%), intestine weight (26.98%), intestine length index (10.41%) and intestinal somatic index (13.92%). All fish survived in all groups after exposure to *A. hydrophila* and enteritis morbidity showed a quadratic relationship with MOS addition ($P < 0.05$). It was found that the optimal group (400 mg/kg MOS) experienced a 53.45% decrease in enteritis morbidity, as well as a 42.56% decrease in red-skin morbidity (5, 28).

Intestinal immunological parameters

To investigate the effects of MOS on fish intestines and phenotype after infection of *A. hydrophila*, we tested several major immunological parameters. Figure 1 shows observations of immunological parameters. LZ, ACP activities, C3, C4 and IgM contents showed a quadratic effect in response to MOS supplementation ($P < 0.01$). In comparison with the control, while the MOS supplementation levels increased to 400, 600, 400, 400, and 400 mg/kg diet, in the PI, the LZ and ACP activities, C3, C4, and IgM contents reached their maximum levels, and with higher MOS addition, all of them showed a decreased trend; while the MOS supplementation levels increased to 400, 400, 400, 600, and 600 mg/kg diet, in the MI, then all of them (except C4 contents) showed a decreased trend; while the MOS supplementation levels increased to 600, 400, 600, 600, and 600 mg/kg diet, in the DI, the LZ and ACP activities, C3, C4, and IgM contents reached their maximum levels, and with higher MOS addition, all of them showed a decreased trend.

Intestinal antimicrobial peptide-related gene expression

To determine whether MOS affects the antimicrobial peptides in different segments, the expression of antimicrobial peptide-related genes was systematically measured, and the results are shown in Figure 2. The present study showed exhibited that antimicrobial peptide expression in all segments increased quadratically with MOS increasing levels ($P < 0.05$). Gene expression changes (fold-change) in the PI as compared to the control were as follows: MOS-400 mg/kg showed up-regulation of β-defensin-1 (0.46), Hepcidin (0.71) and LEAP2A (0.73) ($P < 0.05$), while MOS-600 mg/kg showed up-regulation of Mucin2 (0.8), LEAP2B (0.96) and MBL (1.95) ($P < 0.05$). In the MI, MOS-400 mg/kg showed up-regulation of LEAP2B (0.38) ($P < 0.05$) and MOS-600 mg/kg showed up-regulation of β-defensin-1 (0.39) ($P < 0.05$), MOS-600 mg/kg showed up-regulation of Mucin2 (0.68), Hepcidin (0.35), LEAP2A (0.64), and MBL (1.78) ($P < 0.05$). In the DI, MOS-400 mg/kg showed up-regulation of Mucin2 (1.88), β-defensin-1 (1.49), LEAP2A (1.00), LEAP2B (0.49) and MBL (1.34) ($P < 0.05$), while MOS-600 mg/kg showed up-regulation of Hepcidin (1.24) ($P < 0.05$).

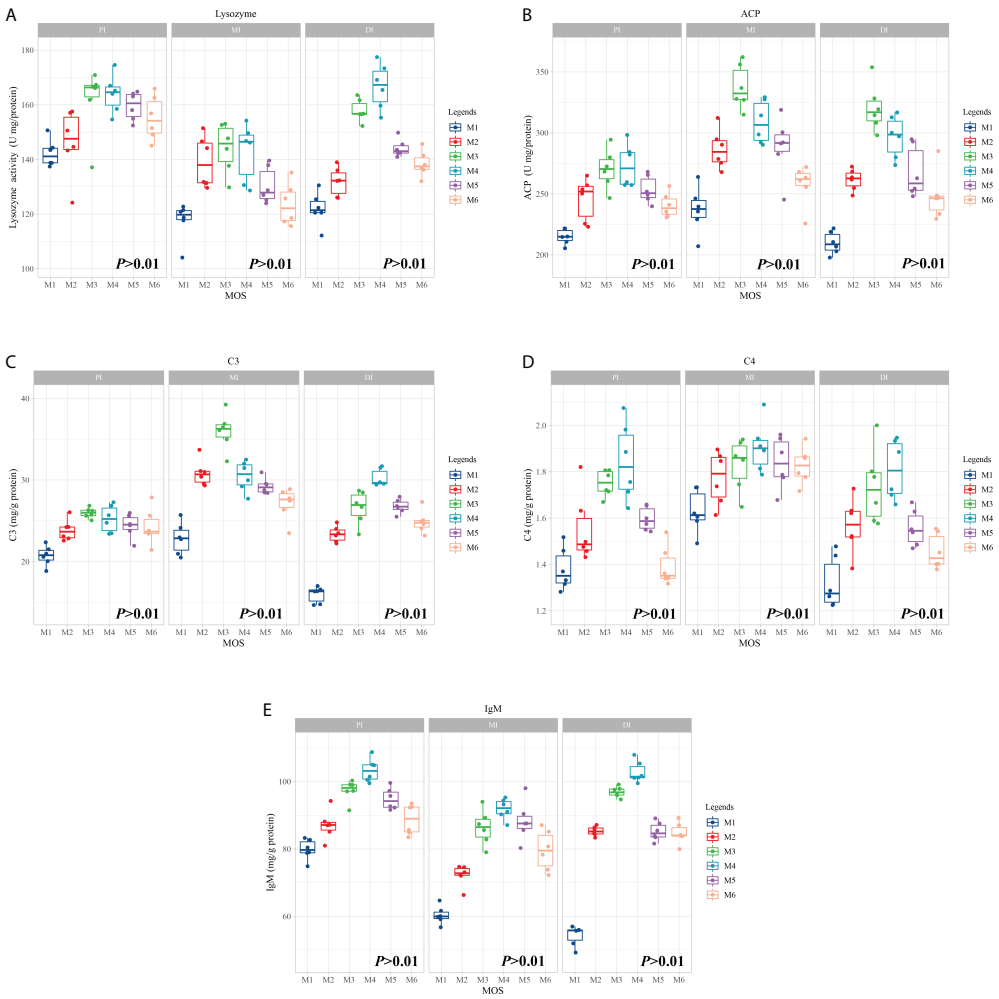

FIGURE 1
Effect of dietary MOS supplementation on immune barrier function in three intestinal segments of grass carp after infection of *Aeromonas hydrophila*. **(A–E)**, immune-related parameters, LZ, Lysozyme activity (U/mg protein); ACP, acid phosphatase (U/mg protein); C3, complement 3 (mg/g protein); C4, complement 4 (mg/g protein); IgM, immunoglobulin M (mg/g protein). N = 6 for each MOS level, different letters. The quadratic effects of MOS supplementation were assessed by using orthogonal polynomial contrasts.

Intestinal inflammatory cytokines-related gene expression

To explore the influence of MOS on intestinal immune response, multiple intestinal inflammatory cytokines-related genes were investigated. As shown in Figure 2, inflammatory cytokines (including pro- and anti-inflammatory cytokines) expression in the PI (except IL-4/13B), MI (except IFNγ2, IL-4/13A and IL-4/13B) and DI (except IL-4/13B) increased quadratically with increasing MOS levels ($P < 0.05$). The maximum fold change in inflammatory cytokines in the PI as compared to control was as follows: MOS-400 mg/kg showed down-regulation of IFNγ2 (0.26), IL-12p35 (0.57) and IL-17D (0.70) and up-regulation of IL-11 (0.93) and IL-4/13A (0.55) ($P < 0.05$); MOS-600 mg/kg showed down-regulation of TNFα (0.47), IL-1β (0.61), IL-12p40 (0.46) and IL-15 (0.38) and up-regulation of IL-8 (0.95), IL-10 (0.73), TGF-β1(0.93) and TGF-β2 (1.37) ($P < 0.05$); MOS-800 mg/kg showed down-regulation of IL-6 (0.71) ($P < 0.05$). In the MI, MOS-400 mg/kg showed down-regulation of TNFα (0.74) and IL-1β (0.6) and up-regulation of IL-8 (0.92) and IL-10 (0.76) ($P < 0.05$); MOS-600 mg/kg, showed down-regulation of IL-6 (0.58), IL-12p35 (0.47) and IL-17D (0.56) ($P < 0.05$); MOS-800 mg/kg showed down-regulation of IL-12p40 (0.48) and IL-15 (0.58) and up-regulation of TGF-β1 (0.68), TGF-β2 (0.53), IL-4/13A (0.89) and IL-11 (0.81) ($P < 0.05$). In the DI, MOS-400 mg/kg showed down-regulation of IL-6 (0.46), IL-12p35 (0.55) and IL-15 (0.2) and up-regulation of IL-8 (1.05), TGF-β1 (0.97), IL-4/13A (0.76) and IL-11 (0.69) ($P < 0.05$); MOS-600 mg/kg showed down-regulation of TNFα (0.58), IL-1β (0.47), IFNγ2 (0.54), IL-12p40 (0.28) and IL-17D (0.53) and up-regulation of IL-10 (1.07) and TGF-β2 (0.83) ($P < 0.05$). Hance, the three intestinal segments

FIGURE 2
Heatmap of MOS supplementation changed expression of immune-related parameters in three intestinal segments of grass carp after infection of *Aeromonas hydrophila*. The signal values of up-regulation (red) and down-regulation (blue) were expressed and ranged from 0.5 to 2.5 folds.

were significantly influenced by dietary MOS supplementation in terms of decreased pro-inflammatory cytokine gene expression and increased anti-inflammatory cytokine expression. However, MOS supplementation did not affect IL-4/13B (in the PI, MI and DI) and IFNγ2 (only in the MI).

Intestinal immunomodulatory key signaling molecules

To explore the influence of MOS on the regulative pathway of the intestinal inflammatory response, immunomodulatory key signaling molecule-related genes were investigated. As seen in Figure 2, the present study has found that intestinal immune response is closely related to NFκB and TOR signalling pathways after MOS supplementation. A quadratic relationship was found with increasing MOS levels ($P < 0.05$) in the NFκB and TOR signalling pathways-related molecules gene expression in the PI (except NFκBp52, IKKα, 4EBP2, TLR1, TLR4 and TRIF), MI (except NFκBp52, IKKα, 4EBP2, Raf, TLR4 and TRIF) and DI (except NFκBp52, IKKα, 4EBP2, TLR1, TLR4 and TRIF). The maximum fold change in signal molecular in the PI as compared to control was as follows: MOS-400 mg/kg showed down-regulation of c-Rel (0.58) and MAPK2α (0.47) ($P < 0.05$); MOS-600 mg/kg showed down-regulation of NFκBp65 (0.61), IKKβ (0.50), IKKγ (0.57), 4EBP1 (0.54), p38MAPK (0.62), Raf (0.73), MAPK6 (0.63), TLR2 (0.44), TLR5 (0.73), IRAK4 (0.63), IRAK1 (0.54) and TRAF6 (0.59) ($P < 0.05$); MOS-800 mg/kg showed down-regulation of MyD88 (0.58) ($P < 0.05$); MOS-600 mg/kg showed up-regulation of IκBα (0.74), TOR (0.73) and S6K1 (0.79) ($P < 0.05$). In the MI, MOS-400 mg/kg showed down-regulation of NFκBp65 (0.61), c-Rel (0.56), IKKγ (0.38),

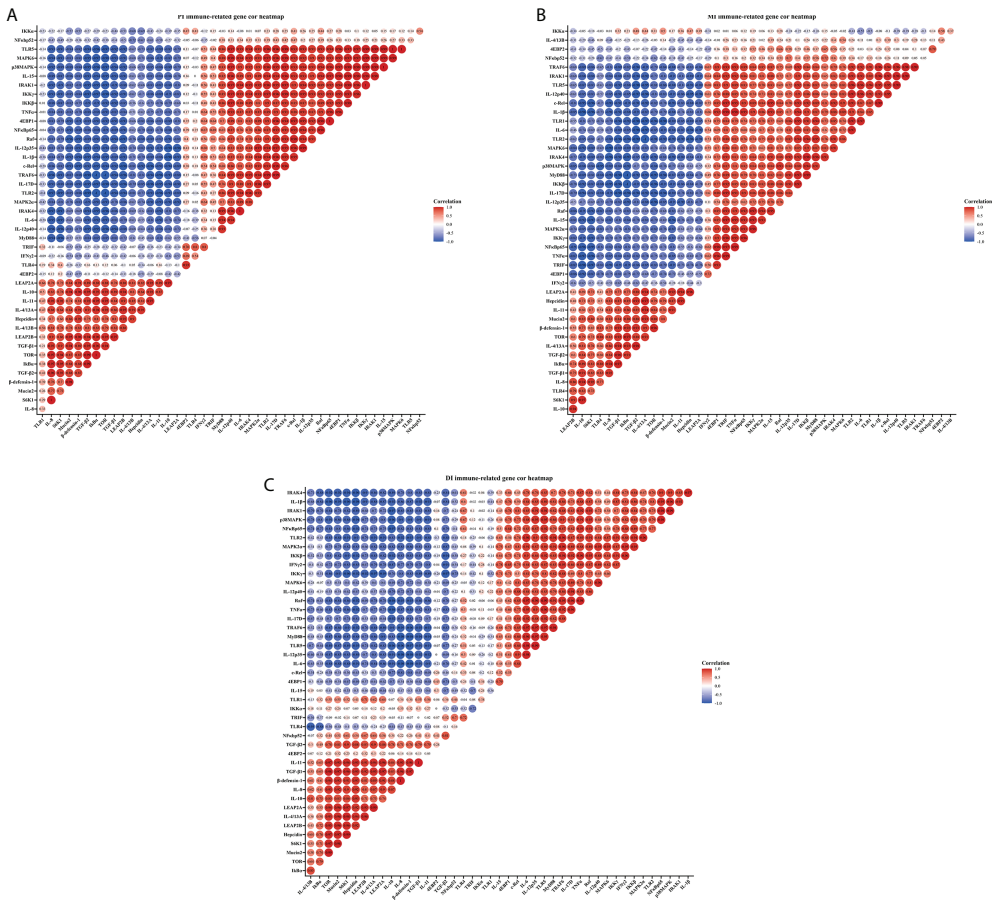

FIGURE 3
Correlation analysis of parameters in three intestinal segments of grass carp after infection of *Aeromonas hydrophila*. Proximal intestine **(A)**, Middle intestine **(B)**, and Distal intestine **(C)** of grass carp. R > 0.7, strong correlation; 0.5 < R < 0.7, moderate correlation; R < 0.5, weak correlation.

p38MAPK (0.58), Raf (0.43), MAPK6 (0.65) and IRAK4 (0.49) ($P < 0.05$); MOS-600 mg/kg showed down-regulation of IKKβ (0.57), 4EBP1 (0.56), MAPK2α (0.47), TLR1(0.32), TLR2 (0.81), TLR5 (0.72), MyD88 (0.45) and TRAF6 (0.66) ($P < 0.05$); MOS-800 mg/kg showed down-regulation of IRAK1 (0.82) ($P < 0.05$); MOS-400 mg/kg showed up-regulation of S6K1 (1.03) ($P < 0.05$); MOS-600 mg/kg showed up-regulation of IκBα (0.86) and TOR (1.14) ($P < 0.05$). In the DI, MOS-200 mg/kg showed down-regulation of c-Rel (0.49) ($P < 0.05$); MOS-400 mg/kg showed down-regulation of IKKγ (0.35), 4EBP1 (0.33), MAPK2α (0.35), MAPK6 (0.22), TLR2 (0.43), MyD88 (0.40) and TRAF6 (0.60) ($P < 0.05$); MOS-600 mg/kg showed down-regulation of NFκBp65 (0.37), IKKβ (0.43), p38MAPK (0.60), Raf (0.51), TLR5 (0.40), IRAK4 (0.39) and IRAK1 (0.53) ($P < 0.05$); MOS-400 mg/kg showed up-regulation of TOR (0.93) and S6K1 (0.71) ($P < 0.05$); MOS-800 mg/kg showed up-regulation of IκBα (0.53) ($P < 0.05$). However, MOS supplementation did not affect NFκBp52, IKKα, 4EBP2, TLR4 and TRIF in the PI, MI and DI, as well as TLR1 in the PI and DI.

Correlation analysis

Correlation analysis was performed to examine the relationship between the expression of immune-related genes involved in intestinal immune response and the signal molecules involved in regulation (Figure 3). These data showed that the studied antimicrobial peptides have a strong and moderate negative correlation with studied anti-inflammatory cytokines mRNA levels, but a strong and moderate positive correlation with studied pro-inflammatory cytokines mRNA levels ($R > 0.7$, strong correlation; $0.7 > R > 0.5$, moderate correlation; $R < 0.5$, weak correlation) in the three intestinal segments. Gene expression of the majority of studied pro-inflammatory cytokines revealed a strong and moderate positive correlation with NFκBp65 mRNA levels, whereas the majority of studied anti-inflammatory cytokines had a strong and moderate positive correlation with TOR gene expression in the three intestinal segments (except IL-4/13B in the MI and IL-15 in the DI). In addition, the mRNA levels of TLR2 and TLR5 showed a strong

and moderate positive correlation with a majority of pro-inflammatory cytokines in the three intestinal segments.

Immunohistochemical analysis of key role protein

Immunohistochemical analysis was performed after the bacterial challenge to observe how MOS supplementation affected intestinal epithelium (IRAK1, MyD88, TRAF6 and NFκBp65) in the three intestinal segments (Figure 4). The IOD of p-IRAK1, MyD88, TRAF6 and NFκBp65 were quantified using Image Pro Plus 6.0 software. In comparison with the control, in Figure 4A, the p-IRAK1 IOD displayed a declining trend among all intestinal tracts ($P < 0.05$) for the MOS-400 mg/kg group, but only in the PI and MI ($P < 0.05$) for the MOS-1000 mg/kg group, with the p-IRAK1 IOD in the MI not having obvious changes ($P > 0.05$); in Figure 4B, while the MyD88 IOD of MOS-400 mg/kg group displayed a declining trend among all intestinal tracts ($P < 0.05$), the MyD88 IOD of 1000 mg/kg group was only significantly decreased in the DI ($P < 0.05$), with no obvious changes in the PI and MI ($P > 0.05$); in Figure 4C, the TRAF6 IOD of MOS-400 mg/kg group decreased among all intestinal tracts ($P < 0.05$) but there was no significant difference for the 1000 mg/kg group ($P > 0.05$); in Figure 4D, the NFκBp65 IOD of the 400 mg/kg

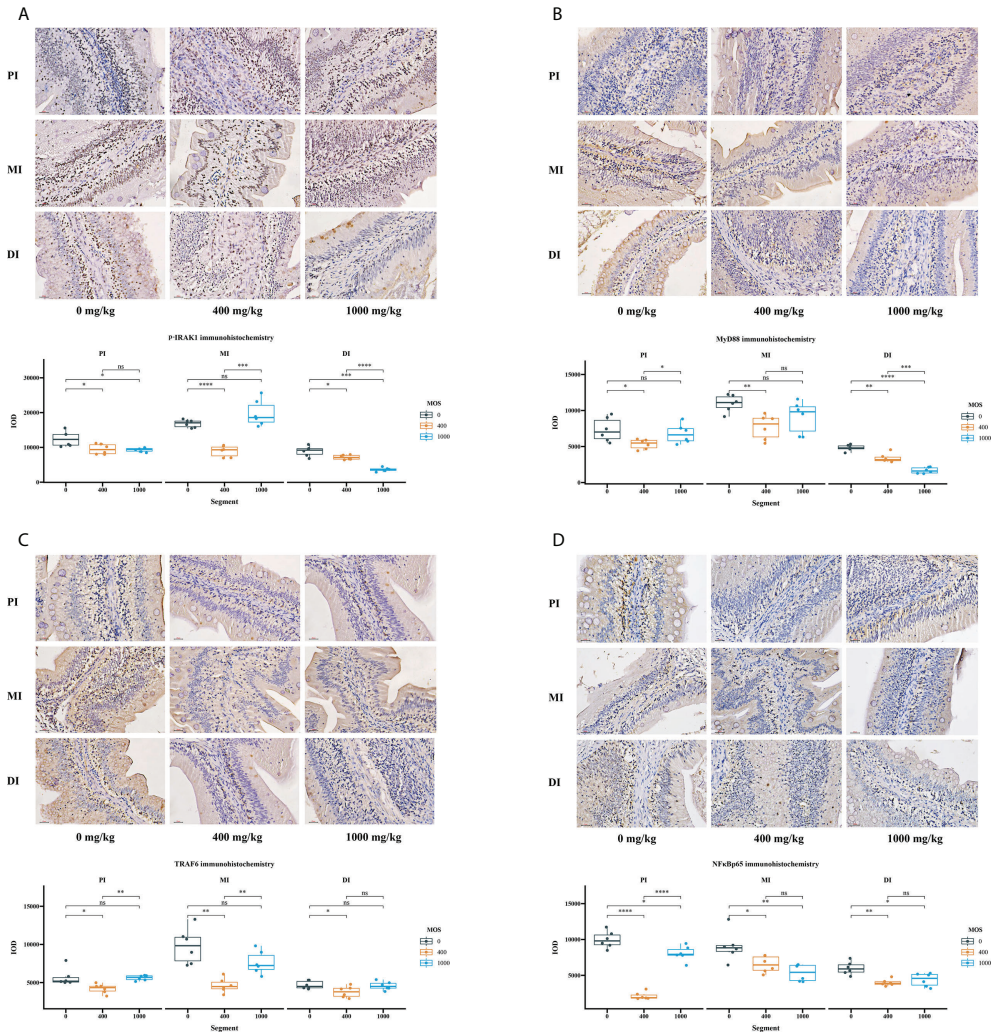

FIGURE 4
The effect of MOS on p-IRAK1, MyD88, TRAF6, and NFκBp65 expression by immunohistochemistry method in three intestinal segments after infection of *Aeromonas hydrophila*. **(A)** p-IRAK1 protein expression, **(B)** MyD88 protein expression, **(C)** TRAF6 protein expression, **(D)** NFκBp65 protein expression; Quantification of the positive area as revealed by Image Pro Plus 6.0. N = 6 for each MOS level. Differences among the variables were assessed using Student's t-tests. Statistical significance: *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; $^{ns}p > 0.05$.

group and 1000 mg/kg group showed a declining trend among all intestinal tracts ($P < 0.05$).

Key role protein levels of intestinal immune response

To determine how MOS affects the immune response in the intestine, several crucial signalling molecules (TOR, NFκBp65, TLR2, MyD88, IRAK1, and TRAF6) were investigated, respectively (Figures 5–7). In Figure 5, in comparison with the control, the NFκBp65 protein levels declined with MOS addition 800 mg/kg among all intestinal segments, and then gradually increased. In Figure 6, the p-TOR Ser 2448 protein levels in the PI, MI, and DI increased quadratically with increasing MOS levels ($P < 0.05$), while the T-TOR protein levels only increased quadratically in the PI and MI. In compare to the control, the p-TOR Ser 2448 protein levels were elevated with MOS addition (600(PI), 600(MI), and 800(DI) mg/kg) for all intestinal segments, before gradually decreased. As shown in Figure 7, with increasing levels of MOS, the MyD88 protein levels in the PI, MI and DI decreased quadratically ($P < 0.05$), and so did protein levels of TLR2, p-IRAK1 and TRAF6 in the PI and MI. Compared with the control group, the TLR2 protein levels declined with MOS supplementation up to 800 mg/kg in the PI, MI and DI, and then plateaued; the MyD88 protein levels declined with MOS supplementation up 600, 800, and 200 mg/kg in the PI, MI and DI, and then plateaued; the p-IRAK1 protein levels were declined with MOS supplementation up to 800, 600, and 1000 mg/kg in the PI, MI and DI respectively, and then plateaued (except in the DI); the TRAF6 protein levels declined with MOS supplementation up to 800, 600 and 1000 mg/kg in the PI, MI, and DI respectively, and then plateaued (except in the DI).

Discussion

The current study was based on the same growth trial used in our previous work in grass carp, which was part of a larger research to investigate the protective effect of MOS supplementation on fish intestinal barrier function (28). As prebiotic zootechnical feed ingredients, MOS derived from *Saccharomyces cerevisiae*'s outer cell wall has been extensively studied to replace antibiotics as a growth promoter and immunopotentiator (2). Among aquatic animals, *A. hydrophila*

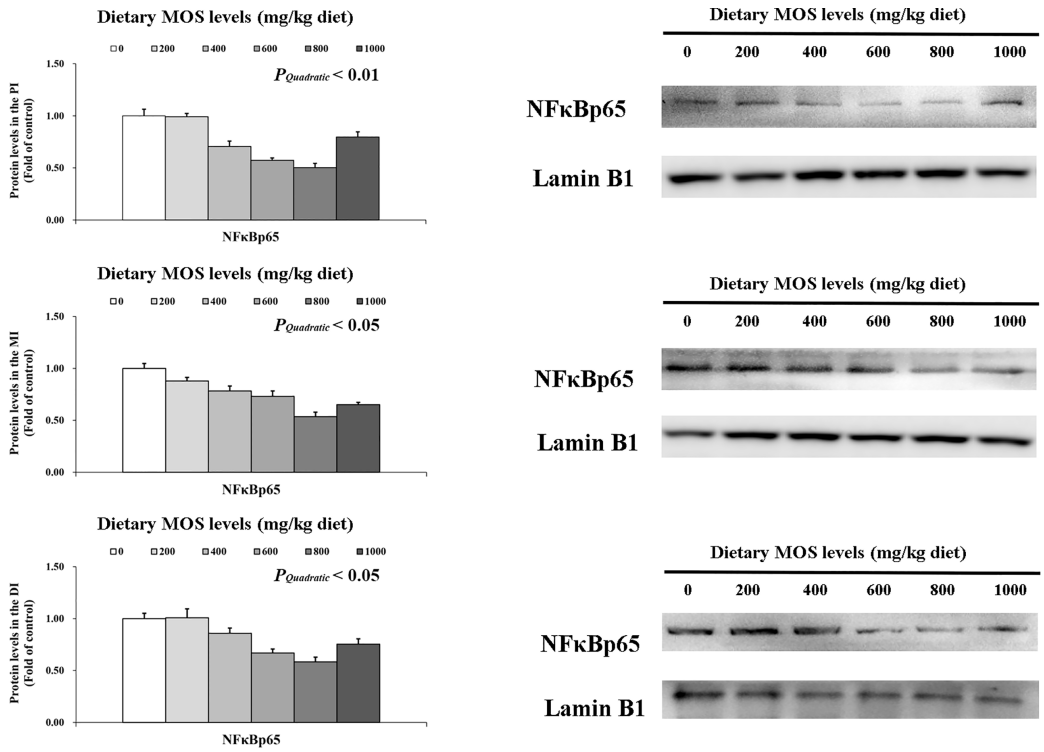

FIGURE 5
Western blot analysis of NFκBp65 protein levels in three intestinal segments of grass carp after infection of *Aeromonas hydrophila*. Data represent means of three fish in each group, error bars indicate S.D. The quadratic effects of MOS supplementation were assessed by using orthogonal polynomial contrasts.

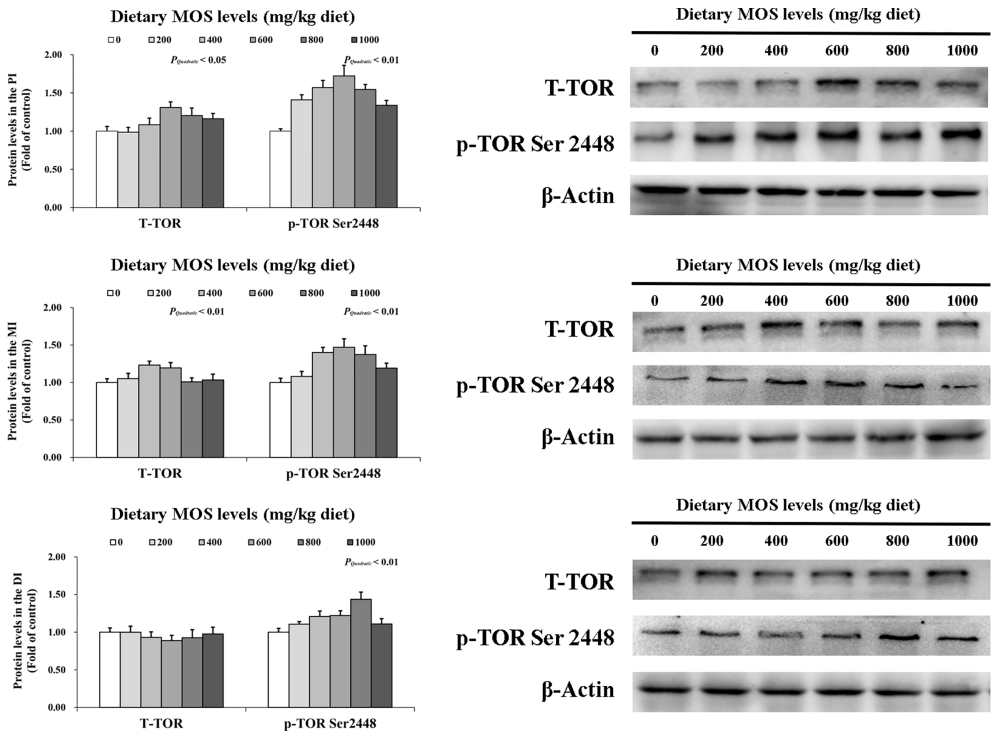

FIGURE 6
Western blot analysis of T-TOR and p-TOR Ser2448 protein levels in three intestinal segments of grass carp after infection of *Aeromonas hydrophila*. Data represent means of three fish in each group, error bars indicate S.D. The quadratic effects of MOS supplementation were assessed by using orthogonal polynomial contrasts.

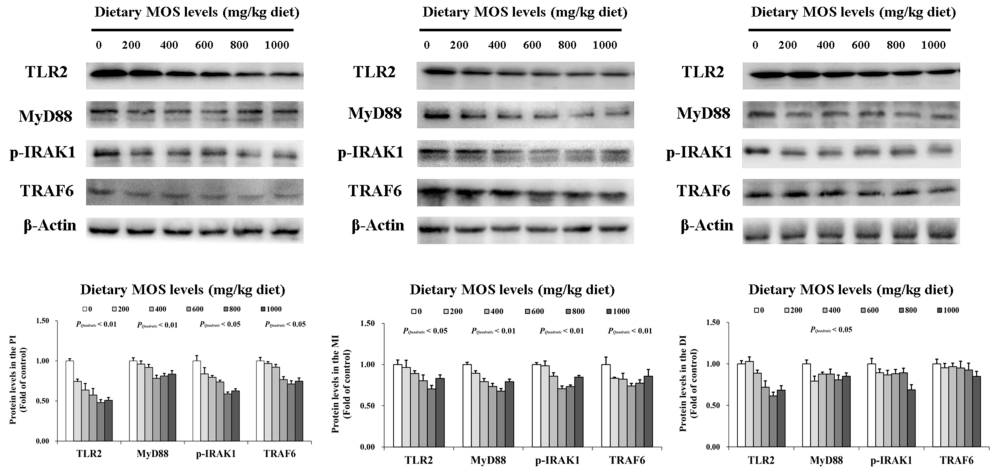

FIGURE 7
Western blot analysis of TLR2, MyD88, p-IRAK1, and TRAF6 protein levels in three intestinal segments of grass carp after infection of *Aeromonas hydrophila*. Data represent means of three fish in each group, error bars indicate S.D. The quadratic effects of MOS supplementation were assessed by using orthogonal polynomial contrasts.

is known for causing severe enteritis and damage to intestinal structures, which could be hazardous to intestinal health (31). According to our previous research, fish growth, intestinal antioxidant capacity, disease resistance, and structural integrity can be enhanced by optimal MOS addition after *A. hydrophila* infection (6, 28). Many researchers consider fish growth and development to be strongly correlated with intestinal barrier function at the moment (32, 33). Nevertheless, the intestinal immune barrier functions also a crucial role in maintaining normal intestinal barrier function (34). As we previously demonstrated, MOS can enhance intestinal antioxidant capacity and structural integrity when confronted with *A. hydrophila* (6, 28). However, these evidences do not provide a sufficient understanding of MOS's protection of intestinal barriers. Consequently, based on the previous works on physical barriers (antioxidant ability and structural integrity), we carried out further exploration of immune barriers and revealed potential molecular mechanisms. Firstly, we investigated the effects of MOS supplementation on antibacterial substances in the intestine of grass carp.

MOS promotes the production of antibacterial substances

As important physical and immune barriers, intestinal epithelial cells (IECs) prevent microbial communities from spreading from the lumen into other parts of the body (35). There is a close relationship between the fish immune barrier and antimicrobial substances, such as antimicrobial compounds and antimicrobial peptides. To our knowledge, similar to mammals, the IECs of bony fish are composed of multiple types of cells with different functions such as goblet cells, Paneth cells, absorptive enterocytes, enteroendocrine cells and so on (35, 36). The first line of defense against microbial invasion is the secretion of glycosylated mucins (Mucin2) from goblet cells to the intestinal tract (37). Other antibacterial substances such as lysozyme and antimicrobial peptides are secreted by Paneth cells to remove and kill pathogenic microorganisms exogenously (38), whereas ACP involves localization in the enterocytes to protect intestinal epithelial cells and releases superficial cells for sloughing (39). Additionally, the complement system recognizes pathogenic microorganisms and rapidly responds with a tailored defense response (40). IECs have been identified as primary producers of complement components across the gastrointestinal tract (41), whereas IgM is the only antibody that responds to pathogens in both the systemic and mucosal compartments, produced by lymphocytes in fish intestines (42).

The current study showed that the MOS-400 mg/kg group elevated LZ and ACP activities, and the C3, C4, and IgM contents, as well as up-regulated hepcidin, LEAP2A, LEAP2B, Mucin2 and β-defensin-1 mRNA levels in all three intestinal segments of grass carp. These data suggested that dietary MOS supplementation promotes the production of antibacterial substances in the intestine of fish. Our previous work has confirmed that different degrees of pathological changes (such as hyperaemia, edema, inflammatory cell infiltration, cell necrosis, etc.) occurred in the intestine of grass carp after being challenged by *A. hydrophila* (28). This indicated that following pathogen infection, the intestinal structural integrity was compromised, and excessive inflammation was triggered, which eventually resulted in IECs necrosis. As a result of the necrosis of IECs, many cells (such as enterocytes, goblet cells, Paneth cells, etc.) will lose their immune function (35, 43). We speculated that MOS could increase the content of antibacterial substances in the intestinal tract through multiple pathways. One such way was that, MOS would bind to pathogenic bacteria and prevent them from colonizing intestinal epithelium, thus protecting IECs. This was consistent with our previous results, in which the *A. hydrophila* population was significantly reduced and intestinal ultrastructure and apical junction complex were significantly improved (6, 28). Another possibility could be that MOS may activate the complement lectin pathway to promote a series of cascade reactions and eventually form a membrane-attacking complex to effectively kill pathogenic microorganisms (40). As we know, molecular patterns (MAMPs) that are associated with microbial growth include mannose-binding lectins (MBLs) that recognize mannose residues on cell surfaces (40). During pathogen invasion, MBL binds to mannose residues on pathogen surfaces and further activates the complement system (41). Our hypothesis was supported by the present study that showed that the expression levels of MBL were significantly up-regulated.

What is noticeable is that the optimum level for most antimicrobial substances is between 400 and 600 mg/kg MOS, before a plateau or gradual declines was observed with greater MOS supplementation. There may be an excessive effect caused by the excessive addition of MOS. It has been reported that MOS supplementation could increase the number of goblet cells in European sea bass (24). Another study has confirmed that abnormal goblet cell proliferation can produce excess mucus, leading to the occurrence of inflammation in LS174T cells (44). Our results also showed that the expression of the inflammatory cytokines in the high dose MOS group (1000 mg/kg) was significantly higher than that of the more optimal MOS groups (400-600 mg/kg), indicating that excessive MOS is not conducive to the recovery of intestinal homeostasis after the challenge. Although we have studied the effect of MOS on multiple antibacterial substances after the *A. hydrophila* challenge, more representative evidence of the immune barrier is needed to support it. As described above, the intestinal immune barrier is also closely associated with the inflammation response, which is primarily mediated by inflammatory cytokines (7, 15). Consequently, we next investigated the relationship between dietary MOS

supplementation and the inflammatory cytokines in the intestine of grass carp.

MOS attenuates the inflammatory response

The cellular components of the intestinal immune barrier include not only the largest proportion of IECs in the superficial layer (mucosal layer), but also the immune cells (such as macrophages, dendritic cells, adaptive, lymphocyte cells, etc.) with special functions in the inner layer (lamina propria) (35). When pathogens penetrate the mechanical barrier of the intestinal wall (including mucus layer, epithelium, and Apical junction complex), a secondary line of defense is initiated (immune cell dominance), triggering an immune response (45). Although almost all intestinal cells can secrete various cytokines, in bony fish, immune cells play a major role (7, 36). After receiving external and internal signals, immune cells contribute to the immune response by secreting pro-inflammatory cytokines (such as TNFα, IL-β, IL-6, and IL-8) and anti-inflammatory cytokines (such as TGF-β and IL-10) (15, 46). It was confirmed that down-regulating pro-inflammatory cytokines and up-regulating anti-inflammatory cytokines mRNA levels in fish could reduce excessive inflammatory response (46). In the current study, by examining the mRNA levels of multiple studied inflammatory cytokines, the data from all three intestinal segments showed that almost all of the studied pro-inflammatory cytokines were down-regulated (except INFγ2 in the MI) with optimal MOS supplementation (400-800 mg/kg), whereas studied pro-inflammatory cytokines anti-inflammatory cytokines were up-regulated (except IL-4/13B in all three segments) with optimal MOS supplementation (400-600 mg/kg). Similarly, MOS reduced TNFα and IL-6 expression in the intestine after *A. hydrophila* challenged in Blunt Snout Bream (*Megalobrama amblycephala*) (47). Thus, we believe that MOS has a positive prebiotic effect on the fish intestinal tract.

Interestingly, MOS supplementation could up-regulate IL-8 expression in all three intestinal segments. To our knowledge, IL-8 is a typical proinflammatory cytokine that is involved in the inflammatory response (48). This result, contrary to our expectations, seems to suggest an increase in local inflammation caused by MOS supplementation. Coincidentally, the results are not unique. It has been reported that MOS up-regulated IL-8 expression in the intestine after *A. hydrophila* challenged in Nile Tilapia (*Oreochromis niloticus*) (49). Another study also showed that the mRNA levels of IL-8 was up-regulated with MOS supplementation after infection (3). Although our results are consistent with these studies, there is no reasonable explanation for this abnormal phenomenon yet. Thus, we make the following assumptions based on some reliable literature. The up-regulation of IL-8 from MOS supplementation in the three intestinal segments of grass carp might be partially related to prostaglandins (PGs). A study on the European sea bass indicated that MOS could increase PGs content in the intestine (24). It was reported that PGs could up-regulate IL-8 mRNA levels in the human colonic epithelial cell (50), which may partly support our hypothesis. It is worth noting that MOS supplementation had no effect on the mRNA levels of IL-4/13B in the three intestinal segments of grass carp. A possible reason for this difference could be partially related to phosphorus digestibility. A study showed that MOS supplementation could increase phosphorus digestibility in the ileum of piglets (18). A previous study from our laboratory also confirmed that phosphorus does not affect IL-4/13B mRNA levels in the head kidney and spleen of grass carp (19), which may partly support our hypothesis. In addition, our data also showed that MOS had no significant effect on INFγ2 in the MI segments. However, existing literature is unable to provide any reasonable explanation, thus subsequent studies are needed to further explore and verify this. It needs to be emphasized that most of the inflammatory cytokines involved in the inflammatory response are typically modulated by classical immune-related signalling pathways (20, 21, 26). Thus, we next investigated the relationships between the dietary MOS supplementation and the immuno-related signalling pathway in the intestine of grass carp.

MOS regulates signalling pathways in inflammatory responses

In mammals, the mRNA levels of pro-inflammatory cytokines are regulated by IKK (including IKKα, IKKβ and IKKγ), which could suppress IκBα degradation, and then inhibit activation of NFκB (including NFκBp52, NFκBp65 and c-Rel) (51). In addition, in human PBMCs, the study showed that mTOR enhances the anti-inflammatory cytokine IL-10 activity (52). It was reported that mTOR could phosphorylate S6K1 and inhibits the initiation factor 4EBP to initiate the translation of distinct mRNAs in mammals (53). Based on the current research progress and the results of previous studies from our laboratory, it is certain that the function of these key inflammatory signalling molecules between fish and mammals is conserved (54). However, there are no reports on prebiotics involving signal molecules related to intestinal immune regulation of fish. To fill this gap in knowledge, we investigated gene expression and protein levels of key signalling molecules in the TOR and NFκB signalling pathways.

Our results found that optimal MOS supplementation down-regulated gene expression of NFκBp65, c-Rel, IKKβ and IKKγ, and it up-regulated IκBα gene expression in all three intestinal segments. Further correlation analysis showed that these pro-inflammatory cytokines (except IFN-γ2 in the MI) gene expression were positively correlated with NFκBp65 and c-Rel mRNA levels, but had a negative correlation with IL-8 mRNA levels in the three intestinal segments. Furthermore,

the mRNA levels of signal molecules NFκBp65, c-Rel, IKKα, IKKβ and IKKγ were negatively correlated with IκBα mRNA levels. In addition, protein levels of NFκBp65 in the three intestinal segments were significantly reduced with MOS supplementation, which further indicated that the NFκB pathway was effectively inhibited. These results indicate that MOS supplementation down-regulated the mRNA levels of the studied pro-inflammatory cytokines (except *IL-8* and IFN-γ2 (in the MI)), which may be partly related to (IKKβ and IKKγ)/IkBα/(NFκBp65 and c-Rel) signalling pathway in the three intestinal segments of fish. Interestingly, we found that dietary MOS supplementation did not affect NFκBp52 and IKKα mRNA levels in the three intestinal segments of grass carp. There could be several possible reasons for these differences. First, MOS supplementation did not affect NFκBp52 mRNA levels and this might be partially associated with IKKα levels also not being affected. A study on human lung carcinoma cells indicated that IKKα could activate NFκBp52 mRNA levels (55). In the present study, since MOS supplementation did not affect IKKα in the intestine, this may partially support our speculation. Second, MOS supplementation did not affect IKKα mRNA levels possibility due to its association with *E. coli* and PKC-ζ. Our previous work showed that MOS could decrease the number of *E.coli*, which are mainly located in the intestinal tract (28). In human HT-29/B6 cells, it has been reported that *E.coli* could increase the protein level of PKC-ζ (56). Another study on rat Kupffer cells showed that PKC-ζ could activate IKKβ and IKKγ but had no effect on IKKα expression (57), thus possibly explaining our observations. In addition, our results showed that studied anti-inflammatory cytokines were up-regulated and positively correlated with TOR mRNA levels with MOS supplementation. Furthermore, the protein levels of p-TOR Ser 2448 in the three intestinal segments were significantly increased with MOS supplementation, which further indicates that the TOR pathway was effectively activated. This result is consistent with the other study in juvenile hybrid grouper intestine that showed up-regulation of TLR3 with MOS supplementation (27). One interesting aspect is that dietary MOS supplementation could down-regulate mRNA levels of 4EBP1 but not 4EBP2 in three intestinal segments, which might be partly due to butyrate and p38MAPK. A study on weaned rabbits indicated that MOS could increase butyrate content in the caecal (58). A previous study from our lab confirmed that butyrate could activate p38MAPK (23). It was reported in humans that activation of p38 MAPK could down-regulate the expression of 4EBP1 but have no effect on the expression of 4EBP2 (59), thus partially supportting our hypothesis. These results indicate that MOS supplementation up-regulated anti-inflammatory cytokines (except 4EBP2) mRNA levels, which may be partly related to TOR/S6K1/4EBP (not 4EBP2) in the three intestinal segments.

In light of the above evidence, it has been established that MOS regulates inflammation primarily by inhibiting the NFκB and TOR pathways. Nevertheless, the mechanism by which mannan oligosaccharides activate these classical pathways is unknown. As far as we know, molecular patterns associated with pathogens (PAMPs) could be recognized by PRRs and consequently stimulate immunity and defense (60). As one of the classic PRRs of immune activation, TLRs have become a focal point of immune response research, which could activate NFκB by a cascade of reactions (26). Therefore, we focused on pattern recognition receptors and selected several TLRs (TLR1, TLR2, TLR4 and TLR5) that are closely related to pathogen interaction for preliminary exploration. The present data displayed that MOS supplementation down-regulated TLR1 (in the MI), TLR2, TLR5 and the downstream related signal molecules (MyD88, IRAK1, IRAK4 and TRAF6) in the three intestinal segments. The results of correlation analysis also show that the TLRs signalling pathway was positively correlated to most of the studied pro-inflammatory cytokines. Our results are similar to the previous study in Ussuri catfish (*Pseudobagrus ussuriensis*), which showed that in the intestine, the gene expression of TLR2 and MyD88 were down-regulated with yeast culture (the main component is MOS) supplementation after being *A. hydrophila* challenged (61). Interestingly, some previous studies involving growth trials of other animals yielded different findings, for instance, in juvenile hybrid grouper, TLR3 was up-regulated (27), and in chickens, both TLR2 and TLR4 gene expressions were up-regulated following MOS supplementation (62). We speculate that the main possible reason for this interesting phenomenon is related to challenge stress. Both studies on hybrid grouper and chickens did not involve a bacterial challenge, whereas the current experiment involved on. Based on previous studies, we found that MOS is an excellent immunostimulant, and the optimal level of addition can maintain a sensitive immune status under normal circumstances, which were validated in multiple bony fish studies (2, 63). However, *A. hydrophila* usually causes excessive intestinal inflammation in fish (1), and our data suggest that MOS supplementation can reduce such a symptom in the intestine of fish. In addition, our study also found that MOS had no significant effect on TLR4 in the three intestinal segments. These results were beyond our expectations. Generally, TLR4 plays a key role in intestinal pattern recognition (recognizing bacteria lipopolysaccharides) during bacterial infection in mammals (26). However, unlike mammals, TLR4 does not recognize LPS in fish (64). This may partly explain why TLR4 expression was not significant in the intestine. However, the specific mechanism involved is not clear, which is worthy of further study in future work. To our knowledge, as a downstream signal molecular, TRAF6 could activate the NFκB signalling pathway by activating IKKβ (26, 65). Our result also showed that TRAF6 has a strong correlation with IKKβ, IKKγ, and NFκB, which partly explains how MOS inhibits TLRs-mediated excessive inflammatory signalling. In conclusion, we believe that the inhibiting effect of TLRs pathway by MOS may

be related to intestinal immune responses under *A. hydrophila* infected condition, but there are still some limitations to the current study, which requires further verification by more experiments. However, it is notable that the existing studies do not investigate the mechanism by which MOS affects TOR signals. A surprising finding has been that PI3K and Akt regulate TOR, and these molecules are also regulated by TLR signals (66). It is contradictory for MOS to inhibit TLRs signal and activate TOR signal. We hypothesized that since intestinal immune barrier function is the result of simultaneous interaction of multiple intracellular communication, there must be crosstalk between other signalling pathways of different signalling molecules leading to different signalling mechanisms. A study in weaned pigs showed that MOS could up-regulate GLUT-2 mRNA levels in the duodenum (67). Another study on mice showed that GLUT-2 has a cooperative mechanism with Akt/mTOR/S6K1 signalling pathway in pancreatic beta cells (68). Our results also showed MOS activated TOR signal, which partially supports our hypothesis. Nevertheless, we still lack an understanding regarding the mechanism. Therefore, we expect to use next-generation sequencing and cell culture-related technologies in the future to reveal how MOS regulates intestinal immune responses through specific subtypes and related signalling pathways in the TLRs family and TOR signal.

Difference in immunological effect of MOS on different organs

We found several two interesting findings when comparing the results of intestinal immunological effect in this study to those of previous studies from our lab on the multiple functional organs (head kidney, spleen, and skin) (4, 5). The content of antimicrobial substances and the expression of inflammatory cytokines in the intestine showed a similar overall trend compared with the head kidney, spleen, and skin, but it was obvious that the content of LZ and ACP in the intestine was significantly lower than that in the head kidney and spleen. The reason for this phenomenon may be related to organ structure. To our knowledge, the head kidney, and spleen of fish contain a large number of immune cells (monocytes), which can produce a large number of antimicrobial substances (LZ and ACP) and inflammatory cytokines when immunologically stimulated (69, 70), which were supporting our hypothesis. Secondly, we also found that MOS had different effects on the expression level of IL-8 in different organs of grass carp after challenged, which was up-regulated in the intestine, insignificant in the head kidney, and spleen, and significantly down-regulated in the skin (4, 5). We speculate that the possible reason for this phenomenon is related to the specific expression of PGs in the intestine. As mentioned above, MOS can increase the intestinal PGs content of European sea bass (24), which can increase the expression of IL-8 in human colonic epithelial cells (50), thus supporting our hypothesis. In addition, studies on greater amberjack also found that different types of MOS could enhance disease resistance by regulating inflammatory cytokines in multiple organs under parasite infection conditions (3). However, this was not completely consistent with our results. In conclusion, we believe that the immunological response of MOS to different organs is related to many factors in fish, including fish species, feeding time, dosage, pathogen, and different structure of functional organs.

Conclusions

Overall, this study presents clear evidence that dietary MOS enhanced grass carp immune barrier function after infection with *A. hydrophila*. Based on the results of our study, dietary MOS supplementation improves the immune barrier function of the intestine in several ways as follows: (1) dietary MOS supplementation could increase intestinal disease resistance, producing more antibacterial compounds and immunoglobulins. (2) dietary MOS supplementation could regulate the dynamic balance of inflammatory cytokines, inhibiting the excessive inflammatory response. (3) MOS supplementation improved fish immune barrier function may partly be related to modulating TLRs/MyD88/NFκB and TOR/S6K1/4EBP signalling pathways.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The animal study was reviewed and approved by Animal Care Advisory Committee of Sichuan Agricultural University.

Author contributions

Z-YL performed formal analysis, investigation and writing original draft. LF performed conceptualization, funding acquisition and supervision. W-DJ performed data curation, validation, project administration and writing review & editing. PW performed conceptualization, methodology, validation, data curation and project administration. YL and JJ performed project administration. S-YK, LT, S-WL, C-BZ performed resources. X-QZ performed conceptualization, designed experiment, supervision and funding acquisition.

All authors contributed to the article and approved the submitted version.

Funding

This research was financially supported by National Key R&D Program of China (2019YFD0900200 and 2018YFD0900400), National Natural Science Foundation of China for Outstanding Youth Science Foundation (31922086), the Young Top-Notch Talent Support Program, Supported by China Agriculture Research System of MOF and MARA (CARS-45), and supported by Sichuan Science and Technology Program (2019YFN0036). The authors would like to thank the personnel of these teams for their kind assistance.

Conflict of interest

Authors S-YK, LT, S-WL, C-BZ were employed by Sichuan Animtech Feed 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1010221/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 15 September 2022
DOI 10.3389/fimmu.2022.997985

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Comparative Immunology, a section of the journal Frontiers in Immunology

RECEIVED 12 August 2022
ACCEPTED 29 August 2022
PUBLISHED 15 September 2022

CITATION
Wang M, Chen Z, Wang Y, Zou J, Li S, Guo X, Gao J and Wang Q (2022) Largemouth bass (*Micropterus salmoides*) exhibited better growth potential after adaptation to dietary cottonseed protein concentrate inclusion but experienced higher inflammatory risk during bacterial infection. *Front. Immunol.* 13:997985. doi: 10.3389/fimmu.2022.997985

Largemouth bass (*Micropterus salmoides*) exhibited better growth potential after adaptation to dietary cottonseed protein concentrate inclusion but experienced higher inflammatory risk during bacterial infection

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Cottonseed protein concentrate (CPC) has been proven to partially replace fishmeal without adverse effects on fish growth performance, while little information is known about the effects on liver health during bacterial infection. In the present study, 15% CPC was included into the diet of juvenile largemouth bass (32.12 ± 0.09g) to replace fishmeal for 8 weeks, with fish growth potential and hepatic inflammatory responses during *Nocardia seriolae* (*N. seriolae*) infection systemically evaluated. After adaptation to dietary CPC inclusion, largemouth bass even exhibited better growth potential with higher SGR and WGR during the last three weeks of whole feeding trial, which was accompanied with higher phosphorylation level of TOR signaling and higher mRNA expression level of *myogenin* (*myog*). At the end of 8-weeks feeding trial, the histological structure of largemouth bass liver was not significantly affected by dietary CPC inclusion, accompanied with the similar expression level of genes involved in innate and adaptive immunity and comparable abundance of T cells in bass liver. *N.seriolae* infection induced the pathological changes of bass liver, while such hepatic changes were more serious in CPC group than that in FM group. Additionally, RT-qPCR results also suggested that largemouth bass fed with CPC experienced much higher inflammatory potential both in liver and gill during *N. seriolae* infection, which was accompanied with higher expression level of genes involved in pyroptosis. Therefore, this study demonstrated that the application of CPC in largemouth bass diet should be careful, which may induce higher inflammatory potential during *N. seriolae* infection.

KEYWORDS

cottonseed protein concentrate, largemouth bass, liver health, Nocardia seriolae, inflammation

Introduction

Aquaculture supplies plenty of high protein food for human beings and has become the fastest animal-protein producing industry among all animal husbandry in the past 30 years (1). Feed expenses have always been the main cost for fish farmers, and fish meal has been traditionally regarded as the highest quality protein raw material for aquafeed due to its high-quality amino acid ratio, good palatability and perfect digestibility (2). However, global fishmeal production cannot reach a stable increase like the aquaculture production and the fishmeal price has significantly increased year by year (3). It is important to search for alternative protein sources to reduce fishmeal utilization in fish diet, and soybean meal has been widely used in advanced aquafeed because of its high protein content and balanced amino acid composition (4). In China and other main aquaculture countries, the soybean self-production capacity is relatively weak and the global soybean application has been pledged to be restricted which are in the expense of deforestation. Thus, it has become an industry consensus to develop new raw materials and reduce the use of fish meal and soybean meal in aquafeed. Many protein sources have also been tested in fish diet, including rapeseed meal, peanut meal and many others (5). However, the main constraint for these alternative sources remains in their much lower production than soybean meal. Cotton is one of the most important crops in the world, with large output but low production cost, which makes the price of cotton products relatively low (6). During the process of cotton production, a large number of by-products such as cottonseed can be produced, and developing them as feed protein raw materials not only has important economic value, but also has important ecological significance for reducing carbon emissions. With cottonseed as the basic raw material, cottonseed protein concentrate (CPC) is produced through low-temperature oil extraction, removal of gossypol and other process stages, based on the "liquid-liquid-solid" extraction technology production processes. The crude protein level of CPC could reach up to 60%, which is rich in arginine, leucine, phenylalanine and other essential fish amino acids. Additionally, some water-soluble non-starch polysaccharides, oligosaccharides, and anti-nutritional factors such as phenol and tannin has been removed to significantly improve its nutritional value and palatability (7). CPC has been proved to effectively replace partial FM without adverse effects on growth of silver sillago (*Sillago sihama*), golden pompano (*Trachinotus ovatus*), hybrid grouper (*Mycteroperca tigris* × *Epinephelus lanceolatus*) (8), red drum (*Sciaenops ocellatus*) (9), and rainbow trout (*Oncorhynchus mykiss*) (10).

With the urbanization, the demand for high-nutritional value and bone-less fish species is significantly increased in many countries including China (11). Largemouth bass (*Micropterus salmoides*), a carnivorous fish species originating from Canada and America, has been widely cultured all over the world. Ever since it was introduced to China in 1980s, the aquaculture production of largemouth bass has significantly increased year by year and has reached 619, 519 tons in 2020 (12). During aquaculture, the liver health of largemouth bass has received much attention. Liver structure was reported to be significantly affected by dietary glucose level (13). Additionally, *N. seriolae* infection, which is rather common in largemouth bass aquaculture, also caused serious hepatic damage (14). In fact, liver receives dual blood supply from the portal vein and hepatic artery, and both acts as the metabolic center and performs immune-regulatory roles (15). The liver is constantly bombarded by a stream of dietary and commensal bacterial products with inflammatory potential even in healthy individuals, which results in persistent, regulated inflammation (16). Such inflammation creates the potential for excessive immune activation during challenge by pathogens or tissue damage, which could be resolved during appropriate immune activation to maintain liver homeostasis. Failure to clear 'dangerous' stimuli or regulate appropriately activated immune mechanisms leads to chronic pathological inflammation and disrupted tissue homeostasis characterized by the progressive development of fibrosis, cirrhosis and eventual liver failure (17). Especially, during fishmeal replacement by plant proteins, the existing anti-nutritional factors in plant sources would be transferred to liver, which may cause continual inflammatory stimulus to liver and even immune fatigue in liver. When fish was exposed to pathogenic infection, the chronic pathological inflammation may occur which results in the liver failure. In largemouth bass, CPC has been reported to replace fishmeal at different ratios in different studies without adverse effects on fish growth performance (18–20). However, the immune status and tissue health have been rarely evaluated, especially the immune status during bacterial challenge.

As mentioned above, *N. seriolae* infection seriously affected the health of largemouth bass and resulted in the damage of internal organs including liver and eventual high mortality (14). *N. seriolae* infection has been reported to induce high inflammatory responses in bass liver (21) while little information is known about the metabolic control of such inflammatory responses in liver. Considering the high production of CPC and its application potential in diet for largemouth bass, it is important to study the influences of CPC inclusion on liver structure and function, especially the inflammatory responses during bacterial infection. Thus, the influences of dietary cottonseed protein concentrate replacing fishmeal on growth potential, liver health status and challenge resistance of largemouth bass (*Micropterus salmoides*) were systemically evaluated in the present study. To our acknowledge, this is the first study to evaluate the effects of CPC on fish hepatic inflammatory responses during nocardiosis.

Materials and methods

Ethics statement

All the fish care and experimental procedures were approved by the Animal Experiment Committee of Huazhong Agricultural University (permit number HZAUFI-2016-007).

Feed formulation, fish husbandry and sampling

Two isoproteic (50%) and isolipidic (12.5%) diets were formulated based on fish meal (FM) or cottonseed protein concentrate (CPC), respectively (Table 1). In order to balance the amino acid composition, krill meal, blood cells, wheat gluten and corn gluten meal were also included in each diet. Attractant (betaine) and taurine were also added to improve the food palatability.

Juvenile largemouth bass were acclimated to our laboratory conditions and experimental feeds for two weeks before being randomly distributed into fibre-glass tanks of 400-L capacity. Experiment was done in wet lab of College of Fisheries, Huazhong Agricultural University. Fish with initial weight of 32.12 ± 0.09 g were distributed into 6 experimental tanks (400-L) which were connected to a circulating water system. The water parameters in circulating water system were kept constant with water flow at 0.5 L/min, water temperature at 26 ± 2 °C, and oxygen content of outlet water at over 85% saturation. Day length and dark ranged over the course of the trial following natural changes. Each diet was randomly allocated to triplicate groups of fish for 8 weeks. Feed was offered by hand to apparent satiety twice per day, with feed consumption was recorded. After 2 weeks, 5 weeks and 8 weeks, all fish were counted and group-weighed under moderate anaesthesia (3-aminobenzoic acid ethyl ester, MS 222; 100 μg/mL) for growth parameters calculation.

At the end of the feeding trial, total fish number and body weight in each tank were counted and measured after being anesthetized by MS222, respectively. Blood samples were collected from the caudal vessels of 9 fish from each group with heparinized syringe. Then fish were perfused with PBS to discard the inference of blood on liver and gill immune responses. Liver and gill tissues were separated, with partial segments stored in paraformaldehyde. Other tissue samples (serum, gill, liver and others) were immediately frozen in liquid nitrogen and then stored at -80°C for further assay.

Bacterial challenge

N. seriolae strain HG2101, was isolated from an infected largemouth bass and preserved in our laboratory. The whole genome sequence has been sequenced by our laboratory to confirm the accurate (unpublished). Before the formal experiment, a preliminary study was conducted to determine the LD50 of this bacterial strain in largemouth bass. Thus the infection concentration in the formal experiment was finally determined as 1.0×10^7 CFU/mL. Before bacterial challenge experiment, the *N. seriolae* was taken out from −80°C refrigerator and recovered on plate medium at 28°C. Then the single colony was cultured in a tryptic soy broth soybean-casein digest medium (pancreatic digest of casein 17.0 g L^{-1}, papaic digest of soybean 3.0 g L^{-1}, dextrose 2.5 g L^{-1}, sodium chloride 5.0 g L^{-1}, dipotassium phosphate 2.5 g L^{-1}) in a shaker incubator at 180 rpm at 28°C. The colony forming units per milliliter (CFU/mL) in the final culture were measured by plate counting under a microscope. Bacterial suspensions at 1.0×10^7 CFU/mL were prepared in normal saline.

For the challenge experiment, largemouth bass in all groups were firstly anaesthetized with MS 222 and then artificially injected with the *N. seriolae* (0.10 mL) by intraperitoneal injection. After challenge experiment, all fish was transferred back to each tank with the same water conditions. Fish samples were obtained from FM and CPC group at 0 day, 1 day, and 3 days post infection (dpi). Fish sampling methods were similar to previous in 2.3.

TABLE 1 Formulation and proximate chemical composition of the tested diets (% dry matter).

	FM	CPC
Fish meal	48	35
Cottonseed protein concentrate	0	15
Krill meal	3	3
Wheat gluten	4	4
Blood meal	2	2
Corn gluten meal	5	5
Soybean meal	0	0
Wheat meal	6	6
Cassava starch	6	6
Beer yeast	2.5	2.5
Bentonite	13.1	9.8
Fish oil	4	5
Lecithin high potency	2	2
Vitamin mineral premix	2	2
Alanine	1.5	0.5
Taurine	0	1
Betaine	0.5	0.5
Choline chloride	0.25	0.25
Calcium bis	0	0.3
Calcium Propionate	0.1	0.1
Ethoxyquin	0.05	0.05
Proximate composition		
Crude protein	47.15	47.35
Crude fat	10.68	10.86

Histological assay

The liver and gill tissues of largemouth bass in two groups at different time points post infection were fixed with polyformaldehyde. After fixing, the tissues were transfered into gradient alcohol for dehydration, xylene for transparent, and then paraffin for section slicing. The obtained 5-μm sections of liver tissues were then used for H.&E. staining, following the producers' introduction. The stained slides were examined under a light microscope (Olympus, DP72) equipped with a camera (Nikon E600) and CellSens Standard Software (Olympus) for image acquisition.

TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was conducted to precisely detect cells experiencing programmed cell death (PCD) with the one-step TUNEL Assay kit (Beyotime, China) following the manufacturer's instructions. Briefly, tissue sections were twice dewaxed in xylene for 5-10 minutes. Then tissues were immersed into anhydrous ethanol for 5 minutes, 90% ethanol for 2 minutes, 70% ethanol for 2 minutes, and distilled water for 2 minutes. Tissues were treated with DNase-free proteinase K (Beyotime, 20μg/mL) at room temperature for 15-30 minutes and then washed with PBS for three times to remove excess proteinase K. Afterwards, the labelling solution containing terminal deoxynucleotidyl transferase, buffer, and fluorescein dUTP was added to tissues for labelling reaction at 37 °C for 60 minutes in a humidity chamber. Following incubation, excess labelling solution was washed off smears with PBS, and then cell smears were mounted with fluorescent microscopy mounting solution. Images were captured and analyzed using a CCD camera (Olympus, Japan).

RNA extraction and RT-qPCR analysis

Total RNA samples were extracted from liver and gill tissues using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. The purity and concentration of RNA were detected with Nanodrop 2000 spectrophotometer (Thermo scientific, USA), accompanied with the integrity of RNA checked by 1.0% agarose gel electrophoresis. One microgram of the resulting total RNA was reverse transcribed into cDNA using the SuperScript III RNaseH-Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) and oligo dT primers (Promega, Charbonnie `res, France) according to the manufacturers' instructions.

Quantitative RT-PCR was carried out on an iCycler iQTM real-time PCR detection system (BIO-RAD, Hercules, CA, USA) using the Eva Green 2 × qPCR Master mix (ABM, Canada). The program was as follows: 95 °C for 5min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. In addition, a melt curve analysis was performed after amplification to verify the accuracy of each amplicon. 18S was employed as a non-regulated reference gene and no changes in 18S gene expression were observed in our investigations (data not shown). The relative quantification of the target genes was determined *via* the comparative CT method ($2^{-\Delta\Delta Ct}$ method). Primer used in the present study was shown in Table 2.

Western blotting analysis

Proteins from liver homogenates were separated on SDS-PAGE. For each assay, all samples were analyzed at the same time on four-wide gels to eliminate inter-assay variation. Proteins were electrophoretically transferred to polyvinylidene difluoride transfer membranes (Pall Corporation), which were incubated with appropriate primary antibodies, washed and exposed to an appropriate secondary antibody. Blots were developed using an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology) and visualized. Primary antibodies for Phospho-S6 (Ser235/236) (cat # 4856) and S6 (cat # 2217) used in the present study were purchased from Cell Signaling Technology (U.S.A.) and primary antibody for β-actin (AC004) was purchased from ABlonal Biotechnology (China).

Immunofluorescent assay

In order to detect T cells, liver paraffin sections were stained with Alexa Fluor 647 anti-Lck (pAb, 1: 900) overnight at 4°C. After washing four times, all sections were stained with DAPI (4′,6-diamidino-2-phenylindole, 1 μg/mL, Invitrogen) before mounting. Images were captured and analyzed using a CCD camera (Olympus, Japan).

Calculations and statistical analysis

Mean final weight (MFW, g) = total final weight/fish number; Weight gain ratio (WGR, %) = 100*(final body weight – initial body weight)/initial body weight; Specific growth rate (SGR, %/day) = 100* (ln final body weight – ln initial body weight)/days; Feed intake ratio (FI, %/day) = 100 *dry feed intake/[(initial body weight + final body weight)/2]/days; Feed conversion ratio (FCR) = dry feed intake/(final body weight – initial body weight).

All statistical analyses were performed using SPSS 17.0. Data about fish growth performance, feed utilization and western blotting were analyzed using student's t-test. All gene-expression results were tested for normality using the Shapiro Wilk's-W's test, and normally distributed data were analyzed by Factorial (two-way) analysis of variance (ANOVA) to determine the main effects of period and group, and their interactions on gene expression. When significant interaction of period and group were observed, data were analyzed by one-way ANOVA followed by Tukey's multiple range tests to inspect differences among all the treatments. When only a significant interaction

TABLE 2 Primers used in the present study.

Gene Name	Abbreviation	Gene ID	Forward Sequence	Reverse Sequence
18S ribosomal RNA	*18s*	119916718	GCAAAGCTGAAACTTAAAGGAATTG	TCCCGTGTTGAGTCAAATTAAGC
myocyte enhancer factor 2a	*mef2a*	119893304	GCAGTTTGGGGAAGGTCATA	GAGCTGATGCCGGAGTAGAC
myocyte enhancer factor 2b	*mef2b*	119900933	GCACCAACACGGACATACTG	TTCTCACAGCCTGCAGACAC
myogenin	*myog*	119886574	AGTTGGGGTGACAGGAACAG	GCCTCGTTCACCTTCTTCAG
myogenic differentiation 1	*myod1*	119894171	TGCCGCTGATGATTTCTATG	GAGGAGGAAGAGGAGGAGGA
myostatin a	*mstn*	119900535	TTTAGCGTTCAGCAATGACG	GCCACGCAAACCAATTTACT
NACHT, LRR and PYD domains-containing protein 3-like	*nlrp3*	119896601	TCAATCACGCAAAGTATGG	CTCAAGTGGGCAAAGCAG
NLR family, CARD domain containing 3	*nlrc3*	119916443	TGTACTGCAGCCATCTCAGG	AGAGCCGATGTTGTTGTTCC
caspase3	*casp3*	119888653	GCTTCATTCGTCTGTGTTC	CGAAAAAGTGATGTGAGGTA
caspase-1-like	*casp1*	119883616	ATAGCAGCAGAGCAGACA	AGGACAGACCCAGTTCAT
apoptosis-associated speck-like protein containing a CARD	*asc*	119897170	AAAGGCAATAGCAGACGC	AAGTGGAAACCAGGATGT
gasdermin-E-like	*gsdme*	119897735	TTACTTGGTATCTGGTGGTG	ACATTCATCTGTCGTGGC
interleukin 1, beta	*il-1β*	119914255	ACATGACGGAAGTTCAGGAT	GCTGCCTGCTATAGTTGGTT
interleukin-8	*il-8*	119892024	CGTTGAACAGACTGGGAGAGATG	AGTGGGATGGCTTCATTATCTTGT
interleukin-18-like	*il-18*	119882624	CCCTGTTGCTTATCCTGT	CACCATCTTCTTGCCATC
hepcidin antimicrobial peptide	*hep*	119897237	ACACTCGTGCTCGCCTTTAT	AGAAGCCACAGCCCTTGTTA
serum amyloid A	*saa*	119890486	GTTGAAGCTGCGAAAGGAAC	AGCCACTCTCTGGCATCACT
immunoglobulin T	*igt*		GAAGGTCAACAACGCTGAGTG	TGTTGCTGGTCACATCTAGTCC
immunoglobulin D	*igd*		AAGGGAAACAGTGCTGTGCT	TTGCCAGTGGGGTTTGACTT
immunoglobulin M	*igm*	119887406	CTCAATGACCCCCCCTAA	CAAGCCAAGACACCAAAA
T cell receptor alpha	*tcrα*	119903280	CATGAACCACTGGCTGAGAA	TCAGCAGCAATGACATCTCC
T-cell receptor beta-1	*tcrβ*	119917160	CGGACCATGTGAGTGTGTTC	TGAAGAAGTTGACGGTGCAG
cd8 beta	*cd8*	119885986	CGCAGTATCTCCAGCCAGAT	CCAGGCTTCCACATTTCTGT
LCK proto-oncogene Src family tyrosine kinase	*lck*	119910103	TACAGAATCCGCAACATGGA	CTCCAGCTTCAGGGACTCAC

and significant main effects of period or group were observed, data were analyzed by one-way ANOVA followed by Tukey's multiple range tests to inspect differences among periods within each group and vice versa. When the significance is only with the main effects of period or group, the data were analyzed by the two-way ANOVA followed by Tukey's multiple range tests to assess the main effects of period or group only. Differences were considered significant when $P < 0.05$. All data were expressed as mean ± standard deviation of the mean (SD), except the specific statement.

Results

Largemouth bass growth performance and feed utilization were not affected after CPC replacing dietary fishmeal for 8 weeks

The growth performance of juvenile largemouth bass after 8-weeks feeding trial in the present study is shown in Table 3. Mean final weight (MFW) of juvenile largemouth bass in two groups could reach 95.42 ± 0.64 and 94.78 ± 0.90, respectively, which showed no significant difference between two groups ($P>0.05$). Accordingly, the weight gain ratio (WGR) and specific growth ratio (SGR) of bass were also around 1.96 and 1.80 in two groups, respectively. Moreover, the feed intake ratio (FI) also reached 0.23 ± 0.03 and 0.28 ± 0.06, which difference was also not significant ($P>0.05$). Feed conversion ratio was 0.89 ± 0.13 and 1.06 ± 0.03 for FM and CPC, which were not significantly different ($P>0.05$). Thus, the growth performance and feed utilization of largemouth bass was not significantly affected after CPC replacing dietary fishmeal for an 8-weeks feeding period.

Largemouth bass exhibited better growth potential after adaptation to dietary CPC

In order to have a more systematic overlook of bass growth performance with two diets, the growth parameters were separately evaluated from 0-2 weeks, 3-5 weeks and 6-8 weeks (Figure 1A). In the first two weeks, juvenile largemouth bass in FM group exhibited better MFW, WGR and SGR than bass in CPC group. However,

TABLE 3 Growth parameters of juvenile largemouth bass in the present study.

	Control	CPC	P value
Mean initial weight (MFW, g)	32.2 ± 0.12	32.1 ± 0.04	0.350
Mean final weight (MFW, g)	95.42 ± 0.64	94.78 ± 0.90	0.461
Specific growth ratio(SGR,%/day)	1.81 ± 0.005	1.80 ± 0.01	0.581
Weight gain ratio (WGR, %)	1.96 ± 0.009	1.95 ± 0.02	0.584
Feed intake ratio (FI, %/day)	1.58 ± 0.22	1.88 ± 0.04	0.137
Feed conversion ratio (FCR)	0.89 ± 0.13	1.06 ± 0.03	0.138

there were no significant differences in WGR and SGR between two groups (P>0.05) during the period from 3rd weeks to 5th weeks. Furthermore, both WGR and SGR of bass in CPC group was even significantly higher than that in FM group (P<0.05) in the last 3 weeks, and MFW in two groups showed no significant difference (P>0.05). Thus, largemouth bass exhibited adaptation to dietary CPC and then showed better growth potential in the prolonged rearing period.

In order to confirm the growth potential at 8 weeks after adaptation to dietary CPC, the activation status of TOR signaling pathway was evaluated *via* western blotting analysis. As shown in Figure 1B, the phosphorylation level of S6 was significantly higher in bass liver of CPC group than that of FM group (P<0.05). Additionally, the relative mRNA expression level of genes involved in myogenesis were also examined by RT-qPCR. As shown in Figure 1C, the expression of myocyte enhancer factor 2a (*mef2a*), myocyte enhancer factor 2b (*mef2b*), myostatin a (*mstn*), and myogenic differentiation 1 (*myod1*) showed no significant difference between two groups (P>0.05), while the expression of myogenin (*myog*) in bass liver of CPC group was also significantly higher than that of FM group (P<0.05).

Largemouth bass liver structure along with the programmed cell death was not significantly affected after dietary CPC replacing fishmeal

The liver structure of bass in two groups was evaluated *via* H.&E. staining (Figure 2A). As shown, the bass liver exhibited cuboidal hepatocytes with rounded nuclei which are arranged in

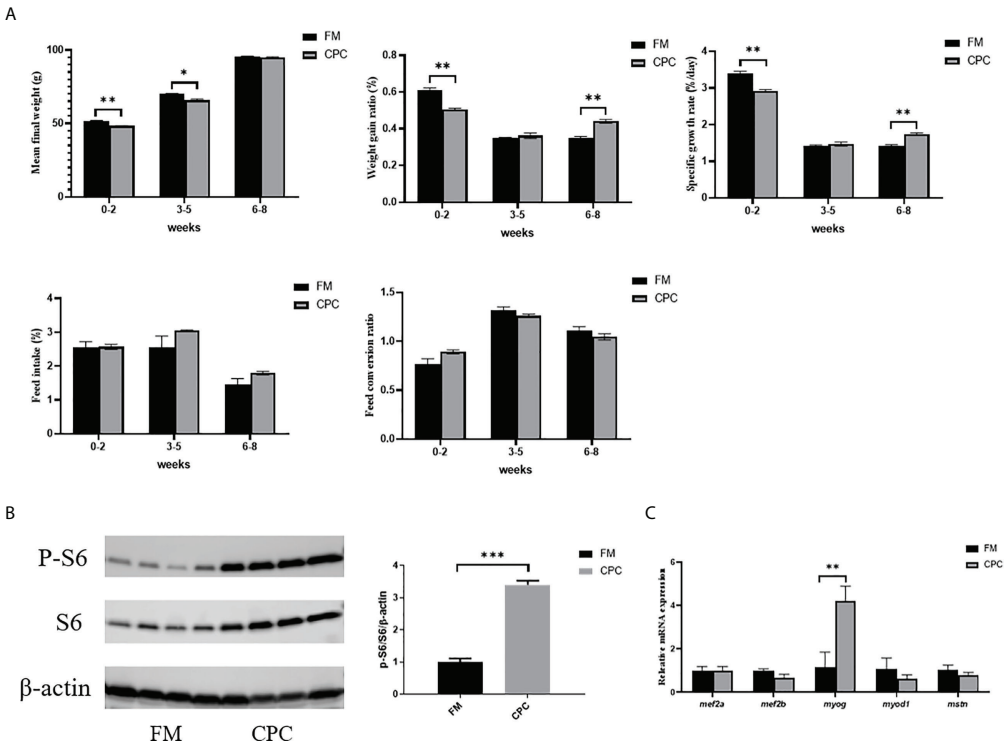

FIGURE 1
Largemouth bass exhibited better growth potential after adaptation to dietary CPC. **(A)** The growth parameters including mean final weight (MFW, g), weight gain ratio (WGR, %), specific growth ratio (SGR, %/day), feed intake ratio (FI, %/day) and feed conversion ratio (FCR) of juvenile largemouth bass fed with FM and CPC in 0-2 weeks, 3-5weeks and 6-8 weeks. **(B)** The relative protein level of S6 and P-S6 in liver of juvenile largemouth bass fed with FM and CPC. **(C)** The relative mRNA expression levels of genes involved in muscle formation (*mef2a, mef2b, myog, myod1, mstn*) in the liver of juvenile largemouth bass in both FM group and CPC group. All data was analyzed with student's t-test and data are means ± SD (n = 6). *P< 0.05; **P< 0.01; ***P< 0.001.

cords and supported by reticulated fibers and connective tissue. The sinusoidal gaps between the cords are also known as hepatic blood sinuses (S), which are the sites of blood filtration into the liver. Moreover, veins (V) and bile ducts (B) are also detected to be accompanied with each other while arterial (A) features were rarely observed. No significant differences were detected in liver interior (left), hepatic blood sinuses (middle) and edge (right) of juvenile largemouth bass in FM and CPC group.

Moreover, the programmed cell death (PCD) including apoptosis and pyroptosis in bass liver was also detected by TUNEL. As shown in Figure 2B, TUNEL$^+$ cells could be mainly detected at the edge of the liver tissue in juvenile largemouth bass of two groups. Additionally, few cells experiencing PCD could be detected in the interior of liver tissue. Statistical analysis showed that the number of TUNEL$^+$ cells in two groups were not significantly different (P>0.05) (Figure 2C).

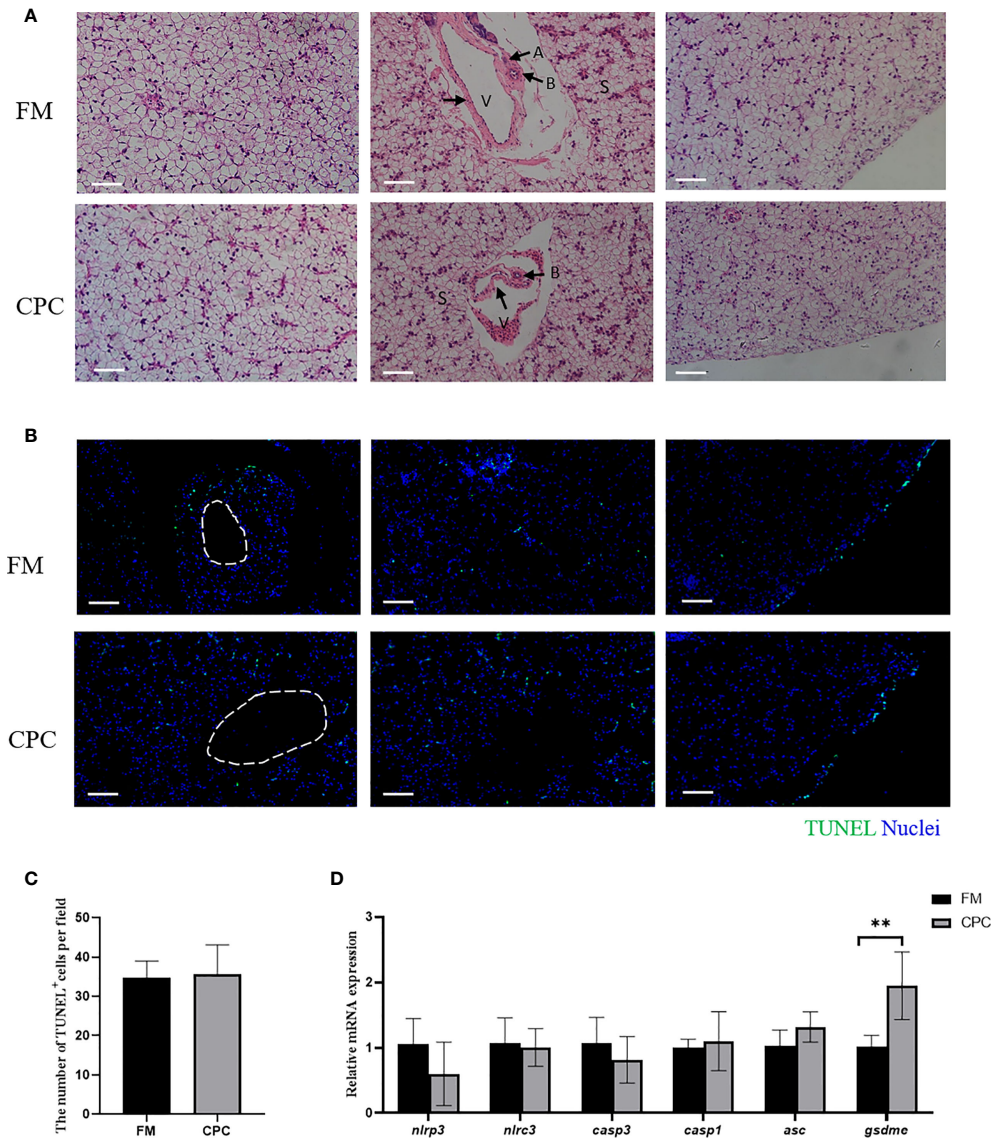

FIGURE 2
Largemouth bass liver structure along with the programmed cell death was not significantly affected after dietary CPC replacing fishmeal. **(A)** The H.E. staining results of juvenile largemouth bass liver fed with FM and CPC for 8 weeks. V: veins; B: bile ducts; A: arterial; S: sinusoid. Scale bars, 50μm. **(B)** TUNEL assay with immunofluorescence microscope on liver tissue to detect the cells experiencing programmed cell death. Scale bars, 100μm. **(C)** The calculated number of TUNEL$^+$ cells in liver of two groups. **(D)** The relative mRNA expression levels of genes involved in apoptosis and pyroptosis (*nlrp3, nlrc3, casp1, casp3, asc* and *gsdme*) in the liver of juvenile largemouth bass in both FM group and CPC group. All data was analyzed with student's t-test and data are means ± SD (n = 6). *P< 0.05; **P< 0.01; ***P< 0.001.

Additionally, the mRNA expression levels of genes involved in apoptosis and pyroptosis were also evaluated in bass liver of two groups. As shown in Figure 2D, the expression of *NACHT, LRR and* PYD domains-containing protein 3-like (*nlrp3*), NLR family CARD domain containing 3 (*nlrc3*), caspase-1-like (*casp1*), caspase-3-like (*casp3*) and apoptosis-associated speck-like protein containing a CARD (*asc*) showed no significant difference in two groups ($P>0.05$). However, the expression of gasdermin-E-like (*gsdme)* in CPC group was significantly higher than that in FM group ($P<0.05$).

Largemouth bass hepatic immunity was not significantly affected after CPC replacing fishmeal

The expression of genes involved in the innate immunity of largemouth bass liver in two groups were evaluated by RT-qPCR. As shown in Figure 3A, the expression of interleukins including interleukin-1beta (*il-1β*), interleukin-18-like (*il-18*), and interleukin-8 (*il-8*) showed no significant differencein bass liver of two groups ($P>0.05$). Similarly, the expression of hepcidin antimicrobial peptide (*hep*) was also not significantly affected by dietary CPC inclusion ($P>0.05$). However, dietary CPC inclusion significantly increased the expression of serum amyloid A (*saa*) ($P<0.05$).

Meanwhile, the adaptive immunity in bass liver was also evaluated *via* both detecting the expression of related genes and the relative abundance of T cells. As shown in Figure 3B, the expression of immunoglobulins including *immunoglobulin M* (*igm*), *igd*, and *igt* showed no significant difference in largemouth bass liver of two groups ($P>0.05$). Similarly, the expression levels of T cell receptor alpha (*tcrα*), T-cell receptor beta-1 (*tcrβ*), LCK proto-oncogene Src family tyrosine kinase (*lck*), and cd8 beta (*cd8*) were also not significantly ($P>0.05$) affected by dietary CPC inclusion. Moreover, the relative abundance of T cells in bass liver were also determined using immunofluorescent assay with antibody against Lck, a cytoplasmic protein bound to CD4 and CD8 in T lymphocytes. As shown in Figure 3C, Lck^+ cells could be detected in bass liver tissue of two groups, which are diffusely distributed in the hepatic sinusoids. The number of Lck^+ B cells in bass liver showed no significant difference between two groups ($P>0.05$).

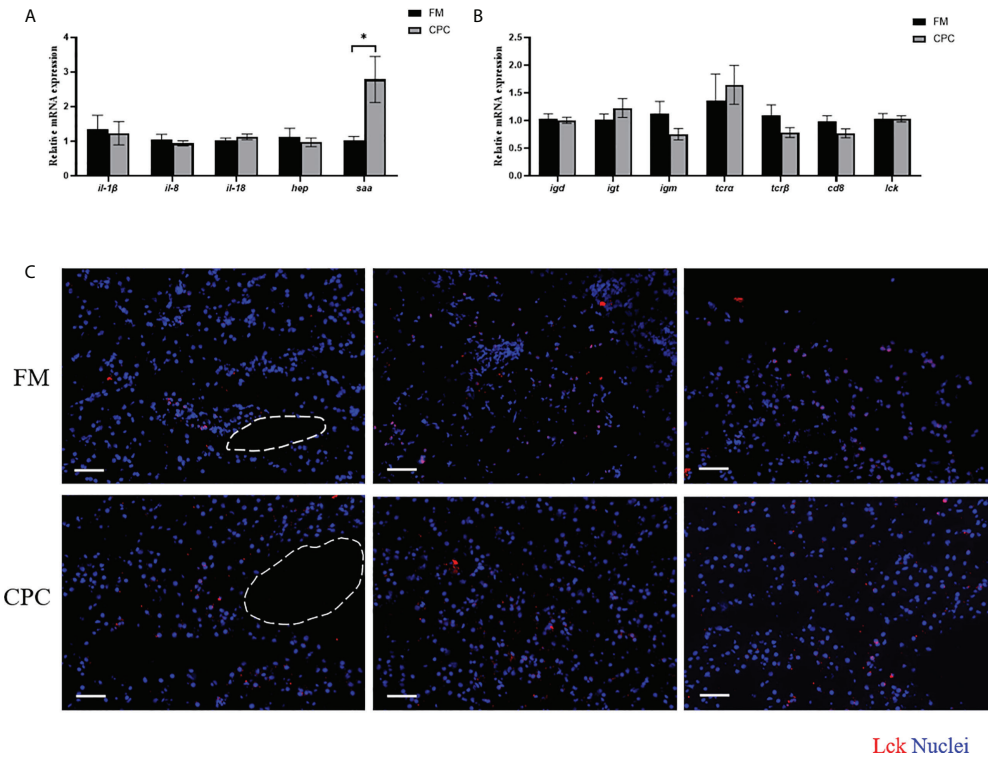

FIGURE 3
Largemouth bass hepatic immunity was not significantly affected after CPC replacing fishmeal. **(A)** The relative mRNA expression levels of genes involved in innate immunity (*il-1β, il-18, il-8, hep* and *saa*) in the liver of juvenile largemouth bass in both FM group and CPC group. **(B)** The relative mRNA expression levels of genes involved in adaptive immunity (*igm, igd, igt, tcrα, tcrβ, lck*, and *cd8)* in the liver of juvenile largemouth bass in both FM group and CPC group. **(C)** Immunofluorence results of T cells in liver of juvenile largemouth bass in both FM group and CPC group with antibody against Lck. Scale bars, 100μm. All data was analyzed with student's t-test and data are means ± SD (n = 6). *P< 0.05; **P< 0.01; ***P< 0.001.

Higher inflammatory responses were induced in both liver and gill of largemouth bass fed with CPC after *N. seriolae* infection

N. seriolae infection induced serious pathological changes in liver of largemouth bass, which were detected *via* H.&E. staining. As shown in Figure 4A, lipogranulomas (black dotted circles) composed of a mixture of lipid droplets (blue arrows), macrophages (black arrows), and lymphocytes (yellow arrows) could be detected in liver of FM and CPC group at 1 dpi. Meanwhile, the number of inflammatory cells (black triangles) in liver of CPC group were more than that in FM group, accompanied with the irregularly arranged cells and ruptured membranes (white dotted circles) in some area. At 3 dpi, the pathological changes were more serious, with chaotic arrangement of hepatocytes and aggravated inflammatory response. Accordingly, a large number of eosinophils infiltrating in liver tissue could be detected in CPC group, which is more serious than that in FM group. Severe cellular vacuolar degeneration (black circles) could also be detected in the CPC group. Moreover, the expression of genes involved in inflammatory responses and pyroptosis in liver were also evaluated *via* RT-qPCR. As shown in Figure 4B, *N. seriolae* infection induced the upregulated expression of *il-1β* in FM group at 3 dpi, while CPC group induced earlier upregulation of *il-1β* expression at 1 dpi ($P<0.05$). Moreover, *N. seriolae* infection induced the upregulated expression of *il-18* in liver of CPC group at 1 dpi and 3 dpi, whose expression was significantly higher than that of FM group ($P<0.05$). Accordingly, the expression of *casp1* and *casp3* at 1 dpi was also significantly higher in liver of CPC group than that of FM group. Similarly, the expression of *gsdme* at both 0 dpi and 1 dpi was also higher in liver of CPC group than that of FM group ($P<0.05$). Thus, dietary CPC inclusion may trigger high inflammatory potential in bass liver during *N.*

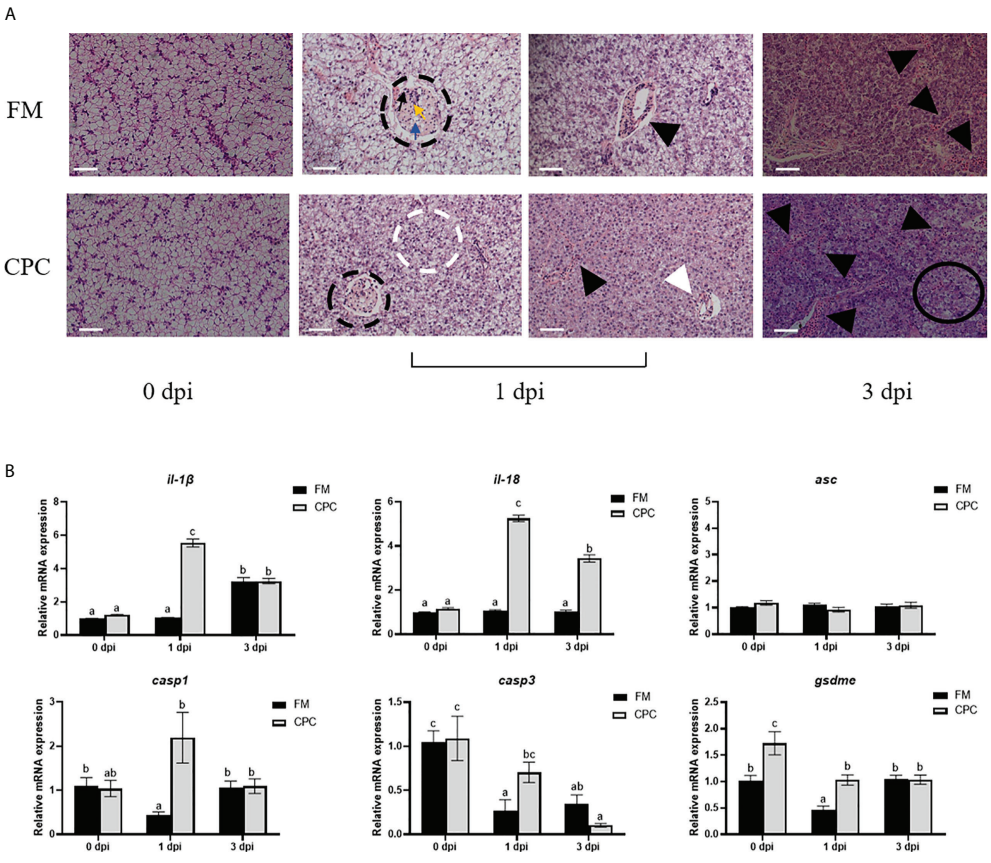

FIGURE 4
Higher inflammatory responses were induced in liver of largemouth bass fed with CPC after *N. seriolae* infection. **(A)** The H.E. staining results of juvenile largemouth bass liver fed with FM and CPC at different days post *N. seriolae* infection (dpi). Blue arrows: lipid droplets; black arrows: macrophages; yellow arrows: lymphocytes; black dotted circles: lipogranulomas; white dotted circles: ruptured cell membranes; white triangles: lymphocytic infiltration; black triangles: inflammatory cells; black circles:cell vacuolar degeneration. Scale bars, 50μm. **(B)** The relative mRNA expression levels of genes involved in inflammation and pyroptosis (*il-1β, il-18, asc, casp1, casp3* and *gsdme*) in the liver of largemouth bass fed with FM and CPC at different days post *N. seriolae* infection (dpi). All data was analyzed with two-way ANOVA and data are means ± SD (n = 6). Mean values with different letters indicated significant difference among groups, $P < 0.05$.

seriolae infection, which is in accordance with the histological structure.

In order to further confirm the pro-inflammatory effects of CPC during bacterial infection, the histological structures and expression of genes involved in inflammatory responses of gill were also evaluated (Figure 5). As shown in Figure 5A, *N. seriolae* infection induced serious histological structural changes in gill, as the primary gill lamella (PL) thickened, which could be detected in two groups. Statistical analysis indicated that the width of PL was significantly higher in CPC group than that in FM group at 1dpi and 3 dpi ($P<0.05$). Meanwhile, the secondary gill lamella (SL) turned to be short and bending after infection in both groups. The ratio of length to width of SL in CPC group was significantly higher than that in FM group before infection, but turned to be much lower in CPC group at both 1 dpi and 3 dpi ($P<0.05$). Moreover, *N. seriolae* infection also induced the upregulated expression of *il-1β* and *casp1* in the gill of largemouth bass in FM group (Figure 5B). The expression of *il-18* and *il-1β* in the gill of CPC group was even significantly higher than that of FM group at 3 dpi, while CPC group also exhibited higher expression of *il-18* at 0 dpi ($P<0.05$). The expression of *casp1* and *asc* in the gill were also significantly higher in CPC group than FM group at both 1 dpi and 3 dpi. Accordingly, the expression of *casp3* and *gsdme* in the gill were

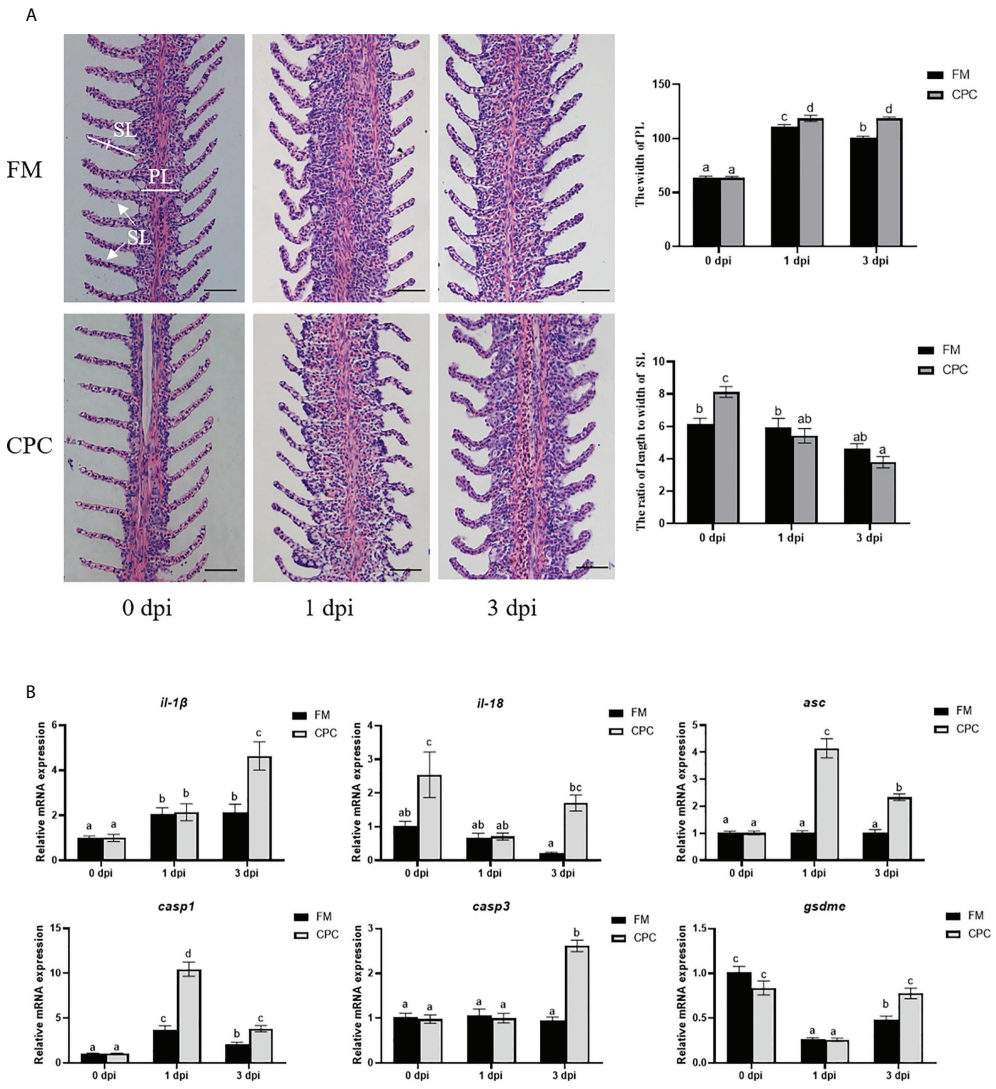

FIGURE 5
Higher inflammatory responses were induced in gill of largemouth bass fed with CPC after *N. seriolae* infection. **(A)** The H.E. staining results of juvenile largemouth bass gill fed with FM and CPC at different days post *N. seriolae* infection (dpi). PL: primary gill lamella; SL: secondary gill lamella. Scale bars, 50μm. **(B)** The relative mRNA expression levels of genes involved in inflammation and pyroptosis (*il-1β, il-18, asc, casp1, casp3* and *gsdme*) in the gill of largemouth bass fed with FM and CPC at different days post *N. seriolae* infection (dpi). All data was analyzed with two-way ANOVA and data are means ± SD (n = 6). Mean values with different letters indicated significant difference among groups, P < 0.05.

also significantly higher in CPC group than FM group at 3 dpi ($P<0.05$).

Discussion

Cottonseed is a sustainable source of plant protein, producing ~10 million metric tons of protein globally (22). In aquaculture practice, cottonseed meal has been widely tested in many fish species, while the existing gossypol seriously restricted its application (23). Reducing the gossypol content to permissible limits (450 ppm, World Health Organization) *via* physical, chemical and biological methods makes cottonseed a good candidate for use in food and feed formulations, with a balanced amino acid composition and functional properties comparable to those of soy protein (24). Recently, the new product, CPC, has been proved to effectively replace partial FM without adverse effects on growth of several aquatic animal species (10, 25). Our present study illustrated the dietary inclusion of CPC (15%) in largemouth bass diet without affecting fish growth performance when fishmeal decreased from 48% to 35% (Table 3). Early studies have also tested the effects of CPC replacing fishmeal in largemouth diet, while the replacing level varied in different studies (18–20). Considering the basic fishmeal level and other protein sources varied in different studies, it will be more convincible to use the real inclusion level of CPC in fish diet. For example, He et al. (18) reported that CPC inclusion of up to 13% showed no adverse effects on weight gain, specific growth rate and protein efficiency ratio of largemouth bass, while Xie et al. (19) also reported that CPC inclusion (23.5%) even promoted largemouth bass growth performance. Another study reported that CPC inclusion level (no more than 36.23%) could replace fishmeal in bass diet (initial body weight of 95.3g) without compromising the growth, flesh composition, texture, flavor characteristics and antioxidant capacity (20). Such phenomenon is rather common in many studies of fishmeal replacement by other protein sources. For example, in hybrid grouper (*Mycteroperca tigris* × *Epinephelus lanceolatus*), dietary CPC could replace fishmeal of up to 60% without significantly altering the growth performance but adversely influenced fish intestinal immune responses and microbial profiles (26), while the same research group showed that dietary CPC inclusion at low levels (15.76% and 18.18%) even promote hybrid grouper growth performance (27). One important question remains why results varied in the different reports. It has been speculated that differential results may originate from the varied fish growth period, the different basic diet formula, and different rearing environment. Here, in order to explain the question, the growth parameters in different feeding period were systemically evaluated. As shown in Figure 1A, bass in CPC group showed lower WGR and SGR than that in FM group in the first two weeks, while no difference was detected between two groups in the next 3weeks. However, both WGR and SGR of bass in CPC group was even significantly higher than that in FM group in the last 3 weeks (Figure 1A), which suggested that largemouth bass may have adapted to CPC diet and exhibited better growth potential at the end of 8 weeks feeding trial. A recent study reported that largemouth bass even showed adaptation of dietary glucose after 8 weeks feeding trial (28). Former studies in rainbow trout have also showed that rainbow trout can be adapted and even trained to plant-sourced proteins and exhibited good performance after several generations of selection (29), which all supported our hypothesis. Additionally, our previous studies have proved that TOR functions as the central regulator of fish growth and nutrient metabolism, and the activation status could be selected as the marker of fish growth potential (30). For example, the inhibition of TOR by rapamycin significantly resulted in the decreased fish body weight, while the adverse growth performance during fishmeal replacement by plant proteins could also be attributed to the inhibition of TOR signaling. In order to further confirm the previous assumption that largemouth bass showed better growth potential after 8-weeks adaptation to dietary CPC, the activation status of TOR signaling pathway was evaluated. In accordance with the higher SGR and WGR from 6-8 weeks, the phosphorylation level of S6 was significantly higher in bass liver of CPC group than that of FM group (Figure 1B). Moreover, the RNA expression level of *myog*, which plays a key role in muscle differentiation by controlling myoblast fusion and myofiber formation, was also significantly higher in bass liver of CPC group than that of FM group (Figure 1C). All these results may suggest that largemouth bass has adapted to dietary CPC and then exhibited even better growth potential with prolonged feeding period. Such results may also explain that fish fed with plant proteins in some studies may exhibit better growth performance than that fed with fishmeal (27), which have adapted to such plant protein sources.

Besides the growth performance, fish health has received attention in recent years, especially during the fishmeal replacement by plant proteins. In the aquaculture practice of largemouth bass, liver health has been given special attention from fish farmers and scientists, which needs a comprehensive overlook of liver structures and functions. As known in mammals, liver is an essential organ controlling nutrient metabolism and immune responses, which is closely related to its unique pattern of vascular distribution, namely, the venous-biliary-artery tract (VBAT) structure (31). Such mammalian-like VBAT structures also exist in some teleost species, such as Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) (32). Here in the present study, the typical VBAT structure was also detected in bass liver *via* H.&E. staining. As shown in Figure 2A bass liver, cuboidal hepatocytes with rounded nuclei were arranged in cords and supported by reticulated fibers and connective tissue. Moreover, veins (V) and bile ducts (B) are also detected to be accompanied

with each other while arterial (A) features were rarely observed. Moreover, no significant difference was found in liver structure of juvenile largemouth bass between two groups. The tissue homeostasis could be due to the controlled proliferation and programmed cell death (PCD) of inner cells (33). Here, TUNEL assay was conducted to evaluate the programmed cell death (Figure 2B). Results also showed that TUNEL$^+$ cells were mainly distributed at the edge of liver with few apoptotic cells in the interior of bass liver and no significant difference was detected between FM and CPC group (Figure 2C). Moreover, the expression levels of several PCD-related genes were also evaluated. As shown in Figure 2C, excepting *gsdme*, the expression of genes including *nlrp3, nlrc3*, *casp1, casp3* and *asc* showed no significant difference between two groups. Thus, H.&E. staining, TUNEL assay and RT-qPCR results all suggested that bass liver structures were not significantly affected after dietary CPC inclusion at 15% for 8 weeks.

As mentioned above, fish liver is also involved in the immune responses, as several types of immune cells have been identified in the hepatic blood sinuses (34). Dietary and commensal bacterial products from the gastrointestinal tract enters liver from portal vein, which exert liver to continuous inflammatory potential (35). In fact, fish is the first bony vertebrate to develop both innate and adaptive immunity among all vertebrates (36), thus both innate and adaptive immune responses in liver were also systemically evaluated. Inflammatory responses are soon induced after pathogenic infection or other damage signals exposure. The expression levels of genes involved in the inflammatory responses including *il-1β, il-8* and *il-18* showed no significant difference between two groups. Moreover, the expression levels of several antibacterial peptide including *hep* and *saa* were also evaluated. The expression level of *saa*, which plays a key role in bacterial clearance and inflammatory regulation, in CPC group was significantly higher than that in FM group, which suggested that bass liver in CPC group was activated to control the inflammatory responses. On the other hand, the adaptive immunity in fish is executed by B cells and its secreted immunoglobulins which are responsible for humoral immunity, and T cells which are responsible for cellular immunity (37). As reported, three types of immunoglobulins (Igs) have been identified in teleosts (36). In the present study, the expression of Igs including *igm, igt* and *igd*, also showed no significant difference between two groups. Moreover, T cells are characterized by the presence of T-cell receptors (TCRs), which consists of an α (TCRα) chain and a β (TCRβ) chain in the conventional T cells. Such T lymphocytes could be distinguished by the expression of mutually exclusive CD4$^+$ or CD8$^+$ co-receptors (38). Additionally, Lck is a cytoplasmic protein bound to CD4 and CD8 in T cells, which participates in antigen-induced T cell activation (39). Here, in the present study, the expression of *tcra, tcr*β*, lck*, and *cd8* also showed no significant difference between two groups, which indicated that dietary CPC inclusion did not significantly affect the expression of T cell markers. Additionally, immunofluorescence study with antibody against Lck also showed that T cells also exist in the liver interior with no Lck$^+$ cells detected in the blood vessel, which indicated the successful perfusion procedure. Importantly, the number of T cells showed no significant difference between two groups. Thus, the innate and adaptive immunity in bass liver under normal status were not significantly affected during dietary CPC inclusion.

Largemouth bass is rather sensitive to *N. seriolae* infection, which caused serious hepatic damage and even mortality (14). In order to further illustrate the immune responses of bass liver during pathogenic infection, all bass was injected with *N.a seriolae*. As shown in Figure 4, the liver pathological structures showed rather serious damages. Lipogranulomas could be detected in liver of FM and CPC group at 1 dpi, while severe cellular vacuolar degeneration (black circles) could also be detected in the CPC group at 3 dpi. Moreover, the number of inflammatory cells (black triangles) in liver increased with the prolonged infection period, and such inflammatory cells in liver of CPC group were more than that in FM group, accompanied with the irregularly arranged cells and ruptured membranes. As mentioned above, fish liver is continuously exposed to dietary and commensal bacterial products, which results in persistent, regulated inflammation (16). When fishmeal was replaced by plant proteins in fish diet, the plant sources-originated anti-nutritional factors would cause further continual inflammatory stimulus to liver. Failure to clear such 'dangerous' stimuli may lead to chronic pathological inflammation and even disrupted tissue homeostasis such as fibrosis, cirrhosis and eventual liver failure (17). Recently, one new type of PCD, pyroptosis which is executed by gasdermin family proteins, has been discovered to closely correlate with the inflammation (40). Under the stimulation of pathogen- and/or damage-associated molecular patterns, pattern recognition receptors (PRRs) such as Nod like receptors could recruit apoptosis-associated speck-like protein containing a CARD (ASC) and pro-caspases to form inflammasomes and then activate caspases, which then cleave gasdermin E in fish to form oligomeric pores, and also promote the maturation and release of inflammatory cytokines such as *il-1β* and *il-18* (40). In the present study, *N. seriolae* infection induced the upregulated expression of *il-1β* at 3 dpi in the FM group, while *il-1β* expression in CPC group was significantly induced much earlier at 1 dpi. Moreover, *N. seriolae* infection induced the upregulated expression of *il-18* in the liver of CPC group at both 1 dpi and 3 dpi, whose expression was significantly higher than that of FM group. Besides the inflammatory cytokines, the expression of *casp1* and *casp3* was also significantly higher in bass liver of CPC group than that of FM group at 1 dpi. Similar results were detected in the expression of *gsdme* in liver at 0 dpi and 1 dpi. Thus, dietary CPC inclusion may trigger high inflammatory potential after *N. seriolae* infection, which is in accordance with the histological structure.

In order to further confirm the pro-inflammatory effects of CPC during bacterial infection, the histological structures and expression of genes in gill involved in inflammatory responses were also evaluated. As shown in Figure 5A, *N. seriolae* infection induced serious histological structural changes in gill, as the primary gill lamella (PL) thickened and the secondary gill lamella (SL) shortened, which could be detected in two groups. Earlier studies in hybrid snakehead (*Channa maculata* ♀ × *Channa argus* ♂) also detected that the gill filament was become short, twisted and shrunk seriously after *N. seriolae* infection (41), with a number of pale yellow nodules could be detected on the serosal surface, mesentery and internal organs (gill, heart, spleen, swim bladder, kidney and liver). Especially, more serious gill damages could be detected in CPC group, with much higher the width of PL but lower ratio of length to width of SL than that in FM group. Moreover, *N. seriolae* infection also induced the upregulated expression of *il-1β*, and *casp1* in the gill of largemouth bass (Figure 5B). Moreover, the expression of *il-18* and *il-1β* in the gill of CPC group was even significantly higher than that of FM group at 3dpi, while CPC group also exhibited higher expression of *il-18* at 0 dpi. The expression of *casp1* and *asc* in the gill were also significantly higher in CPC group than FM group at both 1 dpi and 3 dpi. Accordingly, the expression of *casp3* and *gsdme* in the gill were also significantly higher in CPC group than FM group at 3 dpi. All these results in gill further confirmed that dietary CPC inclusion in largemouth bass significantly increased inflammatory potential and pyroptosis during bacterial infection, which may further influence fish health and survival during infection.

In summary, our present study indicated the potential of dietary CPC as replacement sources for fish meal, and that largemouth bass can adapt to it and even showed better growth potential. However, special concern should be given during the application of CPC in largemouth bass diet as it may induce higher inflammatory risk during *N. seriolae* infection.

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 Animal Experiment Committee of Huazhong Agricultural University (permit number HZAUFI-2016-007).

Author contributions

QW designed and wrote the main context. MW conducted most experimental protocol. ZC and YW joined the experimental analysis. JZ, SL, and XG conducted the experimental analysis. JG supplied the relevant materials. All authors contributed to the article and approved the submitted version.

Funding

This article was funded by National Natural Science Foundation of China (Grant No. 32172996; 31802317) and China Scholarship Council (Grant No. CSC[2021]109).

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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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 07 October 2022
DOI 10.3389/fimmu.2022.1034994

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Comparative Immunology, a section of the journal Frontiers in Immunology

RECEIVED 02 September 2022
ACCEPTED 21 September 2022
PUBLISHED 07 October 2022

CITATION
Chen J, Wang H, Yuan H, Hu N, Zou F, Li C, Shi L, Tan B and Zhang S (2022) Effects of dietary *Clostridium autoethanogenum* protein on the growth, disease resistance, intestinal digestion, immunity and microbiota structure of *Litopenaeus vannamei* reared at different water salinities. *Front. Immunol.* 13:1034994. doi: 10.3389/fimmu.2022.1034994

Effects of dietary *Clostridium autoethanogenum* protein on the growth, disease resistance, intestinal digestion, immunity and microbiota structure of *Litopenaeus vannamei* reared at different water salinities

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The shortage of fishmeal (FM) resources limits the healthy development of aquaculture. Developing new protein sources to replace FM in aquatic feeds is an effective measure to alleviate this situation. However, the application effect of new protein sources is greatly affected by water salinity, which is an important parameter of aquaculture. In this study, the growth, disease resistance, and intestinal digestion, immunity, and microbiota structure of *Litopenaeus vannamei* (initial weight: 0.38 ± 0.01 g) fed on *Clostridium autoethanogenum* protein (CAP) or not at three different water salinities (15 ‰, 30 ‰, and 45 ‰) were compared, aiming to explore the effects of dietary CAP on shrimp when suffering different salinity stresses. The results showed that the growth performance, feed utilization, and survival rate (SR) after pathogen challenge of *L. vannamei* could be significantly improved by dietary CAP when compared with the control at the same salinity and they were also significantly affected by salinity changes when *L. vannamei* was fed on the same protein source. With the increase in salinity, obvious upregulation was observed in the activities and gene expression of digestive enzymes both in *L. vannamei* fed on FM and CAP, with significantly higher levels in *L. vannamei* fed on CAP than in those fed on FM at the same salinity. Meanwhile, the expression levels of immune genes in the CAP group were significantly higher than those in the FM group at different salinities. The intestinal microbiota analysis showed that CAP could increase the relative abundance of beneficial bacteria and decrease the relative abundance of harmful bacteria in the intestine of *L.*

vannamei at the phylum, family, and genus levels, and it was more affected by salinity changes when compared with FM. Besides, the changes in salinity and protein sources led to different changes in the intestinal microflora function of *L. vannamei*. In sum, this study indicated that CAP could improve the growth, disease resistance, digestive capacity, and intestinal microflora of *L. vannamei* with a much more intense immune response and enhance its ability to cope with salinity stress.

KEYWORDS

***Litopenaeus vannamei*, *Clostridium autoethanogenum*, salinity, growth performance, intestinal microbiota**

1 Introduction

Aquaculture can economically provide high-quality animal proteins for the global population (1). In the past 40 years, aquaculture production worldwide has increased rapidly to satisfy the increasing demand for animal protein consumption (2). Up to now, aquatic products have been considered the third-largest source of animal proteins (3). Rapidly increased aquaculture production results in a strong demand for fishmeal (FM), which is the indispensable source of high-quality protein in aquatic feeds (4). The shortage of FM has become one of the biggest problems facing the global aquatic feeds industry, which has seriously affected the healthy and sustainable development of the aquatic feeds industry (5). The threat in the future is that overfishing will lead to an ecological imbalance in the marine ecosystem because FM mainly comes from fishing (6). Thus, the development of suitable novel protein sources will meet the growing needs of the rapidly growing aquaculture industry (7).

Litopenaeus vannamei is the largest shrimp species in the world in consumption and cultivation. The annual production of *L. vannamei* in 2019 was 5.5 million tons (8). Generally, the protein requirements of *L. vannamei* are high and the best protein level for shrimp feed is 35% to 40% (9). FM is also the most common protein source for *L. vannamei* as for most aquatic animals, accounting for 30% of the feeds (10). Therefore, looking for substitutes for FM is of great significance for the healthy development of *L. vannamei* aquaculture. *L. vannamei* is a euryhaline shrimp species with a salinity tolerance range of 0.5–78 ‰ (11, 12). Salinity is a crucial environmental factor for aquatic animal reproduction, growth, development, and survival (13–15). In general, *L. vannamei* could effectively maintain osmotic pressure and ion regulation under different salinities to adapt to the environment (16). However, the survival, growth, and immunity varied according to the different salinities, which was mainly due to the energy digestibility changes (17–20). *L. vannamei* provides sufficient energy to effectively cope with changes in salinity mainly through food intake. If not, it uses its own body energy sources, resulting in slow growth, low survival rate, and so on (21). It has been reported that protein is a fundamental source of energy during salinity changes. *L. vannamei* reared in high salinity required high dietary protein than those reared in low saline waters (22, 23). This does not support sustainable aquaculture growth since high protein levels not only increase the cost of the feeds but also increase protein catabolism, which increases the organic load and environmental pollution (21). Therefore, it is essential to identify cheap protein energy sources to spare dietary protein in the shrimp culture.

At present, the development and application of novel protein sources in shrimp feeds mainly focus on plant protein, animal protein, and single-cell protein (SCP) sources (24). However, the use of plant protein is limited due to its unbalanced amino acid composition, the presence of anti-nutritional factors, and poor taste. At the same time, the nutritional content of animal protein sources usually varies with the season or product batch, and animal protein's biological safety must also be considered. SCP, also known as microbial protein, is not only rich in protein and amino acids but also in vitamins, minerals, nucleotides, and immune polysaccharides and has gotten a lot more favour (25). *Clostridium autoethanogenum* protein (CAP) is a by-product of *Clostridium autoethanogenum* fermentation to produce ethanol. *Clostridium autoethanogenum* can produce ethanol and CAP by using CO produced by industrial tail gas as a carbon source and ammonia water as a nitrogen source. As a high-quality SCP, the protein content of CAP can be as high as 80%. Besides, CAP is rich in essential amino acids, easy for animals to digest and absorb, and contains no anti-nutritional factors. CAP has been used in several aquaculture species, including *Acanthopagrus schlegelii*, *Micropterus salmoides*, *Cyprinus carpio* var. *Jian*, and *L. vannamei*, with the effect of improving growth performance, feed utilization, anti-oxidation, intestinal health, and immune response (26–31). In a basal diet containing 560 g/kg FM, CAP could substitute 30% FM without adverse effects on growth, intestinal histology, and immunity, while higher FM substitution decreased the growth and flesh quality of *L.*

vannamei (27, 32). However, the application effect of CAP on *L. vannamei* at different salinities has not been investigated up to now.

In this study, the ability of *L. vannamei* to utilize CAP at different salinities was investigated from the aspects of growth performance, disease resistance, intestinal digestive capacity, immunity, and microbiota structure, which could provide a theoretical reference for the application of *C. autoethanogenum* as a new protein source in aquatic feeds.

2 Materials and methods

2.1 Diet preparation

Two isoproteic and isolipidic diets were made as shown in Table 1. The control diet was designed using FM (589 g/kg) as the only protein source. Under the premise of using 150 g/kg FM to meet the basic requirement for normal growth (10), 354g/kg CAP was used as the only protein source to replace FM in the experimental diet. All raw materials were crushed and screened through an 80-mesh screen. After the raw materials were mixed by a step-by-step expanding method, they were fully mixed by a V-type mixer (JS-14S, Zhejiang Chint Electrics Co., Ltd., Zhejiang, China). Fish oil, corn oil, and soybean lecithin were added and mixed again, followed by adding some water to the mixture, and then extruded using a twin-screw extruder (M-256, South China University of Technology, Guangzhou, China). The feed pellets were baked at 75°C for 20 min and air-dried naturally, and then stored in the refrigerator at 20°C. CAP was provided by Beijing Shoulang Bio-technology Co., Ltd., Beijing, China. The crude protein, crude lipid, crude ash, and moisture of CAP were 84.21%, 0.19%, 3.27%, and 7.14%, respectively.

TABLE 1 The formula and proximate composition of the diet (dry matter/%).

Ingredients (%)	Groups	
	FM	CAP
Brown fish meal	58.90	15.00
CAP	0.00	35.40
Corn starch	20.00	20.00
Fish oil	0.36	2.52
Corn oil	0.36	2.52
Soyabean lecithin	1.00	1.00
Vitamin and mineral premix[a]	1.20	1.20
Choline chloride	0.50	0.50
Ethoxyquin	0.05	0.05
Attractant[b]	0.10	0.10
$Ca(H_2PO_4)_2$	1.20	1.20
Vitamin C	0.05	0.05
Cellulose microcrystalline	16.28	20.45
Total	100.00	100.00
Proximate composition (%)		
Crude protein[c]	41.39	40.85
Crude lipids[c]	7.53	7.66
Ash	11.56	5.16
Moisture	7.32	7.64

[a]Vitamin and Mineral Premix (kg^{-1} of diet) includes the following contents: thiamine, 5 mg; riboflavin, 10 mg; vitamin A, 5,000 IU; vitamin D3, 1,000 IU; vitamin E, 40 mg; menadione, 10 mg; pyridoxine, 10 mg; biotin, 0.1 mg; cyanocobalamin, 0.02 mg; calcium pantothenate, 20 mg; folic acid, 1 mg; niacin, 40 mg; vitamin C, 150 mg; $FeSO_4{\cdot}H_2O$, 303 mg; KIO_3, 1.3 mg; $Cu_2(OH)_3Cl$, 5 mg; $ZnSO_4{\cdot}H_2O$, 138 mg; $MnSO_4{\cdot}H_2O$, 36 mg; Na_2SeO_3, 0.6 mg; $CoCl_2{\cdot}6H_2O$, 0.8 mg.
[b]The attractant is betaine.
[c]Crude protein and crude lipid contents were measured value.

2.2 Collection and acclimatization of the trialled *L. vannamei*

The *L. vannamei* larvae were provided by Zhanjiang Yuehai Aquatic Fry Co., Ltd. (Zhanjiang, China). 240 *L. vannamei* (initial weight of 0.38 ± 0.01 g) were randomly divided into two equal groups and fed on FM or CAP diets. The FM or CAP group was equally divided into three subgroups at three salinities of low salinity (15 ‰), medium salinity (30 ‰), and high salinity (45 ‰), which were set as previous studies (33, 34). Three biological replicates were set for each subgroup with 40 individuals placed in 300-litre fiberglass tanks. *L. vannamei* were further adapted to low and high salinity. Artificial seawater salt (Jiangxi Yantong Technology Co., Ltd., Jiangxi, China) was used to gradually increase salinity to 45‰ for high salinity, and freshwater was used to gradually decrease salinity to 15‰ for low salinity from the original medium salinity of 30‰, being changed by 2‰ per day. Thus, there were six treatments with three replicates per treatment, that is FM15‰, FM30‰, FM45‰, CAP15‰, CAP30‰, and CAP45‰ groups, respectively. *L. vannamei* were fed four times daily at the following times: 7:00 am, 11:00 am, 17:00 pm, and 21:00 pm. At the beginning of the experiment, *L. vannamei* were fed on an amount of feeds equivalent to 10% of their body weight. 0.1 g of feed was added to the amount of feed per tank per day if the shrimp finished eating within 30 minutes. One-third of the water was replaced each day by water with pre-adjusted salinity from a reservoir. The temperature, ammonia nitrogen, dissolved oxygen, and pH were monitored daily and maintained between 27–30°C, <0.05 mg/L, >6.0 mg/L, and 7.7–8.0, respectively.

2.3 Sample collection

After 24-hour starvation at the end of 8 weeks of feeding, the shrimps were counted and weighed to measure survival rate (SR) and the overall body weight indicators, including the final body weight (FW), weight gain rate (WGR), specific growth rate (SGR), and protein efficiency ratio (PER). Intestines from nine shrimps were randomly collected from each tank into three samples, to

analyze the intestinal digestive enzyme activity, gene expression, and microbiome structure. After being placed in liquid nitrogen for rapid freezing, samples were transferred to −80°C storage for subsequent analysis.

2.4 Growth performance analysis

Based on the recorded data, the indices for the assessment of growth performance, including SR, WGR, SGR, FCR, and PER, were calculated as follows:

$$SR(\%) = \frac{\text{Final shrimp number}}{\text{Initial shrimp number}} \times 100$$

$$SGR\ (\%\ d^{-1}) = \frac{[\text{Ln (Final body weight)} - \text{Ln (Initial body weight)}]}{\text{Days}} \times 100$$

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

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

$$PER\ (\%) = \frac{(\text{Final body weight} - \text{Initial total weight})}{\text{Protein intake}} \times 100$$

2.5 Challenge tests

Vibrio parahaemolyticus were prepared as in our previous studies (35, 36). The *V. parahaemolyticus* cells were centrifuged (5000 g) for 10 min at 4°C and then resuspended by 1 × PBS as an inoculum about 1 × 10^5 colony-forming units (CFU)·μL^{-1}. After sample collection, a total of 30 *L. vannamei* from each group were chosen to perform a challenge test with *V. parahaemolyticus* at a dose of 10^7 CFU/g shrimp. The survival rate was recorded every 4 hours. The differences between the two groups were analyzed by the GraphPad Prism software using the Mantel-Cox (log-rank χ2 test) method.

2.6 Detection of intestinal digestive enzyme activities

50 mg of intestinal tissue was added into 0.01 mol/L PBS (PH = 7.2–7.4) to prepare a homogenate at a proportion of 10% (Tissue: PBS=1: 9). About 20 minutes after centrifugation (2000–3000 rpm/min), the supernatant was collected and examined. The activities of three digestive enzymes, including trypsin, amylase, and lipase, were determined using the enzyme-linked immunosorbent assay (ELISA) kit ml036384, ml036449, and ml036371 from Shanghai Enzyme-linked Biotechnology Co., Ltd., respectively. The relevant operation was carried out strictly according to the manual.

2.7 Gene expression analysis of intestinal digestive enzymes

The total RNA of the intestine was extracted by TransZol Up Plus RNA kits (TransGen, China), and the RNA concentration was determined by Spectrophotometric analysis (Nanodrop 2000). The cDNA was reverse-transcribed from total RNA by Evo M-MLV RT kit with gDNA Clean for qPCR II (Accurate Biotechnology Hunan Co., Ltd, China). The gene expressions of trypsin, amylase, lipase, superoxide dismutase (SOD), lipopolysaccharide (LPS) and beta-1,3-glucan binding protein (LGBP), prophenoloxidase (PPO), phenoloxidase (PO), Crustin (CRU), anti-lipopolysaccharide factor (ALF), penaeidin (PEN), and lysozyme (LYZ) were assessed by the Roche Light Cycler480 thermal cycler (Roche Applied Science, Germany) using the SYBR® Green Premix Pro Taq HS qPCR Kit II (Accurate Biotechnology Hunan Co., Ltd, China). For each target gene, specific primers were designed by Primer 5.0 software according to the known sequences in the NCBI database (Table 2). The results of real-time qPCR were analyzed by the $2^{-\Delta\Delta CT}$ method (37) using elongation factor 1α (EF1α) as a reference gene. Three independent biological replicates were performed for each sample.

2.8 Intestinal microbial analysis

The genomic DNA of the microorganisms was extracted from intestinal samples following the manufacturer's instructions using HiPure Soil DNA Kits (Magen, Guangzhou, China). The V3-V4 region of the 16S rDNA gene was amplified using primers 341F: CCTACGGGNGGCWGCAG; 806R: GGACTACHVGGGTATCTAAT. The PCR program was conducted at an initial denaturation step at 95°C for 5 min, followed by 30 cycles at 95°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 7 min. PCR reactions were performed in a triplicate 50-μL mixture containing 10 μL of 5 × Q5@ Reaction Buffer, 10 μL of 5 × Q5@ High GC Enhancer, 1.5 μL of 2.5 mM dNTPs, 1.5 μL of each primer (10 μM), 0.2 μL of Q5@ High-Fidelity DNA Polymerase, and 50 ng of template DNA. The related PCR reagents were from New England Biolabs, USA. The amplified products were purified by the AxyPrep DNA gel extraction kit (Axygen Biosciences, Union City, CA, USA). Subsequently, amplicons were pooled into equimolar concentrations and sequenced by Guangzhou Genedenovo Biotechnology Co., Ltd., using a Hiseq2500

TABLE 2 PCR primers used in this study.

Gene names	Primers	GenBank no.	Sequences (5'-3')
EF1α	EF1α-F	XM027373349.1	GTATTGGAACAGTGCCCGTG
	EF1α-R		TCACCAGGGACAGCCTCAGTA
Lipase	Lipase-F	XM02373566.1	TCTCCCACTTCAATCGTCA
	Lipase-R		ATGCTTGGAATCGCTCTG
Trypsin	Trypsin-F	JQ277721.1	CTTCCGCCGTGGTCTCAA
	Trypsin-R		TCTGCTCGGTGCCCTCAT
Amylase	Amylase-F	XM027369804.1	GTTCCTTACTCCGCTTTCG
	Amylase-R		CGTAGTCAGTGCCTTGGTTCA
SOD	SOD-F	DQ005531.1	CTTTGCCACCCCTCAAGTATG
	SOD-R		TGCCTCCGCCTCAACCA
GBP	GBP-F	AY723297.1	TACGGAGGAACGACGCTGC
	GBP-R		AAATCATCGGCGAAGGAGC
PPO	PPO-F	AY723296.1	AACTCCATTCCGTCCGTCTG
	PPO-R		CGGCTTCGCTCTGGTTAGG
PO	PO-F	XM027381766.1	AAGCCAGGCAGCAACCAC
	PO-R		CAGAAGTTGAAACCCGTGGC
CRU	CRU-F	AF430071.1	GTAGGTGTTGGTGGTGGTTTC
	CRU-R		CTCGCAGCAGTAGGCTTGAC
ALF	ALF-F	EW713395	TTACTTCAATGGCAGGATGTGG
	ALF-R		GTCCTCCGTGATGAGATTACTCTG
PEN	PEN-F	DQ206401.1	GACGGAGAAGACAATGGAAACC
	PEN-R		ATCTTTAGCGATGGATAGACGAA
LYZ	LYZ-F	AF425673.1	TATTCTGCCTGGGTGGCTTAC
	LYZ-R		CAGAGTTGGAACCGTGAGACC

EF1α, elongation factor 1-alpha, SOD, superoxide dismutase, GBP, beta-1,3-glucan binding protein, PPO, prophenoloxidase, PO, phenoloxidase, CRU, small cysteine and glycine repeat-containing protein, ALF, anti-lipopolysaccharide factor, PEN, Penaeidin, LYZ, lysozyme.

PE250 machine (Illumina, USA). The raw data were deposited in the NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

To obtain high-quality clean reads, FASTP (38) was used to further filter the raw reads and the noise sequence of the raw tags under specific filtering conditions to obtain high-quality clean tags. The clean tags were clustered into operational taxonomic units (OTUs) of ≥ 97% similarity using UPARSE pipeline (38). The representative OTU sequences were classified using the RDP classifier (39) based on the SILVA database (40), with a confidence threshold value of 0.8. Alpha diversity indexes, OTUs, Chao 1, ace, Shannon, Simpson, and Goods coverage were calculated using QIIME (41, 42). Beta diversity indexes and principal coordinates analysis (PCoA) of bray-curtis distances were generated in the R project Vegan package (43). The KEGG pathway of OTUs was analyzed by Tax4Fun (42).

2.9 Statistical analysis

The results were expressed as mean ± standard deviation (mean ± SD), and two-way analysis of variance (ANOVA) was used to test the significance using SPSS 20.0 statistical software. Tukey's multiple comparison method was further used if there were significant differences. For the challenge test, the survival rate was calculated using Log-rank Kaplan-Meier analysis by GraphPad Prism. The differences between all the test results were considered significant at $P< 0.05$ and highly significant at $P< 0.01$.

3 Results

3.1 Growth performance and feed utilization

Analysis of variance analysis (Table 3) showed that the protein source and salinity significantly affected the WGR, SGR, FCR, and PER but not the SR of *L. vannamei*. Besides, the interaction of protein source and salinity had a significant effect on all these five indices of *L. vannamei* ($P< 0.05$).

The protein source changes led to significant changes in the WGR, SGR, FCR, and PER of *L. vannamei* at the same salinity. At the same salinity, a change in protein source resulted in a significant difference in the growth performance and feed utilization of *L. vannamei* between the FM and the CAP groups. At 15‰ salinity, the FCR of *L. vannamei* in the CAP group was significantly lower than that in the FM group, while

TABLE 3 Effects of protein sources and salinity on growth of *L. vannamei*.

Items		SR/%	WGR/%	SGR/(%/d)	FCR	PER (%)
Protein source	**Salinity/‰**					
FM	**15**	88.75 ± 1.77	1757.77 ± 27.12^{b}	5.22 ± 0.03^{b}	1.41 ± 0.12^{B}	1.76 ± 0.16^{A}
	30	90.00 ± 2.50	1784.13 ± 36.33^{Ab}	5.24 ± 0.03^{Ab}	1.49 ± 0.04^{A}	1.66 ± 0.05^{B}
	45	95.83 ± 1.44	1588.92 ± 23.43^{Aa}	5.05 ± 0.12^{Aa}	1.39 ± 0.10^{A}	1.78 ± 0.13^{B}
CAP	**15**	92.50 ± 2.50	1843.40 ± 35.86^{a}	5.30 ± 0.03^{a}	1.04 ± 0.05^{Aa}	2.36 ± 0.10^{Bc}
	30	94.17 ± 1.44	1983.43 ± 59.56^{Bb}	5.42 ± 0.05^{Bb}	1.98 ± 0.09^{Bb}	1.25 ± 0.06^{Ab}
	45	91.17 ± 3.75	1790.54 ± 65.73^{Ba}	5.25 ± 0.02^{Ba}	3.69 ± 0.09^{Bc}	0.67 ± 0.02^{Aa}
***P*-value of two-way ANOVA**						
Salinity		0.1981	<0.0001	<0.0001	<0.0001	<0.0001
Protein source		0.3819	<0.0001	<0.0001	<0.0001	<0.0001
Interaction		0.0161	0.0279	0.0199	<0.0001	<0.0001

Values with different capital letter superscripts indicated significant differences between different protein sources at the same salinity (P<0.05). Values with different small letter superscripts indicated significant difference among different salinities when the protein source was the same (P<0.05). The same as below.

the PER was significantly higher than that in the FM group ($P< 0.05$). The WGR, SGR, and FCR of *L. vannamei* in the CAP group at 30‰ and 45‰ salinity were significantly higher than those in the FM group, while the PER was significantly lower than that in the FM group ($P< 0.05$).

Besides, the alteration of salinity led to different changes in the growth performance and feed utilization of *L. vannamei* in the FM and the CAP groups. The WGR and SGR of *L. vannamei* were significantly different at different salinities both in the FM and the CAP groups. However, only the FCR and PER of *L. vannamei* in the CAP group were significantly different at different salinities. In the FM group, the WGR and SGR of *L. vannamei* at 45‰ salinity were significantly decreased when compared with those at 15‰ and 30‰ salinities. In the CAP group, the WGR and SGR of *L. vannamei* were highest at 30‰ salinity, with a significantly higher level than those at 15‰ and 45‰ salinities. With the increase in salinity, the FCR of *L. vannamei* increased significantly while the PER of *L. vannamei* decreased significantly ($P< 0.05$).

3.2 Survival rates of *L. vannamei* after *V. parahaemolyticus* infection

The survival rates of *L. vannamei* after *V. parahaemolyticus* infection were not significantly different among the FM group at the three different salinities. In the CAP group, the survival rate of *L. vannamei* at 45‰ salinity after *V. parahaemolyticus* infection was highest and significantly higher than that at 30‰ salinity (Figure 1). Under the condition of the same salinity, the survival rate of *L. vannamei* in the CAP group after *V. parahaemolyticus* infection was higher than that in the FM group, with significantly higher levels at 15‰ and 45‰ salinities ($P< 0.05$).

3.3 Digestive enzyme activities in the intestine

As shown in Figure 2, under the condition of the same salinity, the intestinal amylase and lipase activities of

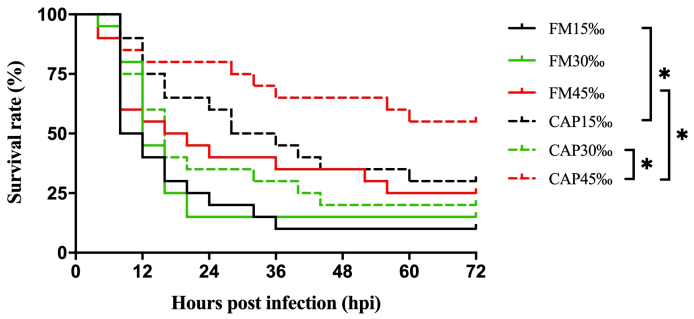

FIGURE 1
Survival rates of L. vannamei after V. parahaemolyticus infection. Differences in survival levels between treatments were analyzed by Kaplan-Meier plot (log-rank χ^2 test). Significant differences in survival rate were marked with asterisks, * indicates $P< 0.05$.

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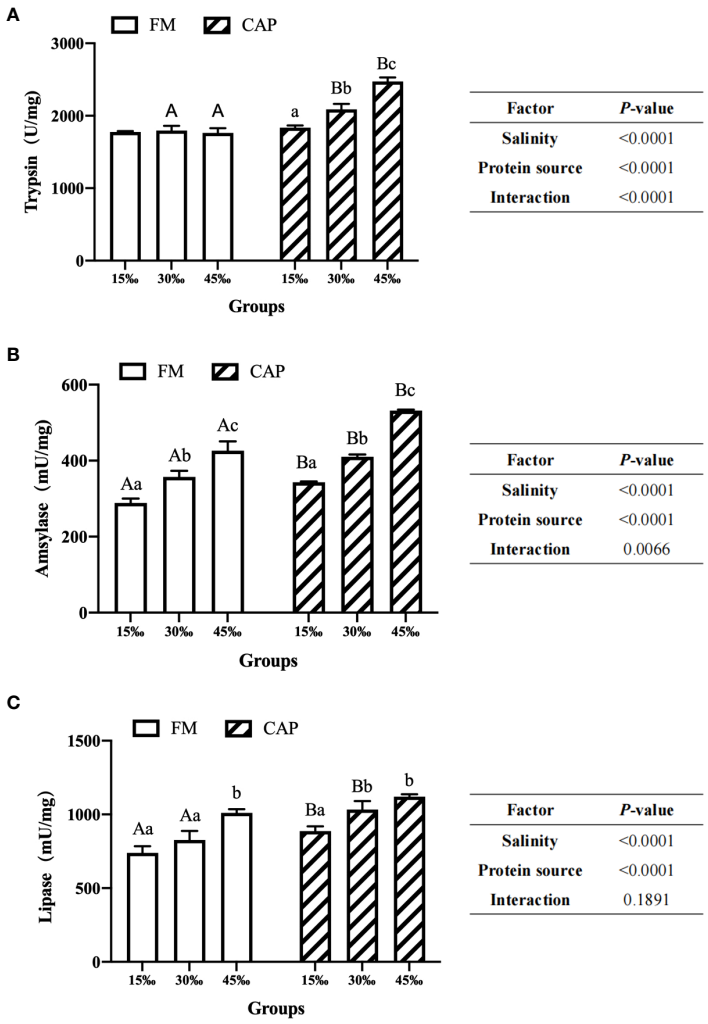


FIGURE 2
Effects of protein sources and salinity on intestinal trypsin **(A)**, amylase **(B)** and lipase **(C)** activities in L. vannamei. Under the same salinity, values with different capital letter superscripts mean significant difference among different protein source (P<0.05); under the protein sources, values with different small letter superscripts mean significant difference between different salinity (P< 0.05).

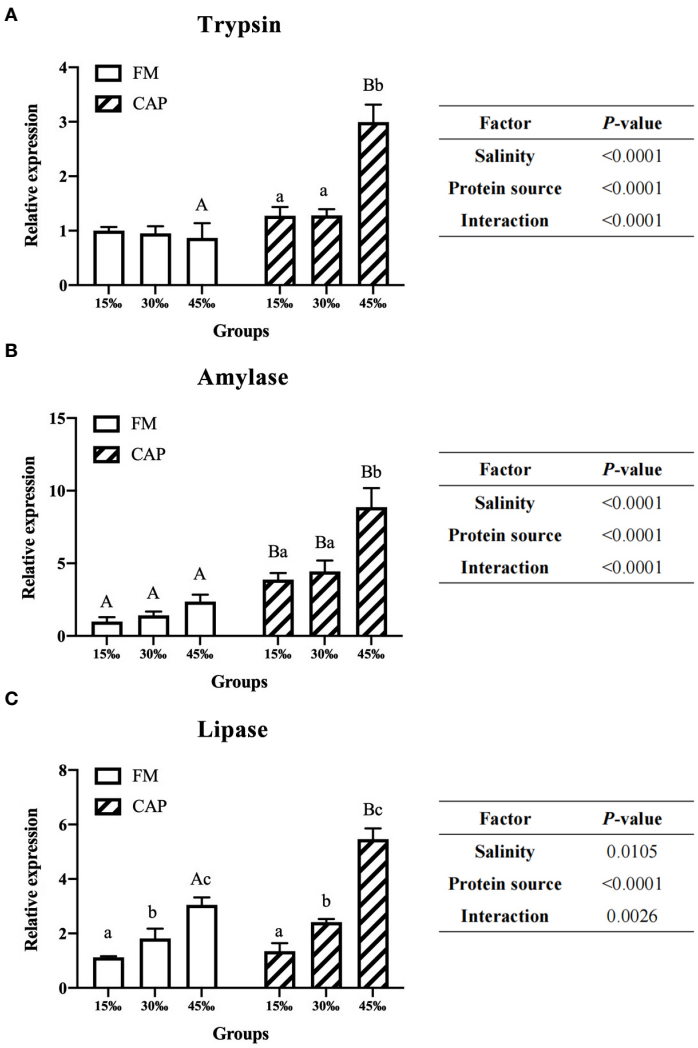
L. vannamei in the CAP group were significantly higher than those in the FM group at 15‰ salinity (P< 0.05). At 30‰ salinity, the activities of all the three intestinal digestive enzymes of *L. vannamei* in the CAP group were significantly higher than those in the FM group (P< 0.05). At 45‰ salinity, the activities of trypsin and amylase but not that of lipase in the CAP group were significantly increased when compared with those in the FM group (P< 0.05).

The activities of three intestinal digestive enzymes, including trypsin, amylase and lipase, were significantly up-regulated with the increase in the salinity both in the FM and the CAP groups, with the only exception being that the trypsin activities in the FM group at different salinities were not significantly changed. In the FM group, amylase activities in the intestine of *L. vannamei* at three different salinities were significantly different, with the highest level at 45‰ salinity. The intestinal lipase activity of *L. vannamei* at 45‰ salinity was significantly higher than those at 15‰ and 30‰ salinities. However, there was no significant difference between the activities of lipase in the intestine of *L. vannamei* at 15‰ and 30‰ salinities. In the CAP group, both the intestinal trypsin and amylase activities of *L. vannamei* were significantly induced by the increase in salinity, reaching the peak at 45‰ salinity. The activities of intestinal lipase in *L. vannamei* at 30‰ and 45‰ salinities were not significantly different but both of them were significantly higher than that at 15‰ salinity.

Two-way ANOVA showed that the protein source and salinity significantly affected the activities of trypsin, amylase, and lipase in the intestine of *L. vannamei*. The interaction of protein source and salinity had a significant effect on the activities of trypsin and amylase but not on that of lipase.

3.4 The gene expression of intestinal digestive enzymes

When compared with those in the FM group (Figure 3), the expression levels of intestinal Trypsin and Lipase genes of *L. vannamei* in the CAP group were significantly higher only at 45‰ salinity, while the intestinal Amylase expression levels were significantly increased at all the studied salinities ($P< 0.05$). The gene expression levels of Lipase but not those of Trypsin and Amylase in the intestine of *L. vannamei* were significantly raised by the increase in salinity in the FM group. However, there was no significant difference in the gene expression of Trypsin and Amylase at different salinities. In the CAP group, the intestinal Lipase expression levels of *L. vannamei* at the three different salinities were significantly different from each other. Both the intestinal Trypsin and Amylase expression at 45‰ salinity were significantly higher than those at 15‰ and 30‰ salinities; there was no significant difference between the FM group and the CAP group. Two-way ANOVA showed that the protein source, salinity, and their interaction had a significant effect on the expression of Trypsin, Amylase, and Lipase in the intestine of *L. vannamei*.



Factor	*P*-value
Salinity	<0.0001
Protein source	<0.0001
Interaction	<0.0001

Factor	*P*-value
Salinity	<0.0001
Protein source	<0.0001
Interaction	<0.0001

Factor	*P*-value
Salinity	0.0105
Protein source	<0.0001
Interaction	0.0026

FIGURE 3
Effects of protein source and salinity on the gene expression of intestinal degistive enzymes in L. vannamei. The detection of gene expression were performed in triplicate for each sample. Expression values were normalized to those of EF1α using the Livak ($2^{-\Delta\Delta Ct}$) method and the data were provided as the means ± SD of triplicate assays. Under the same salinity, values with different capital letter superscripts mean significant difference among different protein source ($P < 0.05$). Under the protein sources, values with different small letter superscripts mean significant difference between different salinity ($P < 0.05$). **(A)** Trypsin gene expression, **(B)** Amylase gene expression, **(C)** Lipase gene expression.

3.5 The expression of immune genes in the intestine

Compared with that in the FM group (Figure 4), the expression of immune genes in the intestine of *L. vannamei* in the CAP group was significantly increased at different salinities ($P<0.05$), with the only exception being that ALF expression was not significantly different between the FM and the CAP groups at 15‰ salinity. In the FM and the CAP groups, a change in salinity led to a significantly increased expression of the detected immune genes except for PO in the FM group and LYZ in the CAP group. In the FM group, the expression levels of PPO and CRU genes in the intestine of *L. vannamei* at different salinities were significantly different, with the highest level at 45‰ salinity. The LGBP expression at 45‰ salinity was significantly higher than that at 15‰ and 30‰ salinities

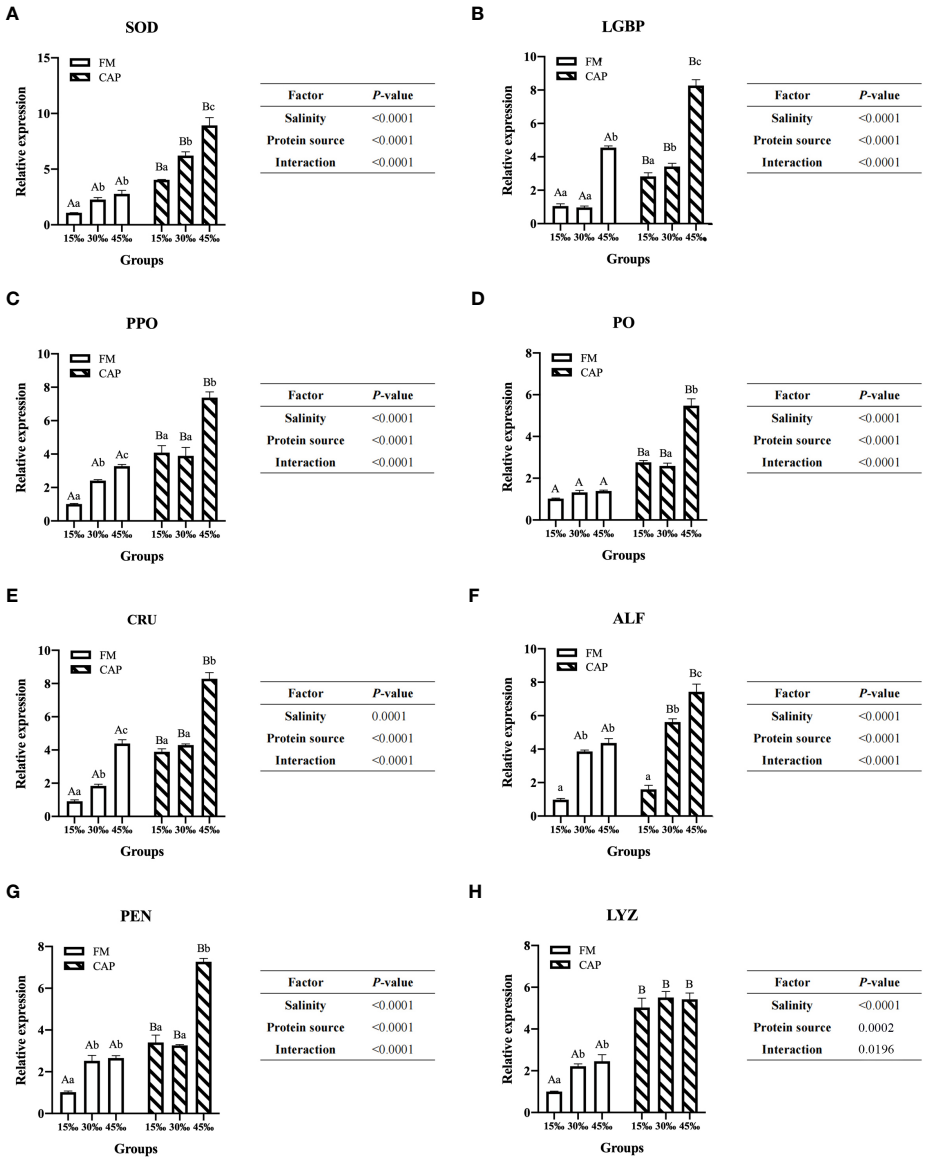


FIGURE 4
Effects of protein source and salinity on intestinal immune gene expression in L. vannamei. The detection of gene expression were performed in triplicate for each sample. Expression values were normalized to those of EF1α using the Livak ($2^{-\Delta\Delta Ct}$) method and the data were provided as the means ± SD of triplicate assays. Under the same salinity, values with different capital letter superscripts mean significant difference among different protein source ($P < 0.05$) The expression of immune genes in the intestine. Under the protein sources, values with different small letter superscripts mean significant difference between different salinity ($P < 0.05$). **(A)** SOD gene expression, **(B)** LGBP gene expression, **(C)** PPO gene expression, **(D)** PO gene expression, **(E)** CRU gene expression, **(F)** ALF gene expression, **(G)** PEN gene expression, **(H)** LYZ gene expression.

(P<0.05), and the LGBP expressions at 15‰ and 30‰ salinities were not significantly different (P>0.05). The expression of SOD, ALF, PEN, and LYZ genes at 30‰ and 45‰ salinities were significantly higher than those at 15‰ salinity, and their expression levels at 30‰ and 45‰ salinity were not significantly different (P>0.05). In the CAP group, the gene expression levels of SOD and ALF in the intestine of *L. vannamei* were significantly different at different salinities, with the highest level at 45‰ salinity. The expressions of intestinal LGBP, PPO, PO, CRU, and PEN at 45‰ salinity were significantly higher than those at 15‰ and 30‰ salinities, but their expressions at 15‰ and 30‰ salinities were not significantly different. Two-way ANOVA showed that protein source, salinity, and their interactions had significant effects on the expressions of all the eight detected intestinal immune genes in *L. vannamei*.

3.6 Intestinal microbiota analysis

3.6.1 Richness and diversity analysis

The raw data of intestinal microbiota analysis in this study have been deposited in the SRA database with the accession number PRJNA870236. As shown in Figure 5, there were 193 core operational taxonomic units (OTUs) among all the tested groups according to Venn diagram analysis. In contrast, 169, 300, 108, 73, 107, and 107 OTUs were unique to FM15‰, FM30‰, FM45‰, CAP15‰, CAP30‰, and CAP45‰ groups, respectively. Clearly, the proportions of shared OTUs within each group were 52.31%, 39.15%, 64.12%, 72.56%, 64.33%, and 64.33%, respectively.

Alpha indices, including Good's coverage, observed species (Sobs), Chao1, abundance-based coverage estimator (ACE), Shannon, and Simpson, were evaluated to investigate the significant differences in the diversity and richness of microbiota in the intestine among different treatments (Table 4). Good's coverage estimates showed that all groups had more than 99% bacterial species. The salinity significantly affected the Sobs, Chao1, ACE, Shannon, and Simpson indices, while the protein source significantly affected the Chao1 and Simpson indices of the intestinal microbiota in *L. vannamei*. Besides, the interaction of protein source and salinity significantly affected the Sobs, Chao1, ACE, and Simpson indices of the intestinal microbiota (P< 0.05). The analysis of beta diversity by PCoA analysis showed that both the samples in the FM and the CAP groups had obvious separation under different salinity conditions, and the two PCoA axes showed 63.60% variation among the groups (Figure 6).

3.6.2 Comparison of the intestinal microbiota composition

As shown in Figure 7A, the top 10 intestinal bacterial phyla of *L. vannamei* sorted from high to low were Bacteroidetes,

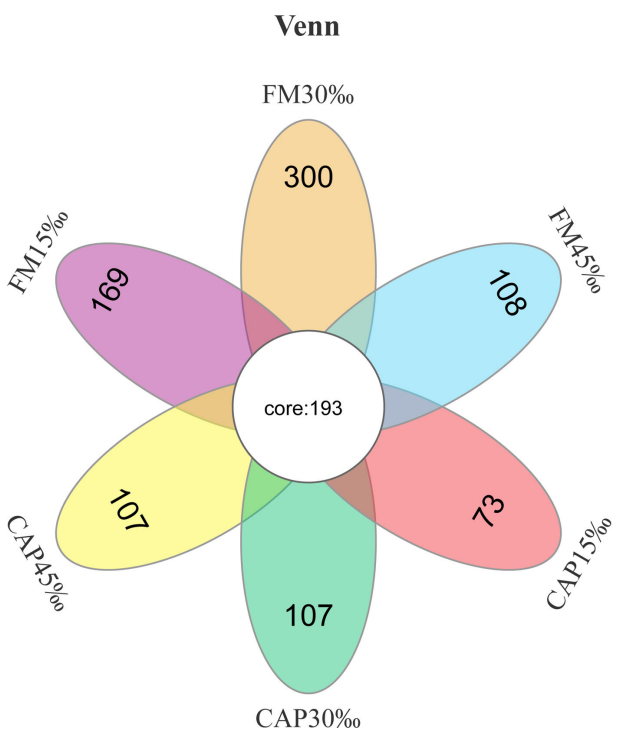


FIGURE 5
Venn diagram of shared and unique OTUs of intestinal microbiota in L. vannamei.

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TABLE 4 Effects of protein sources and salinity on microflora diversity in the intestine of *L. vannamei*.

Items		Good's coverage	Sobs	Chao1	ACE	Shannon	Simpson
Protein source	**Salinity/‰**						
FM	**15**	0.99 ± 0.00	555.00 ± 64.35^{a}	722.04 ± 13.50^{Bb}	635.19 ± 11.89^{a}	3.97 ± 0.09^{a}	0.75 ± 0.10^{Aa}
	30	0.99 ± 0.00	525.00 ± 7.07^{a}	588.28 ± 9.68^{a}	610.46 ± 16.66^{Aa}	4.29 ± 0.04^{ab}	0.82 ± 0.02^{b}
	45	0.99 ± 0.00	738.00 ± 31.95^{Bb}	798.01 ± 20.33^{Bc}	774.83 ± 8.49^{b}	4.89 ± 0.10^{b}	0.91 ± 0.03^{b}
CAP	**15**	0.99 ± 0.00	562.00 ± 14.73	612.40 ± 2.29^{Aa}	636.98 ± 13.29^{a}	4.27 ± 0.42	0.88 ± 0.05^{B}
	30	0.99 ± 0.00	528.33 ± 36.90	619.34 ± 25.69^{a}	648.59 ± 11.87^{Ba}	4.32 ± 0.29	0.88 ± 0.03
	45	0.99 ± 0.00	574.33 ± 70.69^{A}	702.42 ± 7.89^{Ab}	731.50 ± 3.34^{b}	4.58 ± 0.13	0.89 ± 0.03
***P*-value of two-way ANOVA**							
Salinity		0.9255	0.0056	<0.0001	<0.0001	0.0024	0.0009
Protein source		0.0974	0.0844	<0.0001	0.8713	0.9332	0.0052
Interaction		0.3910	0.0348	<0.0001	0.0031	0.1219	0.0021

Values with different capital letter superscripts indicated significant differences between different protein sources at the same salinity ($p<0.05$). Values with different small letter superscripts indicated significant difference among different salinities when the protein source was the same ($p<0.05$).

Proteobacteria, Actinobacteria, Verrucomicrobia, Planctomycetes, Tenericutes, Firmicutes, Patescibacteria, Chlamydiae, and Cyanobacteria. As shown in Figure 7B, the relative abundances of Bacteroidetes in the FM group both at 30‰ and 45‰ salinities were significantly lower than that at 15‰ salinity ($P< 0.05$), while there was no significant difference in those in the CAP group at different salinities ($P > 0.05$). At 15‰ salinity, the relative abundance of Bacteroidetes in the CAP group was significantly lower than that in the FM group. The salinity but not the protein source significantly affected the relative abundance of Bacteroidetes. However, the interaction of salinity and protein source had a significant effect on the relative abundance of Bacteroidetes. The relative abundance of Firmicutes in the FM group at 45‰ salinity was significantly higher than those at 15‰ and 30‰ salinities ($P< 0.05$), while there was no significant difference in the CAP group at different salinities ($P > 0.05$). The salinity, protein sources, and their interaction had a significant effect on the relative abundance of Firmicutes ($P< 0.05$).

At the family level (Figure 8A), the prevalent microbial communities in the intestine of *L. vannamei* consisted of Flavobacteriaceae, Rhodobacteraceae, Vibrionaceae, Psychromonadaceae, and Rubritaleaceae. As shown in Figure 8B, the change in salinity had no significant effect on the relative abundance of Rhodobacteraceae and Rubrialeaceae in the FM group and the relative abundance of Vibrionaceae in the CAP group. In the CAP group, the relative abundance of

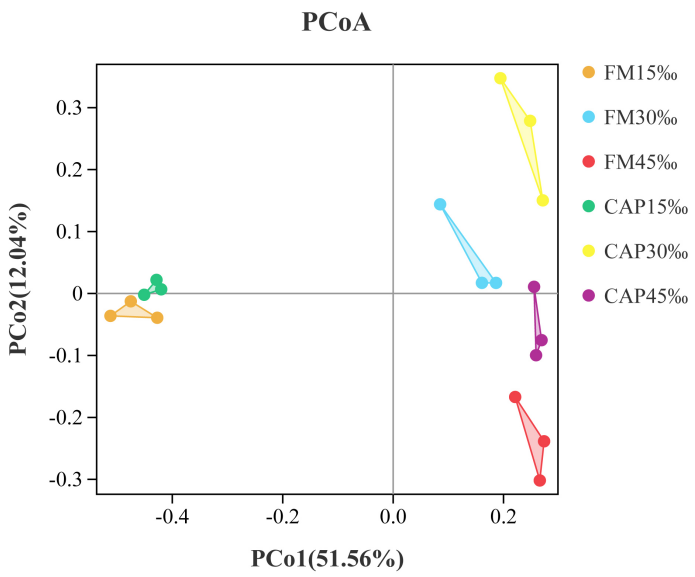

FIGURE 6
Principal coordinates analysis (PCoA) based on Bray analysis of intestinal microbiota in L. vannamei.

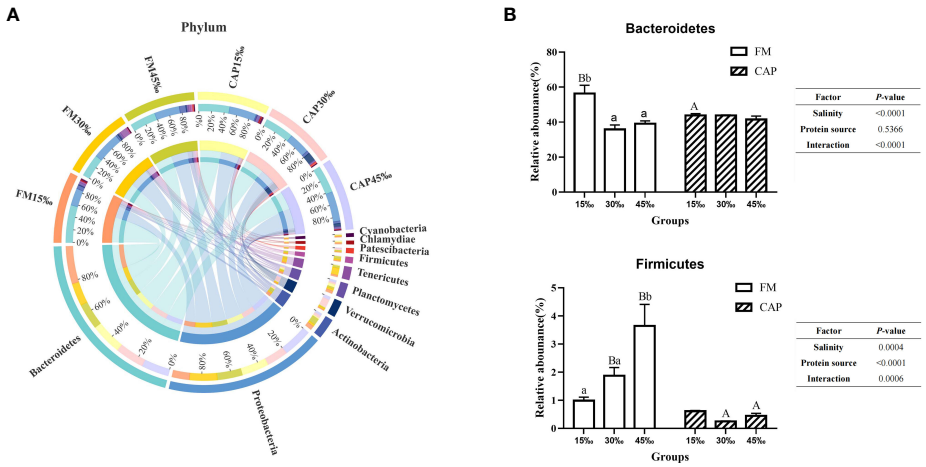

FIGURE 7
Effects of protein sources and salinity on the structure and composition of intestinal microbiota community in L. vannamei at phylum level. **(A)** Mean abundance indifferent groups. One side of the graph is the grouping information, and the other side is the species information. The lines on both sides represent corresponding relationship pairs. The thicker the lines, the greater the abundance value. **(B)** Relative abundance with significant differences in phylum levels. Under the same salinity, values with different capital letter superscripts mean significant difference among different protein source ($P<0.05$); under the protein sources, values with different small letter superscripts mean significant difference between different salinity ($P< 0.05$).

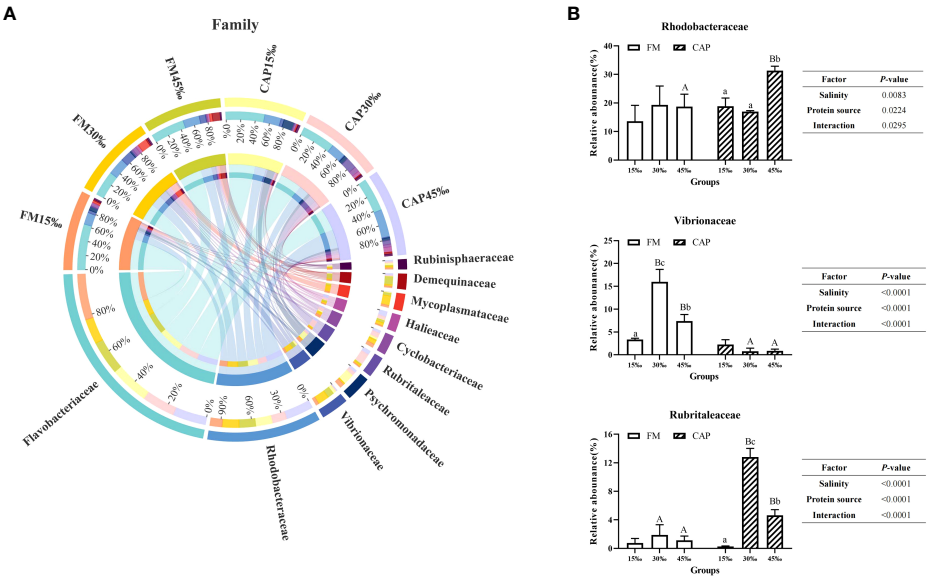

FIGURE 8
Effects of protein sources and salinity on the structure and composition of intestinal microbiota community in L. vannamei at family level. **(A)** Mean abundance indifferent groups. One side of the graph is the grouping information, and the other side is the species information. The lines on both sides represent corresponding relationship pairs. The thicker the lines, the greater the abundance value. **(B)** Relative abundance with significant differences in family levels. Under the same salinity, values with different capital letter superscripts mean significant difference among different protein source ($P<0.05$); under the protein sources, values with different small letter superscripts mean significant difference between different salinity ($P< 0.05$).

Rhodobacteraceae at 45‰ salinity was not only significantly higher than those at 15‰ and 30‰ but also significantly higher than that in the FM group at the same salinity ($P< 0.05$). The relative abundances of Vibrionaceae were significantly different among the FM groups at the three different salinities, with the highest level at 30‰ salinity, and the relative abundances of Vibrionaceae in the CAP group at 30‰ and 45‰ salinities were significantly lower than that in the FM group at the same salinity. In the CAP group, the relative abundances of Rubrialeaceae were significantly different at the three different salinities. When compared with the FM group, the relative abundances of Rubrialeaceae were significantly increased in the CAP group at 30‰ and 45‰ salinities ($P< 0.05$). The salinity, protein sources, and their interaction significantly affected the relative abundances of Rhodobacteraceae, Vibrionaceae, and Rubrialeaceae ($P< 0.05$).

At the genus level (Figure 9A), *Actibacter* was the species with the highest abundance, followed by *Motilimonas*, *Vibrio*, *Halocynthiibacter*, and *Ruegeria*. As shown in Figure 9B, the change in salinity had a significant effect on the relative abundance of *Vibrio* and *Candidatus_Bacilloplasma* in the FM group but not in the CAP group. The relative abundance of *Vibrio* in the FM group at 30‰ salinity was significantly higher than those at 15‰ and 45‰ salinities. The relative abundance of *Vibrio* at 30‰ salinity in the CAP group was significantly lower than that in the FM group. With the increase in salinity, the relative abundance of *Candidatus_Bacilloplasma* in the FM group increased first and then decreased, with the only insignificant difference between 15‰ and 30‰ salinities. At all the three studied salinities, the relative abundance of *Candidatus_Bacilloplasma* in the CAP group was significantly lower than that in the FM group. The change in salinity had a significant effect on the relative abundance of *Rubritalea* in the CAP group but not in the FM group. The relative abundances of *Rubritalea* were significantly different from each other in the CAP group at all the three studied salinities, with the highest level at 30‰ salinity. At 30‰ and 45‰ salinities, the relative abundances of *Rubritalea* in the CAP group were significantly higher than that in the FM group ($P< 0.05$). The relative abundances of *Ruegeria* both in the FM and the CAP groups at 45‰ salinity were significantly lower than those at the 15‰ and 30‰ salinities. When compared with the FM group, the relative abundances of *Ruegeria* were significantly increased in the CAP group at all the three studied salinities ($P< 0.05$). Besides, the salinity and protein sources significantly affected the relative abundances of *Vibrio*, *Candidatus_Bacilloplasma*, *Rubritalea*, and *Ruegeria* ($P< 0.05$), while the interaction of the salinity and protein sources had a significant effect on the relative abundances of *Vibrio*, *Candidatus_Bacilloplasma*, and *Rubritalea* but not on that of *Ruegeria*.

3.6.3 Functional prediction of the intestinal microbial community

Changes in the presumptive functions of intestinal microflora were examined using Tax4fun software to predict the metagenomes. As shown in Figure 10A, the top 10 predicted functions had the following relative abundances: membrane transport (13.28–14.40%), carbohydrate metabolism (12.71–12.99%), amino acid metabolism (12.48–12.95%), metabolism of cofactors and vitamins (7.03–7.20%), energy metabolism (6.98–7.14%), signal transduction (6.43–7.39%), nucleotide metabolism (5.23–5.38%), translation (4.18–4.65%), xenobiotic biodegradation and metabolism (3.96–4.30%), and replication and repair (3.74–4.24%).

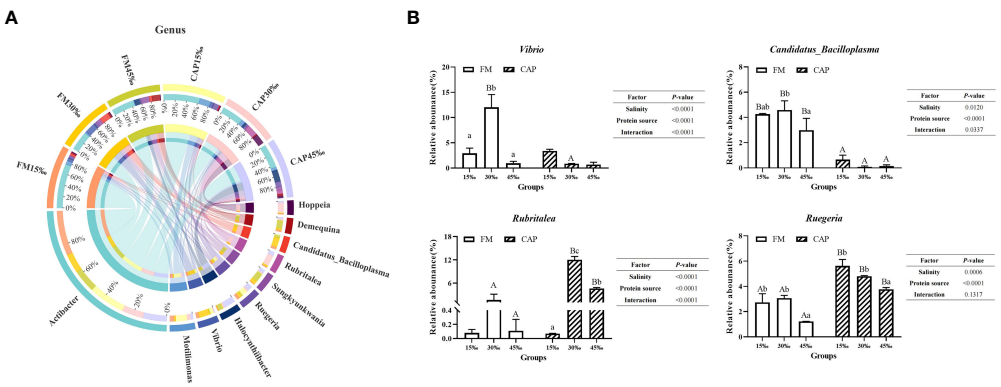

FIGURE 9
Effects of protein sources and salinity on the structure and composition of intestinal microbiota community in *L. vannamei* at genus level. **(A)** Mean abundance indifferent groups. One side of the graph is the grouping information, and the other side is the species information. The lines on both sides represent corresponding relationship pairs. The thicker the lines, the greater the abundance value. **(B)** Relative abundance with significant differences in genus levels. Under the same salinity, values with different capital letter superscripts mean significant difference among different protein source ($P<0.05$); under the protein sources, values with different small letter superscripts mean significant difference between different salinity ($P< 0.05$).

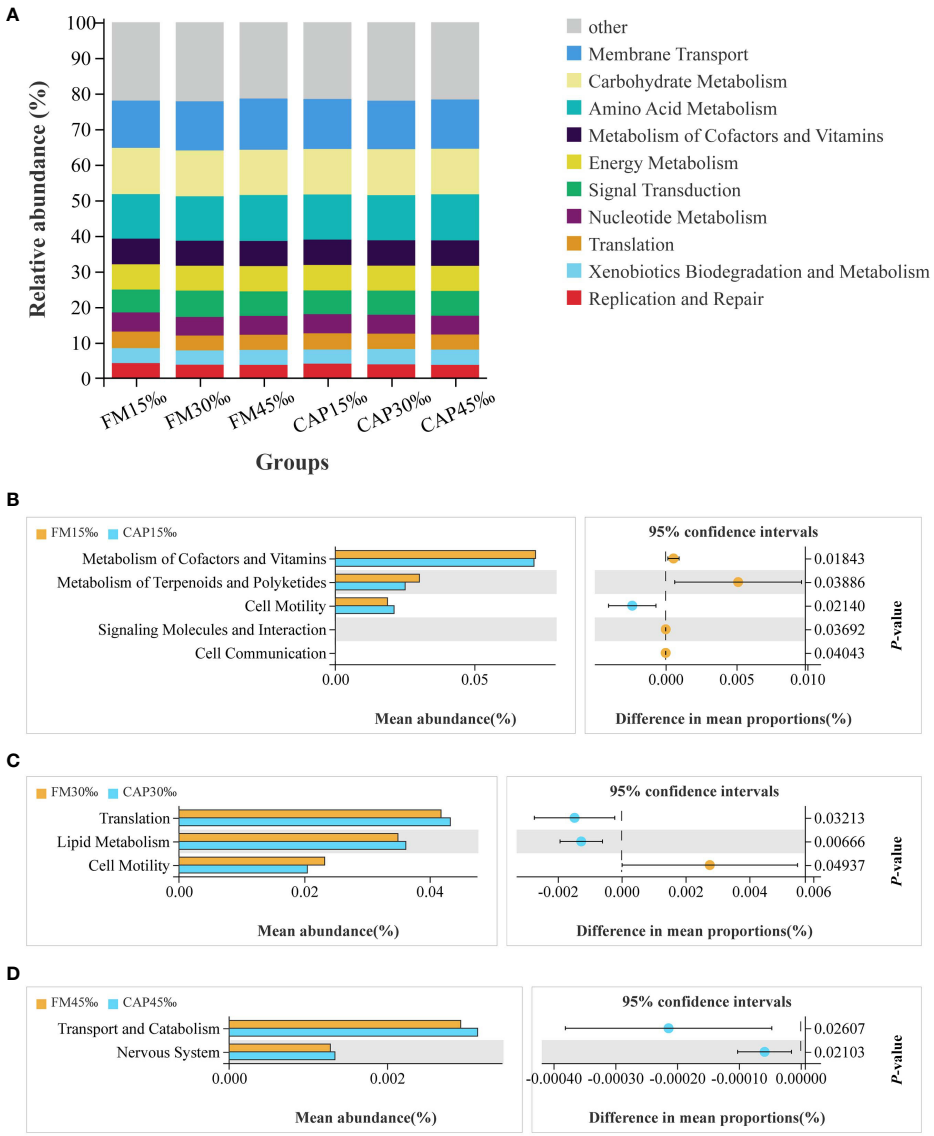

FIGURE 10
Functional prediction of protein sources and salinity in intestinal microbial community. **(A)** Relative abundances of the top 10 predicted functions. **(B–D)** Welch's t-tests of the significantly different functions at level 2.

The results of Welch's t-test showed that there were several predicted pathways for significant enrichment in the microbial community with KEGG level 2 at different salinities of the same protein source ($P< 0.05$). As shown in Figure 10B, the enriched functions related to cell motility were significantly increased, while the functions of metabolism of cofactors and vitamins, metabolism of terpenoids and polyketides, signalling molecules and interaction, and cell communication were significantly decreased in the CAP group when compared with the FM group at 15 ‰ salinity. At 30 ‰ salinity (Figure 10C), the functions of translation and lipid metabolism were significantly increased, while the function of cell motility was significantly decreased in the CAP group when compared with that in the FM group. Comparison between the CAP and FM groups at 45‰ salinity indicated that the functions of transport, catabolism, and the nervous system were significantly increased in the CAP group (Figure 10D).

4 Discussion

There is a great demand for fishmeal in the culture of *L. vannamei*. At present, the substitution of fishmeal has attracted much attention in *L. vannamei* culture due to its shortage (10).

CAP has been used in aquaculture to improve the growth performance, feed utilization, and immune response of *L. vannamei* (27). Salinity is a crucial environmental factor for *L. vannamei* culture and has a great impact on the utilization of protein (44). In this study, the growth, disease resistance, intestinal digestive capacity, immunity, and microbiota structure of *L. vannamei* fed on CAP at different salinities were investigated to evaluate the application value of CAP in shrimp culture.

As a high-quality SCP, CAP has been used for several aquaculture species with various beneficial effects. In *A. schlegelii*, CAP can replace fishmeal in the diet up to 58.20% without adverse effects on growth performance, antioxidation, and digestive enzyme activity (26). In *M. salmoides*, the replacement of fishmeal with CAP did not affect its growth performance and whole-body composition but increased the digestive capacity and antioxidant index. The optimal CAP replacement level was 49.80% with a maximum WGR of juvenile *M. salmoides* (31). Lu et al. found that there is no negative effect on the growth performance and liver health of *M. salmoides* when the level of CAP replacing fishmeal is less than 50%, and excessive CAP inclusion may damage liver health (29). CAP supplementation in practical diets has a beneficial effect on the growth performance of *C. carpio* var. *Jian* (28) and *O. niloticus* (27). In addition, the antioxidant capacity of *C. carpio* var. *Jian* was increased and the whole-body energy homeostasis of *O. niloticus* could be regulated through the AMPK signalling pathway. In *L. vannamei*, CAP could substitute 30% of fishmeal in a diet containing 560 g/kg of fishmeal without adverse effects on growth, intestinal histology, and immunity (27). Consistent with the previous study performed in *L. vannamei*, our study also found that the growth performance, digestive capacity, and immunity of *L. vannamei* could be improved by dietary CAP. The improved growth of *L. vannamei* was evident since the WGR and SGR of *L. vannamei* in the CAP group were significantly higher than those in the FM group at 30‰ and 45‰ salinities. Moreover, the SR, WGR, and SGR of *L. vannamei* were not negatively affected by dietary CAP at all the studied salinities. The digestive capacity of *L. vannamei* was improved since both the activities and the expressions of the digestive enzymes were significantly increased in the CAP group when compared with those in the FM group. Furthermore, dietary CAP significantly increased the SR of *L. vannamei* after a pathogen challenge, which indicated that the immunity of *L. vannamei* was improved by dietary CAP. However, the FCR of *L. vannamei* in the CAP group was significantly higher than that in the FM group, while the comparison of PER between the CAP and the FM groups was opposite at 30‰ and 45‰ salinities. The increased FCR and decreased PER suggested that CAP may not be perfect. Nonetheless, the possibility of using CAP as a safe and effective alternative protein source in aquatic feed is beyond doubt.

The intestinal histology and digestive capacity of fish and shrimp fed on CAP have been investigated before (27, 31). However, there were no reports on the effect of dietary CAP on aquatic animals from the aspect of intestinal microbiota structures. In recent years, the sequencing technology of intestinal microbiota has received extensive attention. Studies have shown that the intestinal microbiota is closely linked to digestion, immunity, metabolism, and the overall health of the host (45, 46). Based on this, the intestinal microbiota structure of *L. vannamei* fed on CAP was compared with that of *L. vannamei* fed on FM. Alpha diversity analysis showed that the diversity and abundance of the intestinal microbiota of *L. vannamei* in the CAP group were significantly different from those in the FM group, suggesting that dietary CAP would change the diversity of intestinal microbiota. Bacteroidetes and Proteobacteria were considered the dominant phyla in the intestine of fish and shrimp (47–50). In this study, the two phyla were the first and second dominant phyla both in the CAP and the FM groups, and there was no significant difference in their relative abundance between the CAP and the FM groups, which further proved that there was no harm in the intestinal microbiota diversity of *L. vannamei* when fed on CAP. An interesting discovery was that the beneficial bacteria significantly increased while the harmful bacteria significantly decreased in the intestine of *L. vannamei* at the phylum, family, and genus levels. The effect was more obvious at the genus level. *Vibrio* is the most common core bacterial group in the intestine of crustaceans (51). Several members in the *Vibrio* genus are conditionally pathogenic bacteria, which seriously affects the survival and health of shrimp, such as *V. parahaemolyticus* (52), *Vibrio harveyi* (53), and *Vibrio alginolyticus* (54). White faeces syndrome (WFS) is a severe disease and has drawn wide attention in shrimp culture. Huang et al. found that *Vibrio* and *Candidatus_Bacilloplasma* were the two overrepresented genera in shrimp with WFS (55). In this study, the relative abundance of *Vibrio* genus in the CAP group was significantly lower than that in the FM group at a salinity of 30‰, and the relative abundance of *C._Bacilloplasma* was significantly increased in the CAP group at 30‰ and 45‰ salinities. On the contrary, the relative abundances of *Rubritalea* and *Ruegeria* were significantly increased by dietary CAP. *Rubritalea* is a genus in the phylum Verrucomicrobia that could degrade the excess mucin produced by the inner wall of the intestine and was helpful for normal growth (56). *Ruegeria* is a global gram-negative marine bacterium that can co-exist with unicellular eukaryotes and inhibit many marine pathogens (57, 58). It could be speculated that since the change in the intestinal microbiota structure of *L. vannamei* was associated with increased disease resistance of *L. vannamei* in the CAP group when compared with the FM group, it may make a valuable contribution to increasing disease resistance. Additionally, the predictive function of intestinal microflora also revealed that

metabolism-related functions account for most of the top 10 functions and are closely related to the promotion of *L. vannamei* growth. However, further study is needed to confirm this speculation.

Several indices can be used to evaluate the immunity of shrimp, including the activities of SOD, LGBP, antibacterial peptide (AMP), and so on. SOD is an important antioxidant enzyme that provided the first line of ROS elimination from cells (59). As a pattern recognition protein (PRP), LGBP is involved in the activation of shrimp immunity by recognizing LPS and β-1,3-glucan from gram-negative bacteria (60). The prophenoloxidase (proPO) system is an enzyme cascade system that is activated upon recognition of pathogens by PRPs like LGBP and plays an important role in the innate immunity of shrimp (61). Crustins, ALFs, and PEN are the three important kinds of AMPs that contribute to the antibacterial capabilities of shrimp as efficient effectors (62). Lysozyme plays an important role in the shrimp's immune defence by destroying peptidoglycan support, resulting in bacterial splitting under osmotic pressure within the bacteria (63). In this study, the relative expression levels of immune genes, including SOD, LGBP, PPO, PO, CRU, ALF, PEN, and LYZ, in the CAP group were higher than those in the FM group, which indicated that the intestinal immunity of *L. vannamei* could be enhanced by dietary CAP. The results were consistent with those of the intestinal microbiota analysis.

Since *L. vannamei* have an open hemolymph circulation system, they adjust their osmotic pressure effectively to adapt to the changes in external salinity (15, 16). Osmotic adjustment is an energy-dependent process. When facing changes in external salinity, providing adequate energy by manipulating the diet is an effective means of improving shrimp's survival abilities (21, 64). Li et al. found that the digestive and immune regulatory abilities of *L. vannamei* remained at a high level, and the expression level of antioxidant-related genes is also significant during salinity stress (44). Obviously, all the regulatory processes in *L. vannamei* required energy consumption. If the energy taken in from the external environment is not enough, *L. vannamei* will use their own body energy, resulting in a rapid reduction in growth. In this study, the growth performance of *L. vannamei* at 30‰ salinity was much better than that at 15‰ or 45‰ salinities, whether the protein source in the feed was FM or CAP. This was probably because the extra energy *L. vannamei* obtained from the feed could be used for growth at a proper salinity rather than coping with the pressure caused by low or high salinities. The activities and expression of intestinal digestive enzymes in *L. vannamei* at 45‰ salinity were significantly higher than those at 30‰ salinity, which further implied that *L. vannamei* need to take in more energy from the external environment in response to salinity stress. What cannot be ignored is that the FCR and PER of *L. vannamei* in the FM group were not obviously affected by the salinity change but they were significantly affected by the increase in salinity. Besides, the SR of *L. vannamei* after a pathogen challenge was only significantly affected by salinity change in the CAP group but not in the FM group. Differently, most of the expressions of immune genes were significantly increased with the increase in salinity both in the FM and the CAP groups. Furthermore, the changes in several main intestinal flora species at different levels of *L. vannamei* affected by salinity increase in the CAP group were significantly different from those in the FM group. All these results indicated that salinity change variably affected *L. vannamei* in several aspects when the protein sources were different.

Finally, our study found that the interaction of salinity and protein source significantly affected *L. vannamei* in most of the aspects studied, including growth performance, activities and expression of digestive enzymes, expression of immune genes, and abundance of special microbiota species at the phyla, family, and genus levels. The results suggest that the change in salinity had a significant impact on the effect of dietary CAP on *L. vannamei*, which could provide a theoretical reference for the practical application of CAP in aquatic feeds.

In conclusion, on the one hand, dietary CAP could effectively improve growth performance, disease resistance, intestinal digestive capacity, immunity, and microbial structure but not feed utilization in *L. vannamei* under the same salinity condition. On the other hand, the change in salinity had much more obvious effects on *L. vannamei* fed on CAP than the control in the aspects of growth performance, disease resistance, intestinal digestive capacity, immunity, and microbiota structure. In any case, CAP could be used as a safe and effective alternative protein source in shrimp feeds.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: NCBI [accession: PRJNA870236].

Ethics statement

This study was reviewed and approved by Guangdong Ocean University.

Author contributions

JC: Conceptualization, Investigation, Formal analysis, Writing-Original Draft. HW, HY, NH, FZ, CL, LS and BT: Methodology, Resources. SZ: Review and Editing, Supervision, Project administration, Funding acquisition. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the National Key R&D Program of China (Grant No. 2019YFD0900200), National Natural Science Foundation of China (Grant No. 32072988), General Program of Natural Science Foundation of Guangdong Province, China (Grant No. 2020A1515010319) and Guangdong Postgraduate Education Innovation Project (Grant No. 202266).

Acknowledgments

We thank Beijing Shoulang bio-technology Co., Ltd. for providing *Clostridium autoethanogenum* protein and Guangzhou Genedenovo Biotechnology Co., Ltd (Guangdong, China) for the services of sequencing and bioinformatics analysis (http://www.omicsmart.com).

Conflict of interest

Author FZ and CL were employed by Beijing Shoulang Bio-Technology Co., Ltd., Beijing, China.

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.

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 14 October 2022
DOI 10.3389/fimmu.2022.1036821

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Comparative Immunology, a section of the journal Frontiers in Immunology

RECEIVED 05 September 2022
ACCEPTED 28 September 2022
PUBLISHED 14 October 2022

CITATION
Liu J-X, Guo H-Y, Zhu K-C, Liu B-S, Zhang N and Zhang D-C (2022) Effects of exogenous taurine supplementation on the growth, antioxidant capacity, intestine immunity, and resistance against *Streptococcus agalactiae* in juvenile golden pompano (*Trachinotus ovatus*) fed with a low-fishmeal diet. *Front. Immunol.* 13:1036821. doi: 10.3389/fimmu.2022.1036821

Effects of exogenous taurine supplementation on the growth, antioxidant capacity, intestine immunity, and resistance against *Streptococcus agalactiae* in juvenile golden pompano (*Trachinotus ovatus*) fed with a low-fishmeal diet

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Taurine has various biological functions in fish, playing an essential role in growth, resistance to oxidative stress, and intestine immunity. Here, we evaluated the effects of exogenous taurine added to low-fishmeal diets on the growth, anti-oxidative stress, intestine immunity, and *Streptococcus agalactiae* resistance in juvenile golden pompano (*Trachinotus ovatus*). Our study showed that exogenous taurine supplementation of 1.2% (T3 group) greatly enhanced the weight gain rate and specific growth rate (SGR) of juvenile golden pompano, significantly upregulating growth-related factor expression in the brain and liver, as well as the levels of growth-related parameters in the serum. Polynomial regression analysis using SGR estimated the optimal dietary taurine level for golden pompano at 1.18%. Moderate exogenous taurine also increased the muscular thickness and villus length within the intestine, maintained intestinal physical barrier stability, activated the Nrf2/Keap-1/HO-1 signaling pathway, increased intestinal antioxidant enzyme gene expression and antioxidant enzyme activity in the serum, and upregulated immunoglobulin and complement levels in parallel with declining reactive oxygen species (ROS) levels in the serum. Antioxidant factor expression was also upregulated in the intestine. Furthermore, supplementation suppressed NF-κB signaling and intestinal pro-inflammatory cytokine gene expression, increased anti-inflammatory cytokine gene expression, and improved intestine immunity.

Finally, taurine supplementation improved the survival rate of golden pompano challenged with *S. agalactiae*. Overall, our findings provide additional information and support for the rational use of taurine in healthy aquatic animal farming.

KEYWORDS

Trachinotus ovatus, taurine, intestine immunity, growth, antioxidant capacity, *Streptococcus agalactiae*

Introduction

As a source of high-quality protein for aquaculture, the limited availability of fishmeal has led to soaring prices, resulting in its partial replacement with plant and animal protein for large-scale economic fish farming (1–5). Currently, fishmeal substitutes include animal (including chicken (1) and feather meal (2)) and plant (including soybean meal (3), fermented soybean meal (4), soybean protein concentrate (5), and corn gluten meal (2)) protein. However, replacing fishmeal with other plant and animal proteins in excess can cause a range of negative effects on aquatic animals. For example, low-fishmeal ratios can cause reduced growth performance (6), altered intestinal microflora (7), structural damage to the intestine (8), oxidative stress (9), and reduced intestine immunity (10, 11) in fish, leading to greater susceptibility to bacteria and viruses. Therefore, it is essential to explore ways to mitigate the negative effects of low-fishmeal feeds on growth performance, antioxidant capacity, and intestine immunity of aquatic animals.

Taurine, chemically known as 2-aminoethanesulfonic acid ($C_2H_7NO_3S$), is a β-sulfur-containing amino acid that promotes growth, improves antioxidant capacity, and protects against inflammation. As a functional amino acid, taurine has been widely studied and applied in aquatic animal nutrition (12). Numerous studies have shown that exogenous taurine in low-fishmeal diets can effectively enhance the growth of *Monopteros albus* (13, 14), *Pagrus major* (15), *Dicentrarchus labrax* (16), and *Rachycentron canadum* (17). Taurine is highly effective against oxidative stress, reducing the oxidative stress effect of Cd on the liver and kidneys of *Clarias batrachus* (18). Studies on *Ictalurus punctatus* (19) showed that taurine could significantly upregulate the mRNA expression of antioxidant enzymes through the Nrf2/Keap-1 signaling pathway to attenuate intestinal oxidative damage and effectively avoid weakened intestine immunity caused by oxidative damage.

Intestine immunity plays a vital role in the regulation of immune homeostasis. Therefore, intestinal health is essential for healthy aquatic animal husbandry (20). The intestinal health of aquatic animals depends on the combined effects of the intestinal physical barrier, antioxidant capacity, and immune barrier (21). Taurine was shown to improve intestinal barrier integrity, increasing villus length and muscular thickness in *M. albus* (13) under a high-fat diet. This resulted in a more regular arrangement of the intestinal villi, which was beneficial for maintaining intestinal health. Taurine is also a good facilitator of intestine immunity in aquatic animals. Exogenous taurine was effective in reducing the intestinal inflammatory response and improving intestinal health in *I. punctatus* (19), *Ctenopharyngodon idella* (22), and *D. labrax* (23).

Golden pompano (*Trachinotus ovatus*) has a wide distribution, high adaptability, and tasty meat. Owing to the maturation of artificial broodstock and culture techniques, it has become one of the most essential marine fish in China for large-scale culture (24, 25). However, golden pompano fed low-fishmeal feed are prone to decreased growth performance, oxidative stress, intestinal structural damage, dysbiosis, and reduced intestine immunity (26–28). In recent years, frequent outbreaks of bacterial diseases during culture have occurred, most often caused by *Streptococcus agalactiae* (29, 30), *Vibrio harveyi* (31–33), and *Vibrio vulnificus* (34, 35) infections, resulting in significant economic losses. Therefore, in the context of a shortage of fishmeal resources and rising prices, the development of feed additives that promote the growth of golden pompano, maintain intestinal health, and improve intestine immunity are vital for its healthy culture.

This study aimed to determine the effects of exogenous taurine added to low-fishmeal feeds on growth, intestinal health, and resistance against *S. agalactiae* in juvenile golden pompano to provide a theoretical basis for taurine supplementation during their culture.

Materials and methods

Ethical statement

All experiments in this study were approved by the Animal Care and Use Committee of the South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences

(No. SCSFRI96-253) and performed according to the regulations and guidelines established by this committee.

Experimental diets

Taurine with a purity of 99.99% was purchased from Guangzhou Nutriera Biotechnology Co., Ltd. (Guangzhou, China). The feed formulations used in these experiments are listed in Table 1. Five isonitrogenous and isoenergetic diets were formulated according to the nutritional requirements of the golden pompano. Protein sources included fishmeal, chicken meal, soybean protein concentrate, fermented soybean meal, and corn protein meal. Lipid sources included fish and soybean oils. Taurine was added at 0.00% (T0), 0.40% (T1), 0.80% (T2), 1.20% (T3), and 1.60% (T4), respectively. First, all ingredients were added to the powder through a 40-mesh sieve. Second, fish oil, soybean oil, and water were slowly added and mixed to create a sinkable pellet feed using the Valva-60D-III twin-screw puffing and granulating machine (Valva Machinery Co., Ltd., Guangzhou, China). Finally, the diets were dried in an oven at 45°C until the moisture content was less than 10% and saved at 4°C (27). The amino acid composition of experimental diets is shown in Table 2.

Experimental procedure

The culture experiments were conducted in offshore cages (100 cm × 100 cm × 200 cm), in the Dapeng New District of Shenzhen, China. Juvenile golden pompano and *S. agalactiae* were obtained from our laboratory. All test fish were reared on T0 group feed without exogenous taurine for seven days before the start of the experiment. Subsequently, 1,050 fish (10.05 ±

TABLE 1 Formulation and nutrition level of the experimental diets (% dry matter basis).

Parameters	Group				
	T0	T1	T2	T3	T4
Ingredients (%)					
Fishmeal [a]	20.00	20.00	20.00	20.00	20.00
Chicken meal [a]	10.00	10.00	10.00	10.00	10.00
Soy protein concentrate [a]	10.00	10.00	10.00	10.00	10.00
Squid paste	5.00	5.00	5.00	5.00	5.00
Soybean meal [a]	12.00	12.00	12.00	12.00	12.00
Fermented soybean meal [a]	5.00	5.00	5.00	5.00	5.00
Corn gluten meal [a]	6.00	6.00	6.00	6.00	6.00
High gluten flour [a]	18.37	17.97	17.57	17.17	16.77
Fish oil [a]	6.00	6.00	6.00	6.00	6.00
Soybean oil [a]	3.00	3.00	3.00	3.00	3.00
$Ca(H_2PO_4)_2$ [a]	1.50	1.50	1.50	1.50	1.50
Choline chloride [a]	0.30	0.30	0.30	0.30	0.30
Vitamin mix [a,b]	1.00	1.00	1.00	1.00	1.00
Mineral mix [a,c]	1.00	1.00	1.00	1.00	1.00
L-lysine monohydrochloride [a]	0.50	0.50	0.50	0.50	0.50
DL-Methionine [a]	0.20	0.20	0.20	0.20	0.20
Threonine [a]	0.10	0.10	0.10	0.10	0.10
Ethoxyquin [a]	0.03	0.03	0.03	0.03	0.03
Taurine [a]	0.00	0.40	0.80	1.20	1.60
Nutrition level [d]					
Crude Protein (% dry matter)	42.79	42.74	42.69	42.63	42.58
Crude Lipid (% dry matter)	13.42	13.40	13.38	13.37	13.35
Moisture (% dry matter)	10.15	10.76	11.24	10.98	11.32
Ash (% dry matter)	8.53	8.65	8.33	8.71	8.39
Taurine	0.27	0.68	1.09	1.49	1.87

[a]Ingredients are provided by Guangzhou Nutriera Biotechnology Co., Ltd.
[b]Vitamin mix provides the following (Per kilogram content): vitamin A (8×10^6 IU), vitamin D3 (2×10^6 IU), vitamin E 40 000 mg, vitamin B 17 000 mg, vitamin B6 12 000 mg, vitamin B12 100 mg, vitamin K3 10 000 mg, D-pantothenic acid 35 000 mg, folic acid 1 000 mg, nicotinamide 90 000 mg, Biotin 200 mg, inositol 80 000 mg.
[c]Mineral provides the following (Per kilogram content): Fe 10 000 mg, Cu 1 200 mg, Zn 7 000 mg, Mn 5 500 mg, Co 250 mg, I2 250 mg, Se 50 mg, K 60 000 mg, Na 24 000 mg, Mg 60 000 mg
[d]Nutrition level is measured.

TABLE 2 Amino acid composition of the experimental diets(g·100g^{-1}).

Parameters	Group				
	T0	T1	T2	T3	T4
Aspartic acid	4.21	4.94	4.94	4.75	4.18
Threonine	1.93	2.26	1.94	2.16	1.90
Serine	2.08	2.49	2.46	2.30	2.05
Glutamic acid	8.38	10.2	8.94	9.64	8.54
Glycine	3.21	3.57	3.63	3.58	3.16
Alanine	2.86	3.30	2.82	3.21	2.81
Cysteine	0.56	0.50	0.56	0.56	0.46
Valine	1.99	2.27	2.13	2.34	2.03
Methionine	1.10	1.28	0.20	1.14	0.98
Isoleucine	1.65	1.90	1.77	1.96	1.74
Leucine	3.94	4.61	3.92	4.44	3.91
Tyrosine	1.25	1.45	1.30	1.43	1.30
Phenylalanine	2.14	2.54	2.47	2.44	2.16
Lysine	3.18	3.67	3.51	3.59	3.15
Histidine	1.06	1.24	1.20	1.23	1.05
Arginine	2.71	3.18	2.60	3.10	2.73
Proline	3.14	3.57	3.57	3.37	3.04
Taurine	0.27	0.68	1.09	1.49	1.87

0.05 g) were divided into 5 diet groups and placed in 15 cages (3 cages per diet group, 70 fish per cage). Each diet was administered four times daily, at 8:00, 10:00, 14:00, and 16:00 (feed ratio of 2:2:3:3), until apparent satiation for a total of eight weeks. Feeding status of the golden pompano was observed daily, and water quality conditions, water temperature, dissolved oxygen concentration, pH, and salinity were measured daily. Although several fish died during the trial, there were no major disease outbreaks.

Sample collection

The number of golden pompano in each cage was counted at the end of the culture period. After 24 h of fasting, nine fish per cage were randomly taken, placed in buckets containing eugenol (100–200 mg/L; Shanghai Medical Instruments Co., Ltd., Shanghai, China) for anesthesia, and then weighed. First, three fish per cage were snap-frozen in liquid nitrogen and then transferred to -80°C to analyze the routine nutrient composition. Second, blood was drawn from the three fish per cage using 2 mL syringes treated with 1% sodium heparin solution and centrifuged (3,500 × *g*, 4°C) for 10 min, after which the supernatant was transferred into a 1.5 mL cryotube and stored at -80°C for analysis of serum, growth, antioxidant, and immunological parameters. After collecting the serum, the brain, liver, and intestine of golden pompano were quickly collected, rapidly frozen in liquid nitrogen, transferred to -80°C, and stored for use in RNA extraction and gene expression analysis. Finally, the midguts of the remaining three fish per cage were removed and placed in a sampling bottle containing 4% paraformaldehyde solution and transferred to 4°C after 24 h for storage and used for histological analysis.

Growth performance

The parameters of growth performance were calculated as per the following formulas:

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

$$\text{Specific growth rate (SGR, \% / day)} = 100 \text{ x (ln final fish body weight-ln initial fish body weight)/number of days}$$

$$\text{Feed conversion ratio (FCR)} = \text{dry diet intake/net weight gain}$$

$$\text{Condition factor (CF,g/cm}^3\text{)} = 100 \text{ x final fish body weight/final fish body length}^3$$

$$\text{Survival rate (SR, \%)} = 100 \text{ x final fish number/30}$$

$$\text{Hepatosomatic index (HSI,\%)} = 100 \times \text{liver weight} / \text{final fish body weight}$$

$$\text{Viscerasomatic index (VSI,\%)} = 100 \times \text{viscera weight} / \text{final fish body weight}$$

$$\text{Feed intake (FI,\%/day)} = 100 \times \text{dry diet intake} / [(\text{initial fish body weight} + \text{final fish body weight})/2] / \text{number of days}$$

Organism composition

The organism composition of whole fish analysis was performed as follows. The content of crude protein and crude lipid were determined using Kjeldahl nitrogen determination and Soxhlet extraction, respectively. The moisture and ash content of whole fish was detected *via* constant weight and muffle furnace cautery method, respectively (AOAC, 2000).

Serum biochemical and immunological parameters analysis

The serum levels of growth hormone (GH), triiodothyronine, thyroxine, insulin-like growth factor receptor-1 (IGF-1), and insulin-like growth factor receptor-2 (IGF-2) were determined using kits (Jian Cheng Bioengineering Institute, Nanjing, China). The total antioxidant capacity (T-AOC), catalase (CAT), glutathione peroxidase (GSH-PX), superoxide dismutase (SOD), and lysozyme (LZM) activities, as well as the levels of malondialdehyde (MDA), reactive oxygen species (ROS), complement 3 (C3), complement 4 (C4), and immunoglobulins (IgA, IgG, and IgM) assays were performed with the respective kits (Beijing Sin-Uk Institute of Biological Technology, Beijing, China).

Midgut histological observation

As previously described by Ding et al. (36), we subjected golden pompano midguts to hematoxylin and eosin (H&E) staining to observe histological structures. First, the midgut was fixed in 4% paraformaldehyde solution for 24 h and dehydrated *via* ethanol grading. Samples were then washed in xylene and paraffin-embedded. After the paraffin wax was completely solidified, it was cut into 5-μm-thick sections using a slicer. The sections were transferred to a water bath at 40°C, and the paraffin wax was melted, allowing the samples to adhere to the slides, which were then dried in an oven at 40°C. They were removed after 24 h, H&E staining and sealing were used to prepare intestinal histological sections. Histological sections were observed under a 200 × light microscope (Leica, Wetzlar, Germany) and intestinal morphological parameters were measured by Image-Pro Plus 6.0 software (National Institutes of Health, Bethesda, USA). The field of view of each slide was divided equally into eight sections, and the length of intact intestinal villi, muscular thickness, and goblet cell number on each intestinal villus were randomly measured in each section. The mean values were used for analysis and preparing graphs in GraphPad Prism 8 (San Diego, California, USA).

Quantitative reverse-transcription PCR

To evaluate the regulatory effects of taurine on growth, intestine immunity, and resistance to oxidative stress in the golden pompano, we selected the following genes for qRT-PCR. First, we analyzed *growth hormone* (*GH*) and *neuropeptide Y* (*NPY*) mRNA expression in the brain. We then determined *insulin-like growth factor receptor-1* (*IGF-1*) and *insulin-like growth factor receptor-2* (*IGF-2*) mRNA expression in the liver. Finally, *CAT*, *GSH-PX*, *SOD*, *nf-e2-related nuclear factor2* (*Nrf2*), *Kelch-like ECH-associated protein-1* (*Keap-1*), *heme oxygenase-1* (*HO-1*), *nuclear factor kappa B* (*NF-κB*), *inhibitor protein-κB* (*IκB*), *IκB kinase* (*IKK*), *tumor necrosis factor-α* (*TNF-α*), *interleukin 1β* (*IL-1β*), *interleukin 8* (*IL-8*), and *interleukin 10* (*IL-10*) mRNA expression were determined in the intestine. *EF-1α* was selected as the housekeeping gene (37), and the primer sources for all tested genes are shown in Table 3, where the primers of the *NPY* were designed by the Primer Premier 6 (Premier Biosoft, Canada).

In this experiment, RNA was extracted from the brain, liver, and intestine by the HiPure Universal RNA Mini kit (Magen Biotech Co., Ltd., Guangzhou, China). RNA mass and concentration were determined using NanoDrop 2000 (Thermo Scientific, USA) and 1% agarose gel electrophoresis. cDNA was prepared using a PrimeScript™ RT kit and gDNA Eraser (TaKaRa, Dalian, China). qRT-PCR was performed using the SYBR® Green Premix Pro Taq HS qPCR Kit. The reaction conditions for qPCR were as described by Ma et al. (37). To eliminate the effect of chance, each sample was analyzed four times in duplicate, and three results were selected to calculate the relative expression levels of the target gene *via* the $2^{-\Delta\Delta CT}$ method (43).

Streptococcus agalactiae challenge

According to a previous study by Gao et al. (25), the LC50 of golden pompano after 120 h of *S. agalactiae* challenge corresponded to a *S. agalactiae* concentration of 2.0×10^7 CFU/fish. After eight weeks of feeding, 20 similarly sized and healthy

TABLE 3 Real-time PCR primer sequences.

Primers	Forward primer sequences (5′-3′)	Reverse primer sequences (5′-3′)	Source
GH	CAGCCAATCACAGACAGCC	GGAACTCCCAAGACTCCACTAA	Liu et al. (38)
IGF-1	CGCTAAATCTCACTTCTCCAAAA	CTCATCAAACCCTTAAACACCAC	Yang et al. (39)
IGF-2	CAATCTCTCCAACCAAATAACCC	CTTTTTTTCTCCCTCCAAACTCT	Yang et al. (39)
NPY	AAAAGACCACCCTGCCTCTG	ATGGCTAAGGAGGAGGGGTT	GenBank: OP292223
CAT	GGATGGACAGCCTTCAAGTTCTCG	TGGACCGTTACAACAGTGCAGATG	Liu et al. (24)
SOD	CCTCATCCCCCTGCTTGGTA	CCAGGGAGGGATGAGAGGTG	Liu et al. (24)
GSH-PX	GCTGAGAGGCTGGTGCAAGTG	TTCAAGCGTTACAGCAGGAGGTTC	Liu et al. (24)
HO-1	AGAAGATTCAGACAGCAGCAGAACAG	TCATACAGCGAGCACAGGAGGAG	Xie et al. (40)
Nrf2	TTGCCTGGACACAACTGCTGTTAC	TCTGTGACGGTGGCAGTGGAC	Liu et al. (41)
Keap-1	CAGATAGACAGCGTGGTGAAGGC	GACAGTGAGACAGGTTGAAGAACTCC	Liu et al. (41)
IL-1β	CGGACTCGAACGTGGTCACATTC	AATATGGAAGGCAACCGTGCTCAG	Liu et al. (24)
IL-8	CCGATCAACAGGGACTTCAA	GAGGACCGAGGGTTCAGACAG	Zhang et al. (42)
IL-10	AGTCAGTCTCCACCCCCATCTT	GCCCACTGGAGTTCAGATGCT	Zhang et al. (42)
TNF-α	GCTCCTCACCCACACCATCA	CCAAAGTAGACCTGCCCAGACT	Liu et al. (24)
NF-κB	CGTGAGGTCAGCGAGCCAATG	ATGTGCCGTCTATCTTGTGGAATGG	Liu et al. (24)
IKK	CCTGGAGAACTGCTGTGGAATGAG	ATGGAGGTAGGTCAGAGCCGAAG	Liu et al. (24)
IκB	GCTGGTCCATTGCCTCCTGAAC	GTGCCGTCTTCTCGTACAACTGG	Liu et al. (24)
EF-1α	AAGCCAGGTATGGTTGTCAACTTT	CGTGGTGCATCTCCACAGACT	Ma et al. (37)

fish per cage were randomly taken, and 200 μL of bacterial suspension at the concentration of 2.0×10^7 CFU/fish was intraperitoneally injected into each golden pompano using a sterile syringe. The same water temperature, pH, salinity, and dissolved oxygen mass concentration used during the culture trial were maintained throughout the 120 h challenge. The fish in each group were fed under the same conditions as the culture trial, with satiety feeding four times a day (8:00, 10:00, 14:00, and 16:00), and the mortality of each group was recorded every 12 h. The survival rate (%) was calculated at the end of the challenge.

Statistical analysis

One-way analysis of variance (ANOVA) was performed on the experimental data using SPSS 26.0. Tukey's test was used for multiple comparisons when differences were significant ($P < 0.05$). The results are expressed as mean ± standard deviation (mean ± SD). The Kaplan–Meier method was used to construct survival curves after *S. agalactiae* challenge, and differences in feed between the control and test groups were compared using the log-rank test.

Results

Growth performance

As can be seen in Table 4, with the increase of exogenous taurine supplementation, the FBW, WGR, and SGR were greatly increased ($P < 0.05$). Especially in the T3 group, all indexes contained the highest values, indicating that exogenous taurine might greatly enhance the growth performance of golden pompano. In addition, FI, FCR, and HSI also demonstrated a significant decline with increasing exogenous taurine. However, exogenous taurine supplementation had no effect on SR, VSI, and CF ($P > 0.05$). As can be seen in Figure 1, the optimal taurine level in the diet of golden pompano was estimated at 1.18% *via* polynomial regression analysis using SGR.

Organism composition

Table 5 showed that exogenous taurine greatly increased crude protein content in the T3 group compared to that in the control group ($P < 0.05$). The highest moisture content was observed in the control group, which was extremely higher compared to all other groups ($P < 0.05$). The crude lipid and ash contents were not influenced by exogenous taurine ($P > 0.05$).

Serum growth-related parameters

With an increase in taurine content, the GH levels in serum showed a trend of increase (0.4–1.2%) and subsequent decrease (1.2–1.6%, Figure 2A). The lowest serum GH level was identified in the control group, which was much lower than those of the experimental groups ($P < 0.05$). The levels of IGF-1 and IGF-2 in serum increased with increasing exogenous taurine

TABLE 4 Growth performance of *T. ovatus* fed diets with different dose taurine supplementation after 8 weeks.

Parameters	Group					*P* valve
	T0	T1	T2	T3	T4	
SR (%)	95.24 ± 0.82	95.24 ± 0.82	96.67 ± 0.82	96.67 ± 2.18	95.71 ± 1.43	0.507
IBW (g)	10.15 ± 0.02	10.12 ± 0.05	10.11 ± 0.05	10.05 ± 0.03	10.08 ± 0.04	0.784
FBW (g)	68.64 ± 5.22^{a}	77.91 ± 2.09^{bc}	80.79 ± 0.97^{cd}	87.98 ± 7.83^{d}	72.10 ± 3.85^{ab}	<0.001
WGR (%)	583.03 ± 51.95^{a}	675.23 ± 20.82^{ab}	703.91 ± 9.64^{ab}	775.40 ± 77.92^{b}	617.41 ± 38.30^{a}	<0.001
SGR (%/day)	3.43 ± 0.13^{a}	3.65 ± 0.05^{bc}	3.71 ± 0.02^{bc}	3.86 ± 0.16^{c}	3.51 ± 0.09^{ab}	<0.001
FI (%/day)	1.42 ± 0.05^{b}	1.20 ± 0.02^{a}	1.30 ± 0.01^{ab}	1.24 ± 0.06^{ab}	1.32 ± 0.03^{ab}	<0.001
FCR (%)	2.15 ± 0.18^{b}	1.75 ± 0.05^{a}	1.87 ± 0.02^{ab}	1.76 ± 0.19^{a}	1.96 ± 0.11^{ab}	<0.001
HSI (%)	1.28 ± 0.07^{b}	1.07 ± 0.08^{a}	1.05 ± 0.08^{a}	1.00 ± 0.09^{a}	1.03 ± 0.07^{a}	<0.001
VSI (%)	5.50 ± 0.34	5.37 ± 0.40	5.18 ± 0.52	5.55 ± 0.16	5.69 ± 0.65	0.170
CF (g/cm^{3})	2.69 ± 0.31	3.36 ± 0.56	3.27 ± 0.66	2.81 ± 0.47	2.83 ± 0.52	0.062

values in the same row with different superscripts are significantly different (P < 0.05).

supplementation and were significantly higher in the T3 and T4 than in the other groups ($P < 0.05$, Figures 2B, C). The lowest triiodothyronine and thyroxine levels were observed in the control group, which were significantly lower than those in the other groups ($P < 0.05$, Figures 2D, E).

Growth-related genes

GH mRNA levels in the brain of golden pompano greatly increased with the increase of exogenous taurine supplementation (Figure 3). *GH* mRNA expression was greatly higher in the T3 and T4 groups than in the control group ($P < 0.05$). The *NPY* mRNA levels in the brain of golden pompano showed a trend of increasing (0–1.2%) and then decreasing (1.2–1.6%) with the increase of exogenous taurine supplementation. T3 fish exhibited the highest *NPY* expression, which was remarkably higher than the expression of the control group ($P < 0.05$). *IGF-1* and *IGF-2* expression in the liver showed a trend of increasing with the addition of exogenous taurine supplementation. The expression of *IGF-1* and *IGF-2* in T3 and T4 fish was the highest and much higher compared with theT0 fish ($P < 0.05$).

Serum antioxidant capacity and non-specific immune parameters

Antioxidant enzyme activity in serum increased with increasing exogenous taurine supplementation (Figure 4). The T-AOC enzyme activity in the T3 and T4 groups were greatly higher compared with theT0 group ($P < 0.05$, Figure 4A). The T-AOC enzyme activity in the T2 fish was similar to the control group, with no significant differences ($P > 0.05$). Still, the highest enzyme activities of CAT and SOD were found in the T2 fish, which were significantly higher compared with theT0 group ($P < 0.05$, Figures 4B, C). Compared with theT0 group, GSH-PX activity were higher while exogenous taurine supplementation

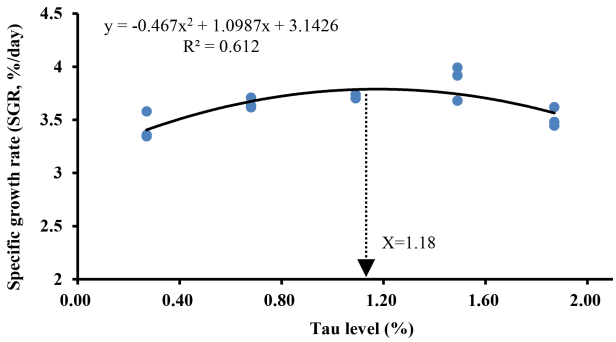

FIGURE 1
Estimation of the optimal dietary taurine level for *T. ovatus* by means of polynomial regression analysis using the SGR.

10.3389/fimmu.2022.1036821

TABLE 5 Effect of dietary taurine level on organism composition of *T. ovatus*.

Parameters	Group					*P* valve
	T0	T1	T2	T3	T4	
Crude protein (g·100g^{-1})	17.37 ± 0.15^{a}	17.40 ± 0.10^{a}	18.00 ± 0.26^{ab}	18.17 ± 0.45^{b}	17.30 ± 0.26^{a}	0.008
Crude lipid (g·100g^{-1})	6.03 ± 0.31	5.80 ± 0.20	5.97 ± 0.38	5.97 ± 0.31	5.93 ± 0.31	0.909
Moisture (g·100g^{-1})	73.30 ± 0.59^{b}	70.67 ± 0.40^{a}	70.83 ± 0.70^{a}	69.87 ± 0.45^{a}	69.80 ± 0.10^{a}	<0.001
Ash (g·100g^{-1})	4.30 ± 0.10	4.09 ± 0.17	4.06 ± 0.23	4.17 ± 0.17	4.07 ± 0.15	0.435

values in the same row with different superscripts are significantly different (P < 0.05).

from 0.8% to 1.6% ($P < 0.05$, Figure 4D), yet the enzymatic activity at 1.2% was markedly lower compared with 0.8% and 1.6% ($P < 0.05$).On the contrary, the MDA and ROS levels in the serum decreased with increasing exogenous taurine supplementation (Figures 4E, F). The MDA and ROS levels of the T3 group recorded the lowest value and were significantly lower than those of the control group ($P < 0.05$). Figure 5 showed that the highest activity of LZM (Figure 5A) and the highest content of C4 (Figure 5F) were identified in T3 and T4 fish, which considerably higher than that in the control group ($P < 0.05$). IgA and C3 levels were significantly higher in the T3 group than in the other groups ($P < 0.05$, Figures 5B, E). IgM levels were higher in the T3 and T4 fish compared to those in the control group ($P < 0.05$, Figure 5D). IgG levels were higher at 0.8–1.6% exogenous taurine compared to the T0 group ($P < 0.05$), yet significantly lower at 1.2% compared to 0.8% and 1.6% exogenous taurine ($P < 0.05$, Figure 5C).

Midgut histological observation

Figures 6A–E are midgut sections of fish C0-C4, respectively. The villus length increased (0–0.8%) and then decreased (0.8–1.6%) with increasing exogenous taurine supplementation (Figure 6F). Villus length in the T2 group was the highest, and a significant increase occurred in the T2 group compared to that in the control group ($P < 0.05$). Muscle thickness increased with increasing taurine content (Figure 6G). The highest muscle thickness was observed in the T3 and T4 groups. The lowest muscle thickness recorded in the control group was lower than that in all other dietary groups, and the difference was highly significant ($P < 0.05$). By contrast, the number of goblet cells per intestinal villi exhibited a significant decline with increasing exogenous taurine supplementation; the control group recorded the highest number of goblet cells, and the difference was highly significant ($P < 0.05$, Figure 6H).

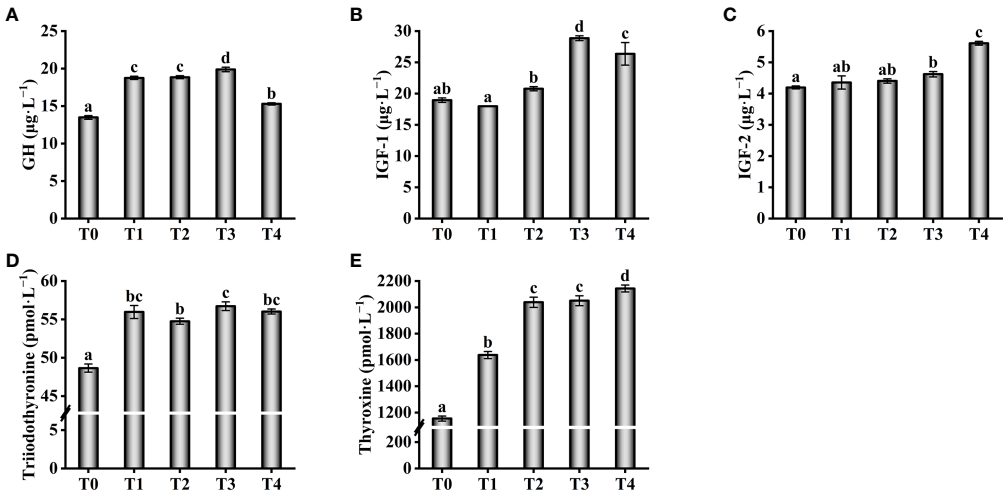

FIGURE 2
Effect of growth-related parameters such as GH **(A)**, IGF-1 **(B)**, IGF-2 **(C)**, Triiodothyronine **(D)** and Thyroxine **(E)** in the serum of *T. ovatus* fed diets with different dose taurine supplementation after 8 weeks. Mean values (n = 9) within values in the picture above with different superscripts are significantly different ($P < 0.05$).

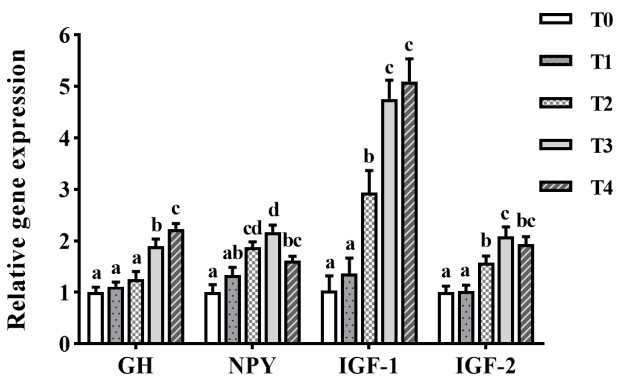

FIGURE 3
The expression profiles of growth-related genes in the liver and the brain of *T. ovatus* fed diets with different dose taurine supplementation after 8 weeks. Mean values (n = 9) within values in the picture above with different superscripts are significantly different ($P < 0.05$).

Antioxidant enzyme expression in the intestine

As shown in Figure 7A, *CAT*, *SOD*, and *GSH-PX* mRNA levels increased with taurine content. The maximum expression of *CAT* and *GSH-PX* was observed with exogenous addition of 1.2–1.6% taurine. *SOD* mRNA level in the T4 group (1.6% taurine) was the highest, and much higher than that in the control group ($P < 0.05$). With the increasing taurine content, *HO-1* and *Nrf2* expression initially increased (0–1.2%) and then decreased (1.2–1.6%, Figure 7B). The maximum expression of *HO-1* and *Nrf2* was observed with the exogenous addition of 0.8–1.2% taurine and was significantly higher than that in the control fish ($P < 0.05$). Conversely, the higher the exogenous taurine addition, the lower the expression of *Keap-1* mRNA. The T0 group showed the highest *Keap-1* expression and was greatly higher than other groups ($P < 0.05$).

Intestine immunity-related gene expression analysis

The mRNA levels of *TNF-α*, *IL-1β*, and *IL-8* drastically decreased with increased taurine content (Figure 8A). The

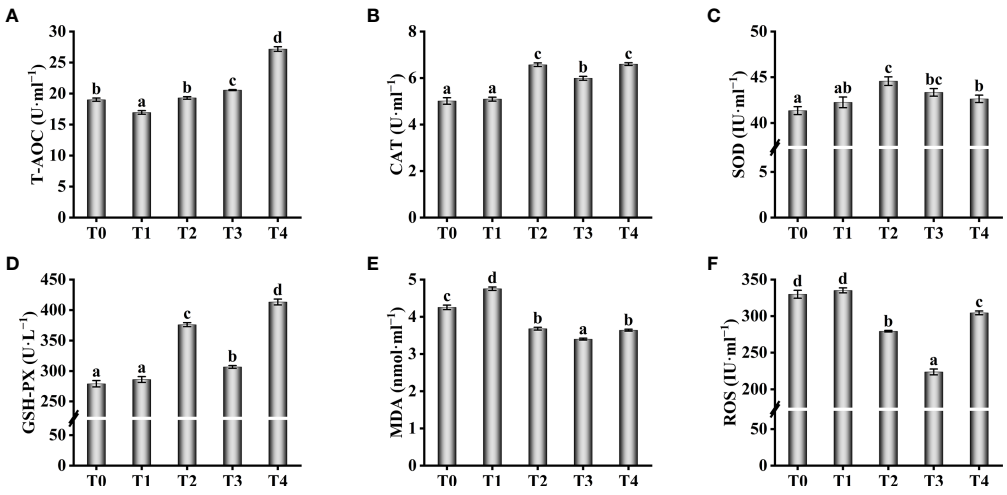

FIGURE 4
Effect of antioxidant capability such as T-AOC **(A)**, CAT **(B)**, SOD **(C)**, GSH-PX **(D)**, MDA **(E)** and ROS **(F)** in the serum of *T. ovatus* fed diets with different dose taurine supplementation after 8 weeks. Mean values (n = 9) within values in the picture above with different superscripts are significantly different ($P < 0.05$).

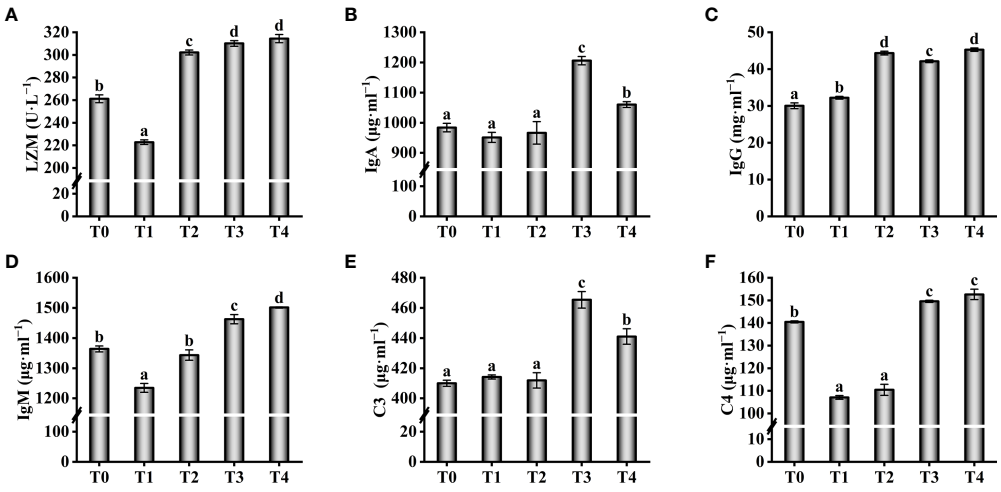

FIGURE 5
Effect of immunological parameters such as LZM **(A)**, IgA **(B)**, IgG **(C)**, IgM **(D)**, C3 **(E)**, C4 **(F)** in the serum of *T. ovatus* fed diets with different dose taurine supplementation after 8 weeks. Mean values (n = 9) within values in the picture above with different superscripts are significantly different ($P < 0.05$).

highest mRNA expression of *TNF-α* was observed in the control fish, which was much higher than those in the other experimental groups ($P < 0.05$). The highest mRNA expression of *IL-1β* was found in T0 and T1 fish, which was greatly higher than those of the other experimental groups ($P < 0.05$). With the increase of taurine content, *IL-10* mRNA expression first increased (0–0.8%) and then decreased (0.8–1.6%). The lowest mRNA expression of *IL-10* was found in the control fish, which was much lower than that in the other groups ($P < 0.05$). *NF-κb* and *IKK* mRNA expression decreased drastically with higher taurine content

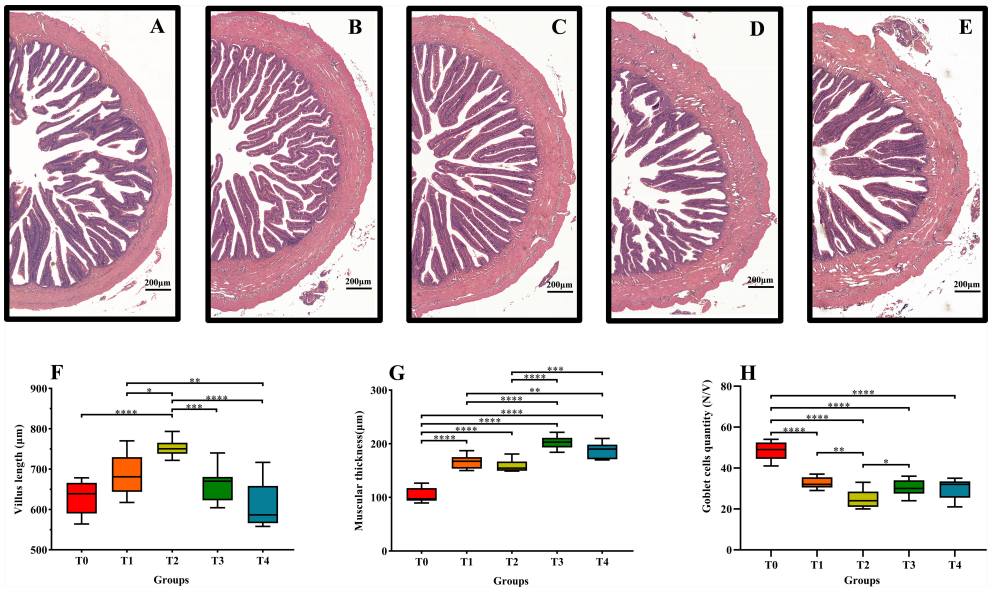

FIGURE 6
Effects of dietary taurine on mid-gut morphology of *T. ovatus*. **(A)**: 0% taurine; **(B)**: 0.40% taurine; **(C)**: 0.80% taurine; **(D)**: 1.20% taurine; **(E)**: 1.60% taurine. Scale bar: 200 μm. The villus length **(F)**, muscular thickness **(G)**, and goblet cells quantity **(H)** of mid-gut in *T. ovatus*. data are presented as mean ± SD (n = 9).Asterisks *, **, ***,and **** indicate statistically significant difference between treated group and control group at $P < 0.05$, $P < 0.01$ $P < 0.001$, and $P < 0.0001$, respectively.

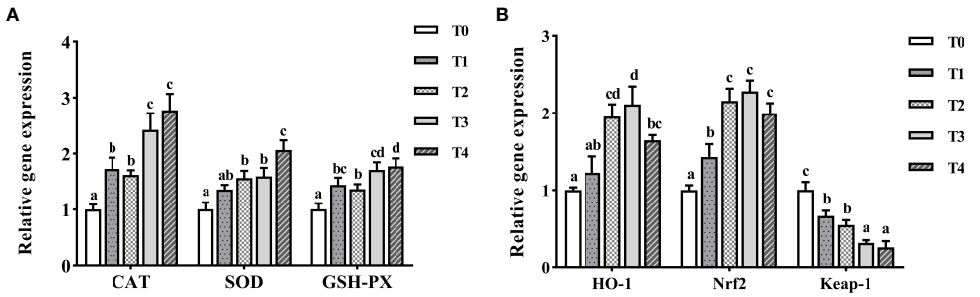

FIGURE 7
The expression profiles of antioxidant genes **(A)** and signaling pathway **(B)** in the intestine of *T. ovatus* fed diets with different dose taurine supplementation after 8 weeks. Mean values (n = 9) within values in the picture above with different superscripts are significantly different ($P < 0.05$).

(Figure 8B). *NF-κB* mRNA expression with the exogenous addition of 1.2–1.6% taurine was considerably lower than that in the control group ($P < 0.05$). The *IKK* mRNA level was significantly higher in the T0 and T1 groups than in the other groups ($P < 0.05$). Conversely, *IκB* gene expression increased with increasing exogenous taurine supplementation in all test groups compared to that in the control group ($P < 0.05$).

Streptococcus agalactiae challenge

As shown in Figure 9, after 120 h of *S. agalactiae* challenge, the survival rates of T0 (0), T1 (0.4%), T2 (0.8%), T3 (1.2%), and T4 (1.6%) fish were 48.33%, 56.67%, 63.33%, 66.67%, and 66.67%, respectively. Survival tended to increase with dietary taurine content. The survival rate of T3 fish was the highest and was significantly higher than that of the control group ($P < 0.05$), indicating that exogenous taurine can promote golden pompano immunity and pathogen resistance.

Discussion

Effects of exogenous taurine supplementation to low-fishmeal diets on the growth and body composition of juvenile golden pompano

Previous studies have shown that exogenous taurine supplementation to low-fishmeal diets may enhance the growth of fish (13–17). For example, exogenous taurine extremely increased the WGR and SGR of *Scophthalmus maximus L* (44), which greatly affected its growth. In the present study, exogenous taurine increased crude protein content and decreased HSI, improving WGR and growth performance in golden pompano, which is generally consistent with the findings of Ma et al. (37) from our laboratory. Various factors affect fish weight gain, including digestive enzyme activity, digestive tract characteristics, and growth-related hormone alterations.

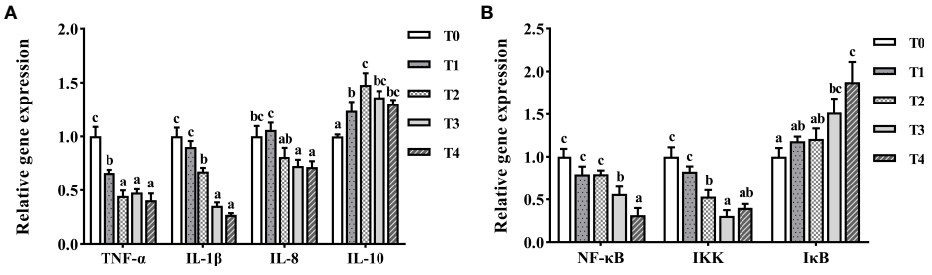

FIGURE 8
The expression profiles of inflammatory genes **(A)** and signaling pathway **(B)** in the intestine of *T. ovatus* fed diets with different dose taurine supplementation after 8 weeks. Mean values (n = 9) within values in the picture above with different superscripts are significantly different ($P < 0.05$).

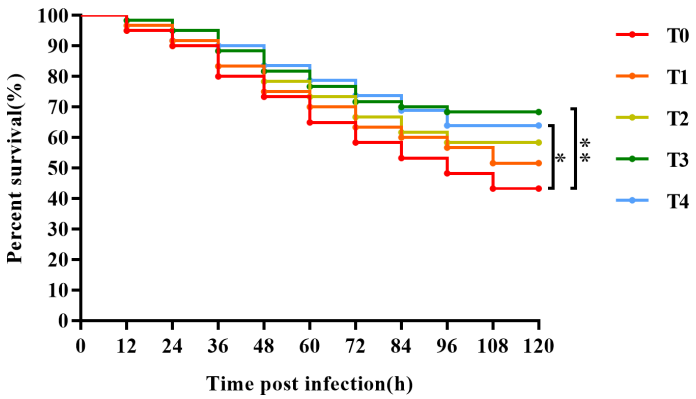

FIGURE 9
The Kaplan-Meier survival analysis of *T. ovatus* after *S. agalactiae* infection. Asterisks *, and ** indicate statistically significant difference between treated group and control group at $P < 0.05$, and $P < 0.01$, respectively.

Thyroxine is thought to promote growth and GH secretion (45). GH stimulates tissue growth, organismal anabolism, and protein synthesis by upregulating the synthesis and secretion of IGF-1 and IGF-2 (46). IGF-1 and IGF-2 play important roles in the growth and development of scleractinian fish (47). Studies on rats have shown that taurine can increase triiodothyronine and thyroxine levels by enhancing thyroid function (48). In addition, taurine can directly stimulate GH secretion in the brains of rats (49). In the present study, we observed that taurine supplementation increased the serum levels of growth-related parameters, such as GH, triiodothyronine, thyroxine, IGF-1, and IGF-2 in fish. NPY plays a vital physiological role in growth, development, reproduction, hormone release, and other activities of organisms. Studies on *Paralichthys olivaceus* have shown that NPY promotes GH release and can substantially increase feeding and growth rates (50). In rats, taurine was demonstrated to downregulate *NPY* expression within the hypothalamus, reducing appetite and food intake (51). In contrast, taurine intake greatly upregulated *GH* and *NPY* mRNA expression in the brain as well as *IGF-1* and *IGF-2* mRNA levels in the liver of the golden pompano. Thus, exogenous taurine intake not only enhances appetite in golden pompano but also promotes feeding and growth.

Effects of exogenous taurine to low-fishmeal diets on oxidative stress resistance and non-specific immunity in juvenile golden pompano

Previous studies have demonstrated that taurine can increase antioxidant enzyme activity and reduce ROS levels in aquatic animals (18, 52). For example, exogenous taurine at 0.4–0.8% drastically increased the T-AOC, SOD, and GSH-PX activities while reducing MDA levels in *Eriocheir sinensis* (52). Similarly, the present study showed that exogenous taurine increased the T-AOC as well as CAT, GSH-PX, and SOD activities and intestinal gene expression, while decreasing MDA and ROS levels in golden pompano serum. Under normal conditions, ROS levels are mainly regulated by Nrf2 and its inhibitory partner Keap-1 (53), with increased transcription of *Nrf2* leading to a decrease in intracellular ROS (54). Nrf2 can also directly regulate *HO-1* promoter activity and rapidly increase antioxidant enzyme expression in fish. For example, taurine chloramine (TauCl), which is derived from taurine, can induce *HO-1* mRNA expression by activating the Nrf2/Keap-1/HO-1 axis, resulting in an enhanced antioxidant capacity (55, 56). Previous studies in our laboratory have shown that Nrf2/Keap-1/HO-1 is also active in the golden pompano and can provide protection against oxidative stress induced by acute ammonia exposure and copper (40, 41). In the present study, we observed that an increase in dietary taurine content significantly promoted and then suppressed intestinal *HO-1* and *Nrf2* mRNA levels, with significant upregulation of *Keap-1* mRNA levels, altogether providing evidence of an enhanced antioxidant capacity mediated *via* the Nrf2/Keap-1/HO-1 axis. However, it should be noted that SOD activity in serum and liver *HO-1* mRNA expression were not the highest and serum MDA levels were not at the lowest level under 1.6% (T4) taurine supplementation, thus suggesting that excessive exogenous taurine is not necessary.

Oxidative stress damages tissues and decreases the non-specific immune capacity (18). Serum LZM, C3, C4, IgM, IgA, and IgG are considered essential indicators of immune function (19, 57). Previous studies have indicated that taurine can increase IgM, C4, and C3 levels in juvenile *I. punctatus*, improve antioxidant capacity, and protect against oxidized fish oil (19). Exogenous taurine improved LZM activity, increased

total immunoglobulin as well as C3 and C4 levels in *Carassius auratus*, reducing the adverse effects of high ammonia levels (57). Similarly, our study showed that exogenous taurine extremely increased serum LZM activity, increased C3, C4, IgM, IgA, and IgG levels, thus enhancing innate and adaptive immunity in the golden pompano.

Effect of exogenous taurine supplementation to low-fishmeal diets on the intestine immunity of juvenile golden pompano

It is well known that the intestinal immune system, which consists of physical and immune barriers, is central to immune homeostasis in aquatic animals (21). Complete intestinal tissue structure is not only a prerequisite for digestion and absorption, but also a requirement for proper intestinal immune function (58). Taurine has been shown to effectively protect intestinal tissue integrity in fish. For example, a study on *Cyprinus carpi* showed that taurine increased villus length and goblet cell quantity (8). Under high-fat diet feeding, taurine addition increased *M. albus* (13) villus length and muscular thickness, resulting in a more regular arrangement of villi which was beneficial for maintaining intestinal health. Our study revealed that exogenous taurine increased intestinal villus length and muscular thickness, reduced the number of goblet cells, and protected the intestinal mucosal layer, thus maintaining intestinal barrier stability and promoting intestinal health in golden pompano.

When the proportion of fishmeal in feed is too low, oxidative stress is induced in the fish liver and intestine, leading to structural damage, reduced intestine immunity, and inflammation (8–11). As a defensive response against pathogen invasion and tissue damage (59), activation of the inflammatory response is mediated mainly *via* NF-κB, IκB, and IKK. In the classical NF-κB signaling pathway, IκB is degraded in response to IKK induction, resulting in NF-κB activation and the production of pro-inflammatory cytokines, such as IL-1β, IL-8, and TNF-α (60). In fish, the inflammatory response is regulated by a combination of anti-inflammatory cytokines (IL-10, TGF-β, etc.) and pro-inflammatory cytokines (IL-1β, IL-8, TNF-α, etc.) (61). As a functional amino acid, taurine can improve intestine immunity in fish and reduce aberrant inflammatory responses. For example, taurine can inhibit *NF-κB* expression in *I. punctatus*, downregulate pro-inflammatory cytokines (*IL-1β*, *IL-6*, *TNF-α*, and *IL-8*), and upregulate anti-inflammatory *TGF-β* mRNA expression, thereby improving intestine immunity and promoting intestine health (19). Similarly, our study showed that exogenous addition of 0.8–1.2% taurine (T2–T4) greatly reduced intestinal *IL-1β*, *IL-8*, and *TNF-α* mRNA levels, upregulating *IL-10*, which enhanced intestine immunity. This finding provides evidence that taurine protects intestinal health by enhancing anti-inflammatory activity.

This study also investigated the protective effects of taurine against *S. agalactiae*. Taurine exhibited a positive impact on the survival of golden pompano, with exogenous addition of 1.2–1.6% taurine extremely increasing survival after *S. agalactiae* challenge. Similarly, feeding *C. idella* with exogenous taurine effectively reduced the incidence of enteritis caused by *Aeromonas hydrophila* (23). Further, taurine can provide reasonable protection against mechanical stress in zebrafish (62) and *Vibrio alginolyticus* challenge (63). The protective effect of exogenous taurine bacterial challenge may stem from the enhanced intestinal antioxidant capacity of fish mediated *via* the Nrf2/Keap-1/HO-1 axis. Furthermore, exogenous taurine reduces the intestinal inflammatory response in fish by suppressing NF-κB signaling.

Conclusions

In summary, moderate exogenous taurine improved upregulated growth-related gene expression, serum growth parameter levels, growth performance, and crude protein content in the golden pompano. The optimal taurine level in golden pompano diet was estimated at 1.18% *via* polynomial regression analysis using SGR. Moderate exogenous taurine also protected intestinal structural integrity, maintained intestinal physical barrier stability, activated Nrf2/Keap-1/HO-1 signaling pathway, increased intestinal antioxidant enzyme gene expression and serum antioxidant enzyme activity. Further, supplementation suppressed NF-κB signaling and intestinal pro-inflammatory cytokine gene expression, increased anti-inflammatory cytokine gene expression, and improved intestine immunity. Taurine protected juvenile golden pompanos after challenge with *S. agalactiae*. Our findings provide additional information and support for the rational use of taurine in healthy aquatic animal farming.

Data availability statement

The data presented in the study are deposited in the Genbank repository, accession number OP292223.

Ethics statement

The animal study was reviewed and approved by Animal Care and Use Committee of the South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences(No. SCSFRI96-253).

Author contributions

D-CZ designed the experiments, wrote the paper. J-XL conducted the experiment, wrote the paper. H-YG: Data curation. K-CZ: Visualization, Methodology. B-SL: Supervision, Methodology, Software. NZ: Visualization, Investigation. All authors contributed to the article and approved the submitted version.

Funding

This research was financially supported by China Agriculture Research System of MOF and MARA (CARS-47), Central Public-Interest Scientific Institution Basal Research Fund of South China Sea Fisheries Research Institute CAFS (2021SD12), Central Public-interest Scientific Institution Basal Research Fund, CAFS (NO.2020TD29), Key Projects of Joint Fund for Regional Innovation and Development of NSFC (U20A2064), National Marine Genetic Resource Center, Guangdong Provincial Special Fund for Modern Agriculture Industry Technology Innovation Teams (2019KJ143), Financial Fund of Ministry of Agriculture and Rural Affairs of China (NHYYSWZZZYKZX2020).

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 20 October 2022
DOI 10.3389/fimmu.2022.1023235

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Comparative Immunology, a section of the journal Frontiers in Immunology

RECEIVED 19 August 2022
ACCEPTED 30 September 2022
PUBLISHED 20 October 2022

CITATION
Porter D, Peggs D, McGurk C and Martin SAM (2022) Gut Associated Lymphoid Tissue (GALT) primary cells and stable cell lines as predictive models for intestinal health in rainbow trout (*Oncorhynchus mykiss*). *Front. Immunol.* 13:1023235. doi: 10.3389/fimmu.2022.1023235

Gut Associated Lymphoid Tissue (GALT) primary cells and stable cell lines as predictive models for intestinal health in rainbow trout (*Oncorhynchus mykiss*)

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The use of functional feeds for farmed fish is now regarded as a key factor in improving fish health and performance against infectious disease. However, the mechanisms by which these nutritional components modulate the immune response are not fully understood. The present study was undertaken to identify the suitability of both primary gut-associated lymphoid tissue (GALT) leucocyte cells and established rainbow trout cell lines as potential alternative methods to test functional feed ingredients prior to full fish feeding trials that can take months to complete. In addition to the primary GALT culture cells, the two rainbow cell lines RTS11 and RTgutGC which are from macrophage and gut epithelial cells, respectively. The cells were stimulated with a variety of pathogen associated molecular patterns (PAMPs) (PHA and Poly I:C) and recombinant rainbow trout IL-1β (rIL-1β), a proinflammatory cytokine, additionally two forms of β-glucan, a prebiotic commonly used aquafeeds were used as stimulants. From this, the suitability of cell models as a health screen for functional feeds was assessed. GALT leucocytes were deemed most effective to act as a health screen over the 4hr time point demonstrating responses to Poly I:C, PHA, and rIL-1β. RTS11 and RTgutGC also responded to the stimulants but did not give a strong T-cell response, most likely reflecting the nature of the cell type as opposed to the mixed cell populations from the primary GALT cell cultures. When stimulated with both forms of β-glucan, GALT leucocytes demonstrated a strong proinflammatory and T-cell response.

KEYWORDS

GALT, gut associated lymphoid tissue, beta glucan, rainbow trout, immune, functional feed

Abbreviations: BG, β-glucan; GALT, Gut Associated Lymphoid Tissue; MOS, Mannanoligosaccharides; PAMP, Pathogen associated molecular pattern; SAA, Serum Amyloid A; VHSV, Viral Haemorrhagic Septicaemia Virus.

1 Introduction

Aquaculture faces several important challenges, from the global demand for greater production and the health challenges that accompany increased production, to the challenges associated with the need to identify new ingredients and additives in line with global sustainability standards.

Infectious diseases are the largest cause of economic loss in the aquaculture industry and are controlled by a variety of methods including vaccination, selective breeding, biosecurity, and nutritional intervention (1–3). The use of therapeutics such as antibiotics, where vaccines and other approaches are unable to control disease, can lead to an increase in antibiotic resistance in pathogenic species (4, 5). To improve the robustness of the fish in aquaculture, functional feeds have been developed which may contain immunostimulants, prebiotics, probiotics, and other compounds such as key vitamins and minerals to promote fish health. These dietary components interact within the gut at the interface between nutrition, microbiome, and the immune system (6, 7). Subsequently, modulation of the immune system can occur either through direct interaction with immune cells *via* specific receptors, or through metabolites produced by the intestinal microbial communities. This modulation can lead to improved fish health and increased resistance against disease leading to a reduction in mortalities, recovery time and the use of chemotherapeutic treatments (8). However, the mechanisms by which these functional ingredients interact with the immune system is poorly understood with further research and development of assays to measure immune modulation needed (9).

β-glucans are a commonly used prebiotic/immunostimulant in functional aquaculture nutrition, they are composed of polymers of repeating units of D-glucose linked by β-glycosidic bonds and have many branched side chains (10). β-glucans are naturally occurring components of the yeast and certain algae cell walls (11). Many studies, across different fish species, demonstrate that β-glucans can modulate the immune response triggering various immune pathways including complement, anti-viral and proinflammatory signaling whilst also promoting survival against bacterial and viral pathogens in salmonids (12–15). The health promoting activity of β-glucan based functional feed to the viral pathogen Viral Haemorrhagic Septicaemia Virus (VHSV) was demonstrated with the Skretting Protec™ diet where survival against VHSV was increased in rainbow trout fed the functional feed compared to control diet (15). This diet contains several dietary additives including β-glucans, vitamin E, vitamin C and zinc and resulted in increased magnitude of expression of both immunoglobulins (*IgM, IgT and IgD*) and anti-viral genes including, *MX Dynamin Like GTPase 1* (*MX*), *and Interferon-gamma* (*IFN-γ*) following infection in comparison to those fish fed the control diet. Many other studies have described the upregulation of key proinflammatory cytokines *Interleukin 1β* (*IL-1β*), *Tumor Necrosis Factor-alpha* (*TNFα*) and *Cyclooxygenase-2* (*COX-2*) after supplementation with β-glucans which appears to increase resistance to bacterial pathogens (13, 15). The immunostimulatory effects can be considered tissue and species-dependent with the head kidney and spleen showing differing upregulation of inflammatory cytokines in trout (13). To highlight the species differences, in carp, the intestinal response was a decrease in the expression of several inflammatory cytokines (16), possibly reflecting the different trials and challenges performed between research groups, highlighting the complexities of the responses during trials. In carp, the direct response to β-glucans indicates the involvement of C-type lectins (CLEC4C) in the recognition of β-glucan molecules following whole transcriptome analysis by RNA-seq (17). In trout, supplementation with β-glucans showed an upregulation of T-cell activation, both the classical and alternative complement pathways, proinflammatory responses, through genes such as *IL-1β* and *TNFα* and signaling pathways including the P13k-AKT and mTOR signaling pathways (18). Subsequently, an assay to study the effects of β-glucans and other dietary stimulants is needed to categorize responses in different tissues and to identify tolerance to pathogens.

Feeding trials are the gold standard in aquaculture nutrigenomics and involve testing novel ingredients with various parameters; whole tissue histology, performance-based metrics, and transcriptomics/proteomics. However, due to feeding trials using many fish to study just one ingredient, complementary methods have since been developed to identify the mechanisms of action of functional ingredients, which could be especially useful at the early stages of characterization of new products. To examine direct tissue responses several techniques have been developed using explant (19), primary cell cultures (20) or cell lines (21).

In rainbow trout, two cell lines; RTgutGC, an intestinal epithelial cell line (22) and RTS11, a spleen monocyte-macrophage cell line (23) may help explain immunological responses to nutrients. RTgutGC has been used as a health screen for model functional ingredients (24) where nucleotides, mannanoligosaccharides (MOS), and β-glucans were used to identify immunostimulatory effects and intestinal cell barrier function. The RTS11 cells are highly immunologically reactive and have been used extensively in immune function studies (25–27).

Cell lines classically comprise one (clonal) cell type so will not reflect the complexity of the host immune response due to the singular cellular phenotype. To overcome the lack of phenotypes present, primary immune cell cultures from the gut associated leucocyte tissue (GALT) may be a complementary approach for testing functional ingredients as described for rainbow trout (20) and gilthead seabream (*Sparus aurata*) (28). For rainbow trout, flow cytometry and targeted

gene expression have been used to identify specific cellular markers which indicate the presence of T-cells, B-cells, and dendritic cells in primary GALT cultures (20) suggesting this may be a suitable model for testing functional feed components.

The aims of the current study were to develop a primary cell culture model that can be used to assay nutritional ingredients for functional feeds. To assess the suitability of the GALT leucocyte assay comparisons between established cell models for the identification of immune responses in the intestine, the permanent cell lines RTS11 and RTgutGC were used. The data generated using GALT leucocytes and rainbow trout cell lines help to further elucidate the mechanisms by which β-glucans act as immunostimulatory molecules.

2 Materials and methods

2.1 Fish

Rainbow trout (400-500g) were maintained in 250L 1 m-diameter fiberglass tanks with recirculating freshwater at 14°C. Fish were fed twice a day with a commercial diet (Skretting Elite FR 6mm) at 1.5% bodyweight per day and sampled at the same time of day on each occasion used. Fish were killed by schedule 1 method in accordance with the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

2.2 Stable cell line culture

The RTS11 cell line was cultured in flasks (75 cm^2) at 20°C in growth media (Leibovitz L-15 media + 30% FBS + 1% Penicillin/Streptomycin), the cells were generally non-adherent to the flasks prior to any stimulation. Cells were collected and pelleted by centrifugation at 500g for 10 mins at 4°C before being resuspended in fresh stimulation media (Leibovitz L-15 media + 1% FBS + 1% Penicillin/streptomycin) and adjusted to $5x10^5$ cells/ml, then 1 ml was added to 24 well plates. RTgutGC cells were cultured in flasks at 20°C in growth media (Leibovitz L-15 media (Gibco) + 10% FBS + 1% Penicillin/Streptomycin (1% P/S) (Gibco)). Prior to stimulation, RTgutGC cells were trypsinised, once separated from the flask, cells were resuspended in growth media, washed, and plated as described for RTS11. These cells were then immunostimulated as described below in section 2.4.

2.3 Isolation of primary GALT leucocytes

GALT cells were isolated according to the protocol by Attaya et al. (20) with modifications described here. Fish were starved for 24hrs before sampling to reduce the gut contents. In total 24 fish were euthanized for the isolation of GALT leucocytes. Immediately following death, the fish were bled by severing the aorta at the gills. The fish was opened from the ventral side and the hind gut was excised (approx. 2-4 cm in length and ~1g in weight) and placed in 1x PBS on ice (Sigma). The hind gut was rinsed in PBS three times, then cut with a sharp scalpel longitudinally to 0.5-1 cm^2 squares to aid in rinsing. Gut segments were then placed in 30 ml of PBS in 50 ml falcon tubes before being washed for 20 mins at 40 rpm on an orbital shaker. Gut segments were washed one final time in PBS before being added to 50 ml falcon tubes containing 25 ml of predigestion solution (HBSS (Gibco, 14025092) + 0.145 mg/ml DTT (Sigma, D9779) + 0.37 mg/ml EDTA (Fisher, D/0700/53)). Gut segments were washed in predigestion solution for 20 mins at 40 rpm in an orbital shaker. The supernatant (S1) was then filtered into a 50 ml falcon tube using 100 µm nylon cell strainers (Greiner). S1 was washed using PBS and pelleted twice for 5 mins at 500g at 4°C and the supernatant was discarded, cells were then resuspended in 20 ml of growth media (Leibovitz L-15 media (Gibco) + 10% FBS + 1% P/S) and stored in a 20°C incubator. The gut segments were rinsed using washing medium (HBSS + 0.05 mg DNase1/ml (Sigma, DN25) + 5% FBS + 1% Penicillin/Streptomycin) to remove EDTA and DTT before being placed into the digestion solution (washing media + 0.37 mg collagenase IV/ml (Gibco, 17104-019)) for 2 hours on an orbital shaker at 40 rpm. The supernatant from the digestion was then strained through a 100 µm nylon cell strainer into the tube containing the resuspended S1 phase prior to being washed using PBS with 1% P/S. The supernatant was washed three times using PBS + 1% P/S to remove any collagenase, with the supernatant being discarded. The cells were then resuspended in 5 ml of growth media as described above. The cell suspension was then slowly layered on top of a discontinuous percoll gradient (25% and 75%) (GE healthcare, 17-0891-01) and centrifuged for 30 mins at 400g, at 4°C. Cells were then collected from the interface between the 25 and 75% percoll gradients and washed twice as above. Cells were then suspended in stimulation media before being adjusted to $2.5x10^5$ cells/ml before one ml was added to 24 well plates to which stimulants were added as described in 2.4.

2.4 Immune stimulation of cell lines and primary GALT cells

Five different stimuli were used; 100 µg/ml Poly I:C (Sigma, P1530), 10 µg/ml PHA (Sigma, 61764), 20 $µgml^{-1}$ recombinant IL-1β [Provided by Dr. Tiehui Wang (Scottish Fish Immunology Research Centre)] (29), and 100 µg/ml of two molecular forms of β-glucan termed M and F (provided by Skretting AI, Norway). M is M-glucans (Biotec), whilst F is Fibosel (Trouw). The two glucans differ in purification but both are in particulate form and are not highly soluble.

Stock solutions for Poly I:C and PHA were made in L-15 media with aliquots stored in -20°C until use. rIL-1β stock solution was stored in -80°C, and aliquots were diluted in L-15 media when in use. Fresh M-glucans and Fibosel stock solutions were made from a powdered version of each, suspended in L-15 media, and stored at 4°C until use. Stock solutions were then added to wells containing cells diluted in media to give the working solutions. Stimulants were added immediately after plating. All experiments were performed in randomized design with quadruplicate wells that were treated independently through the entire protocol (n = 4).

2.5 GALT leucocyte viability

GALT leucocytes were suspended in growth media and incubated in 24 well plates at $5x10^5$ cells/well in 1ml of stimulation media. These cells were incubated at 20°C for 4 hrs and 24 hrs, where they were counted using a Neubauer chamber with 0.5% trypan blue. The viability was determined using the 0 hr time point as controls.

2.6 Transcriptional analysis

RNA was extracted from cells in individual wells ($2.5x10^5$ in the case of GALT Leucocytes and $5x10^5$ for RTS11 and RTGutGC) using 750µL of TRI Reagent (Sigma) in accordance with the manufacturer's instructions. The RNA pellet was washed using 80% ethanol, dissolved in RNase-free water, and stored at -80°C until use. Quality control of the samples was determined using a Nanodrop spectrophometer and the integrity of the RNA was assessed by an Agilent Bioanalyzer 2100. RNA (500 ng) was used as template for reverse transcription and generation of cDNA using the Qiagen QuantiTect Reverse Transcription Kit following manufacturer's instructions. The cDNA was diluted 10x with RNase-free water and stored at −20°C until use.

Gene expression was determined for proinflammatory cytokines (*IL-1β, Interleukin-8* (*IL8*)), an anti-inflammatory cytokine, *Interleukin-10* (*IL-10*), a well characterized antimicrobial peptide, *Hepcidin antimicrobial peptide* (*HAMP*) and an acute phase reactant, *serum amyloid-alpha* (*SAA*), T-cell markers *Interleukin-4/13* (*IL-4/13*) and *IFN-γ* and anti-viral marker genes *MX and Interferon-1 alpha (IFN-1α)* by qPCR (Table 1). Amplification was performed using Agilent Brilliant III Ultra-Fast SYBR and amplification were run on a Roche Lightcycler 480 machine, PCR cycles were: initial denaturation of 3mins at 95°C followed by amplification by 40 cycles (5s at 95°C, 10s at 60°C and 1s at 72°C). The expression of target genes was normalized to the relative expression of the mean of three housekeeping genes *Elongation Factor-1 alpha (ELF-1α), Ribosomal Protein L4 (RPL4)* and *Ribosomal Protein S29 (RPS29)*. Three housekeeping genes were used to minimize random errors involved with qPCR. The gene expression was calculated using the Genex 5 software (Multid) to generate relative gene expression which was then used to calculate fold change in comparison to the control samples.

2.7 Statistical analysis

Data handling, calculation of fold change and statistical analysis of relative expression using a two tailed students T-test, were performed in Microsoft Excel 2016. Statistical analyses of the fold changes for RTS11 time course experiments were carried out using two-tailed Anova in R. Graphical representation of the data was performed in GraphPad Prizm 5.

TABLE 1 Primers used for qPCR analysis.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product Size (bp)	Accession Number	Source
EF1-α	CAAGGATATCCGTCGTGGCA	ACAGCGAAACGACCAAGAGG	327	AF498320	20
RPL4	CCTTCAGAAACATCCCTGGTATCAC	GGGCAGATTGTAGTCTACCTTGAGAG	182	BT057966	30
RPS13	CCCTCTCAGATCGGTGTGATCC	TCCTTGTCCTTTCTGTTCCTCTCC	191	BT059859	30
RPS29	GGGTCATCAGCAGCTCTATTGG	AGTCCAGCTTAACAAAGCCGATG	167	BT043522	30
MX-1	CGTCCCAGACCTCACACTCATC	TGCCATCTTCAAAGCCTCTGTG	187	OMU30253	31
IFN-1α	GTGTGTCATTGCTGTGACTGGA	TTTGTGATATCTCCTCCCATCTG	95	AJ580911	31
SAA	AGTCATCAGTAATGGCCGGGA	AAAAGCTTGTTTGGAATTTGGTCCT	205	NM_001124436	
HAMP	AGTCCCTCATCCGCTGACAT	CAAATAGCGGCGCTCTCCAAT	93	HQ711993.1	
IFN-γ	GTAGCCTGCCGTTTTGAGCA	TGACGGGAGGAGGAACGTAA	250	NM_001124416.2	
Il-4/13β1	GAGATTCATCTACTGCAGAGGATCATGA	GCAGTTGGAAGGGTGAAGCTTATTGTA	255	HG794522	20
TNFα2	CTGTGTGGCGTTCTCTTAATAGCAGCTT	CATTCCGTCCTGCATCGTTGC	99	AJ401377	20
IL-8	GAAACTCGCCACAGACAGAGAA	AGTGTGTTGTTATCTCGCTGGTAA	114	HG917307.1	
IL-10	ACATCCCTGCTGGACGAAGG	GGCAGCACCGTGTCGAGATA	101	NM_001245099.1	
IL-1β	CTGCACCTAGAGGAGGTTGCG	GAAACGCACCATGTCGCTCT	72	NM_001124347.2	

10.3389/fimmu.2022.1023235

3 Results

3.1 GALT leucocyte viability

GALT leucocytes had a viability of 95.52% at 4hrs and 53.1% at 24hours following extraction compared to 0hrs. This matches GALT leucocyte viability previously reported by Attaya et al. (20) observed a decrease from 93% at 4hrs to 53% at 24hrs. As a result of this, all experiments were performed at the 4hrs time point to remove any artifacts of cell death and related responses. GALT leucocyte extractions were carried out separately for each fish to ensure no non-self-recognition and subsequent modulation of the immune system, to ensure the effects seen were only in response to PAMPs.

3.2 Comparisons of gene expression in RTS11, RTgutGC and primary GALT cell in response to Poly I:C stimulations

To examine the capacity for mounting an interferon/antiviral response, Poly I:C was used as a PAMP as it is an effective inducer of the type I interferon response. At 4hrs following stimulation all the cells showed a significant increase in IFN-1a following the poly I:C stimulation (RTS11 4.2-fold, RTgut 57-fold, GALT 2.92-fold) (Figures 1A–F). The RTS11 were also sampled at 24 and 48hr but there was no significant increase in *IFN-1a* at the later time points. The RTgutGC cells showed the greatest magnitude of response with a ~200-fold

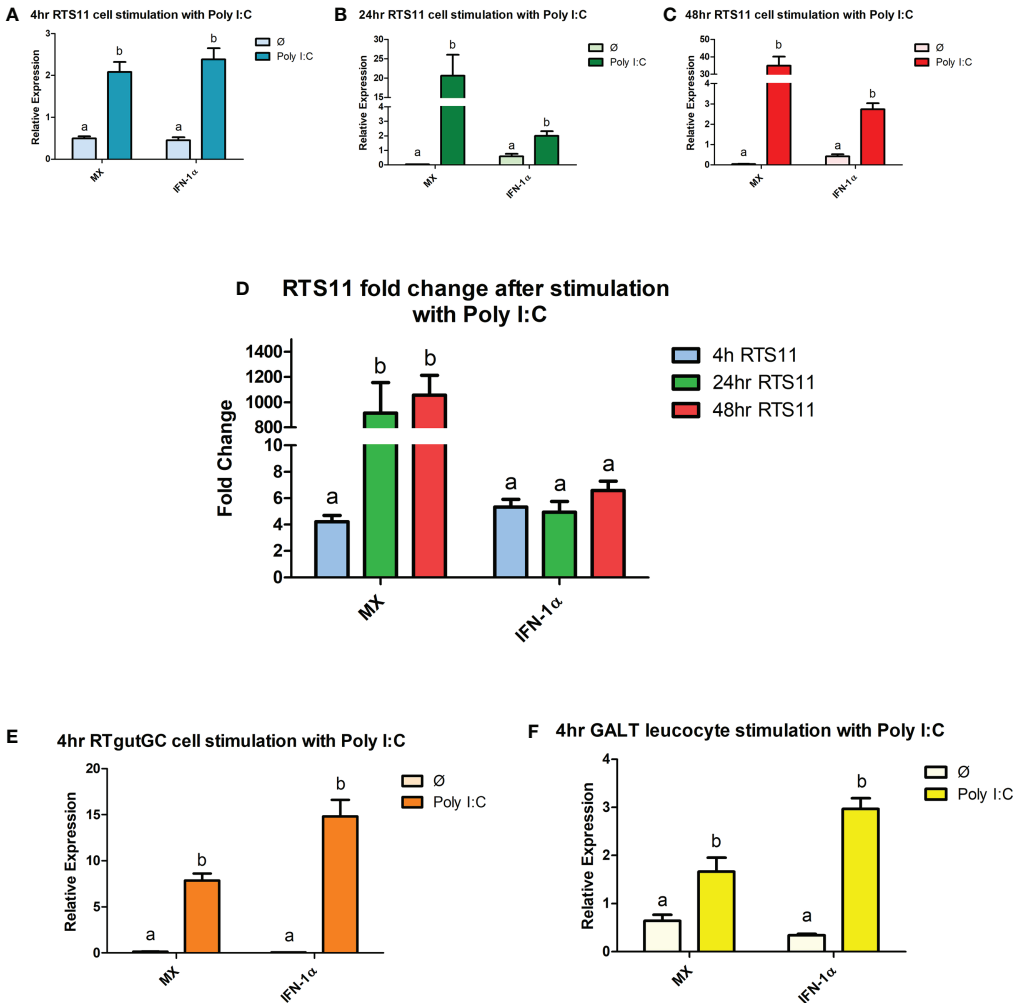

FIGURE 1
RTS11, RTgutGC and GALT leucocyte response to Poly IC. **(A)** RTS11 4hr Poly I:C stimulation showing relative expression. **(B)** RTS11 24hr Poly I:C stimulation showing relative expression. **(C)** RTS11 48hr Poly I:C stimulation showing relative expression. **(D)** RTS11 Fold Change between time points (4, 24 and 48 hr). **(E)** RTgutGC 4hr Poly I:C stimulation showing relative expression. **(F)** GALT leucocyte 4hr Poly I:C stimulation showing relative expression. For relative expression samples were compared between stimulated and unstimulated samples. [a] and [b] denote a significant difference where $p < 0.05$.

increase in *IFN-1α*, whereas the GALT cells showed a ~9-fold increase (not shown in figure). The interferon response gene and *MX* were significantly increased in all the poly I:C stimulated cells confirming the response in these cells at 4hrs post stimulation compared to non-stimulated control cells. The RTgutGC showed the greatest increase in *MX* at 4hrs with an approx. 200-fold increase. At 24 and 48hr the RTS11 cells showed high expression of *MX* compared to the control. These results confirm that GALT leucocytes respond to this viral mimic and give similar responses to the macrophage cell line at the 4hr time point. The background expression of both *MX* and *IFN-1α* is slightly higher in GALT cells compared to RTgutGC and RTS11 hence the fold change expression may appear lower.

3.3 Gene expression in RTS11, RTgutGC and GALT leucocytes in response to PHA stimulation

PHA is a potent stimulator of the proinflammatory response and induces differentiation of T cells. The response to the PHA in the GALT cells revealed a small but significant increase of expression to *IL-4/13* and IFN-γ (Figure 2F), the markers for inflammation, SAA and HAMP were also induced showing both T cell and inflammatory response. The RTgutGC cell line had a minimal response to the PHA stimulant, with only *IL-4/13* showing a difference in expression (Figure 2E). However, in the RTS11 cell line, significant responses were only observed for *SAA* and *HAMP* at both 4hrs and 24hrs post stimulation suggesting the proinflammatory response was being upregulated but not the T-cell response (Figures 2A–D). RTS11 is a macrophage cell line and as a result the T-cell mediated responses were not found to be altered except with *IFN-γ* being significantly decreased at 48hrs.

3.4 Gene expression responses in RTS11, RTgutGC and GALT leucocytes to recombinant trout IL-1β

Recombinant IL-1β is an effective proinflammatory stimulant and drives a high level of gene expression responses. Significant increases in gene expression responses were found in a panel of proinflammatory responding genes, with two cytokines *IL-1β* and *IL-8* and two downstream responding genes, *SAA* and *HAMP*. RTS11, RTgutGC and GALT leucocytes all responded to rIL-1β stimulation (Figures 3A–F). RTS11 showed a significant increase at all time points to *IL-1β, IL-8, SAA*, and *HAMP* with higher responses found at 24 and 48h, especially for SAA, whereas *HAMP* was highest at 24hr and then the magnitude of response dropping at 48hr (Figures 3A–D). The RTgutGC showed the largest expression responses to the rIL-1β after 4hrs in all genes tested, with the GALT leucocytes also exhibiting significant changes at the 4hr time point in *IL-1β, IL-8, SAA*, and *HAMP*, but at a lower magnitude to that of the RTgutGC cells. These results further demonstrate GALT leucocytes' ability to respond to a proinflammatory stimulus as previously demonstrated by PHA. GALT leucocytes show similar responses to the RTS11 cell line suggesting that the responses are being driven by the macrophage response at the 4hr time point.

3.5 Gene expression responses to two β-glucans in cell lines and GALT primary leucocyte cultures

β-glucans are regarded as both prebiotics and as PAMPS, however the precise mode of function of the molecules is not known. Different β-glucan formulations may invoke different responses in cells. Here two different β-glucans are examined for the direct response on two cell lines, a macrophage cell line RTS11, a gut derived cell line RTgutGC and primary GALT leucocytes.

3.5.1 Gene expression responses to M-glucans

The cells that were exposed to M-glucans showed significant increases in gene expression for the proinflammatory marker genes *IL-1β, IL-8*, and *TNFα* in all three cell types (Figure 4). The magnitude of response is highest in the RTS11 cells (Figures 4A–C), which further increases at the later time points for this cell line at 24 and 48h. Secondary responding gene *SAA* indicated a small but significant response at the 4hr time point in the RTS11 cells. At 48hrs post stimulation RTS11 showed significant increases in the responses of both secondary responding genes (*HAMP* and *SAA*). In the RTgutGC cells the proinflammatory cytokines *IL-1β, IL-8* and *TNFα* were all increased significantly as was *SAA* (Figure 4E), a similar response was observed in the GALT cells where *IL-1β, IL-8* and *TNFα* were all increased significantly (Figure 4F). The anti-inflammatory cytokine, *IL-10*, was found to be significantly increased in the RTgutGC cells and RTS11 at 4hrs but not in the primary GALT cells.

There is minimal evidence for an antiviral gene expression response in the RTS11 with only IFN-α being significantly increased at 4h, but the expression was not found during the later time points, the MX gene was not increased here suggesting the M-glucans in the RTS11 did not behave like a viral PAMP (Figure 4D). In the RTgutGC cells a significant rise in MX gene expression was observed, whereas the GALT cells showed no increase in either of these genes. M-glucans may also modulate the T-Cell response with *IL-4/13* being significantly upregulated across all cell types at the 4hr time point, with *IFN-γ* also increased in the RTS11 cells.

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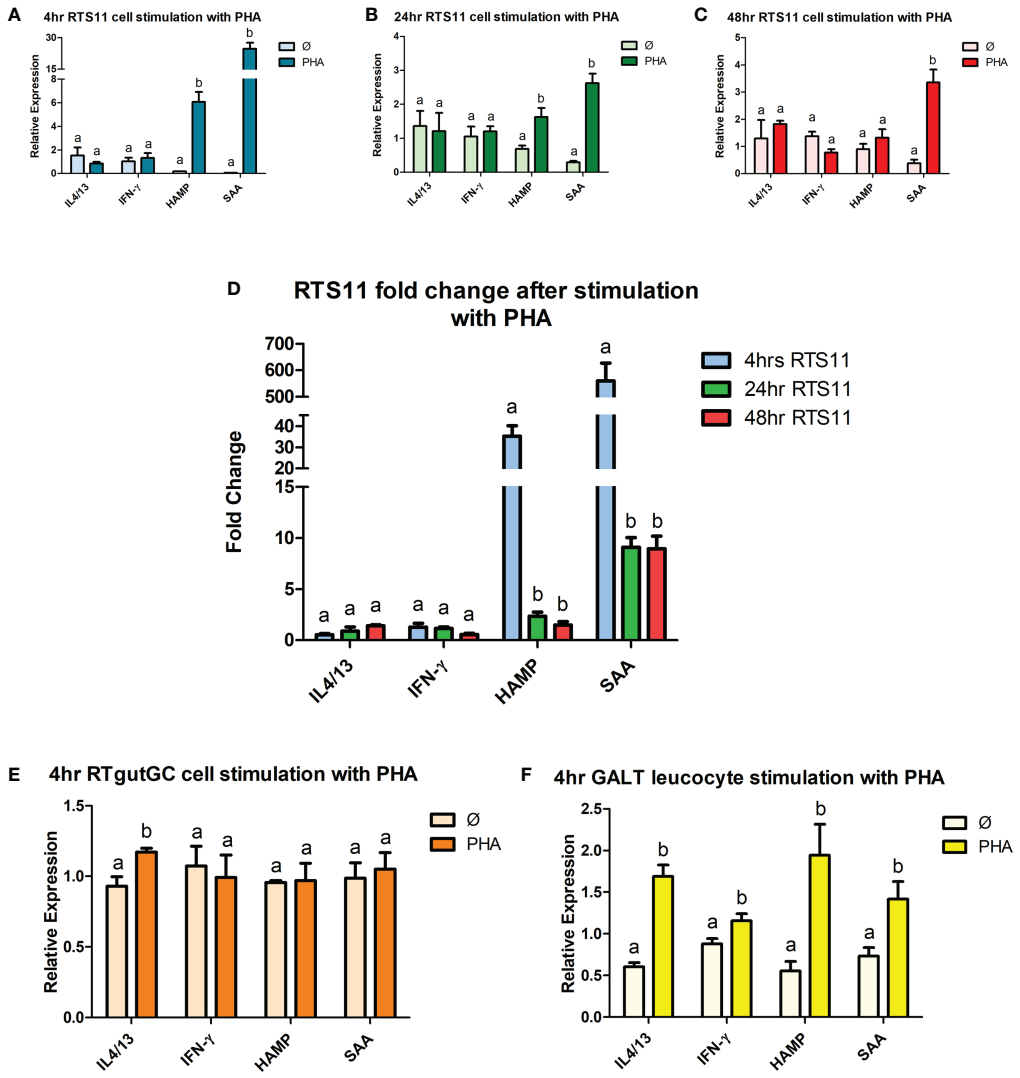

FIGURE 2
RTS11, RTgutGC and GALT leucocyte response to PHA. **(A)** RTS11 4hr PHA stimulation showing relative expression. **(B)** RTS11 24hr PHA stimulation showing relative expression. **(C)** RTS11 48hr PHA stimulation showing relative expression. **(D)** RTS11 Fold Change between time points (4, 24 and 48 hr). **(E)** RTgutGC 4hr PHA stimulation showing relative expression. **(F)** GALT leucocyte 4hr PHA stimulation showing relative expression. *For relative expression samples were compared between stimulated and unstimulated samples.* [a] and [b] denote a significant difference where $p < 0.05$.

3.5.2 Gene expression responses to Fibosel

The three different cell models all indicated a common gene expression response to the Fibosel with *IL-1β*, *SAA* and *HAMP* being significantly upregulated at 4hr post stimulation, the RTS11 cells had the greatest magnitude of response (Figure 5). *TNFα* and *IL-8* were significantly upregulated in both RTS11, at every time point, and RTgutGC after 4hrs. Later time points in RTS11 cells showed a sustained response with *IL-8* and *TNFα* peaking at 48hrs (Figures 5A–D). *IL-10* was significantly upregulated after 4hrs in both RTS11 and RTgutGC. *IL-10* expression showed no significant change at the 24hr time point and was significantly downregulated at the 48hr time point in RTS11 cells. GALT cells showed non-significant increases in expression of *IL-10*. The expression of the secondary proinflammatory responders *SAA* and *HAMP* were both significantly increased in GALT, RTgutGC and RTS11 cells with the response increasing with time post challenge. Interestingly, the proinflammatory markers (*IL-1β*, *IL-8*, *TNFα*, *SAA*, and *HAMP*) all demonstrated a "U-shaped response" over the time course with RTS11 cells (Figure 5D). The gene expression response related to

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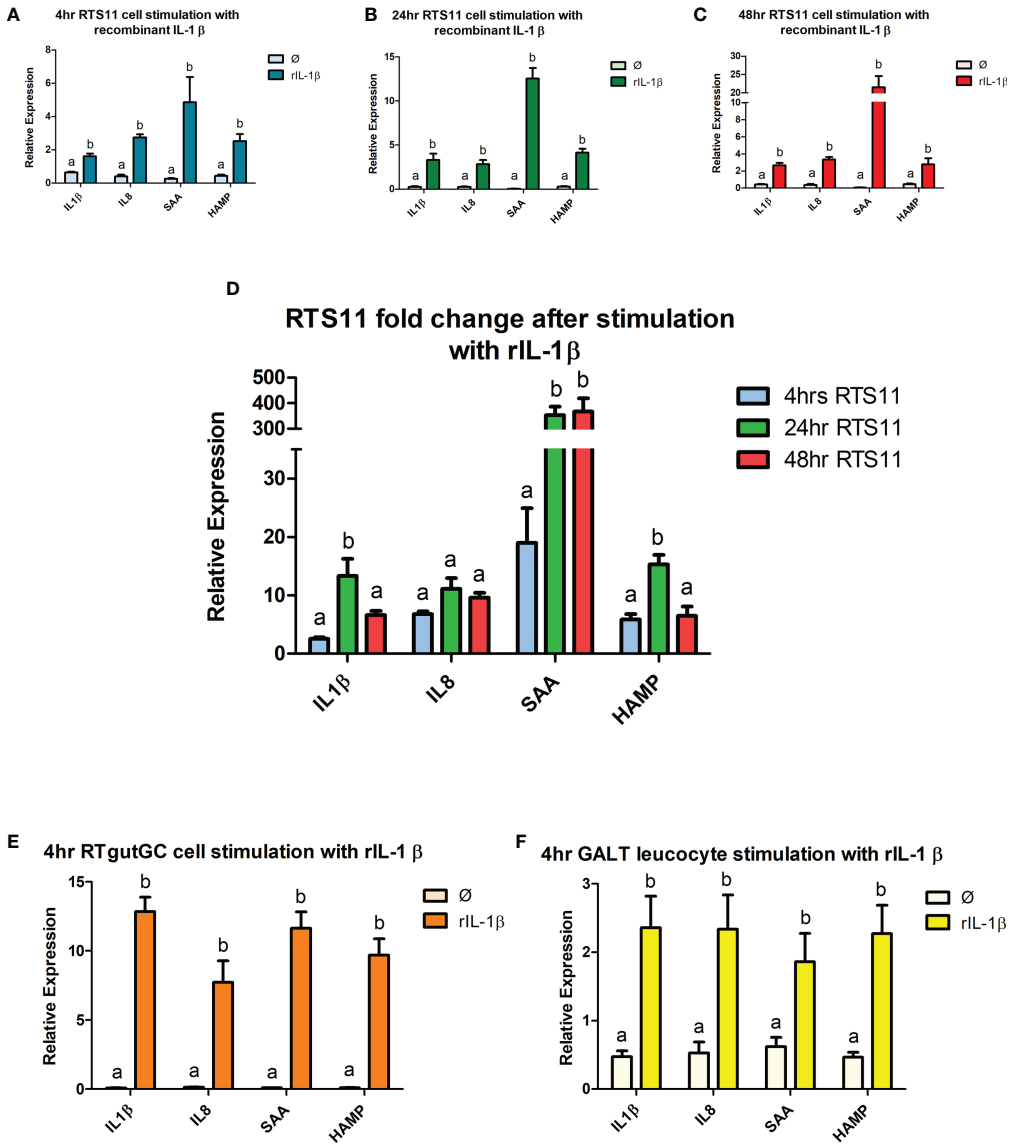

FIGURE 3
RTS11, RTgutGC and GALT leucocyte response to rIL-1β. **(A)** RTS11 4hr rIL-1β stimulation showing relative expression. **(B)** RTS11 24hr rIL-1β stimulation showing relative expression. **(C)** RTS11 48hr rIL-1β stimulation showing relative expression. **(D)** RTS11 Fold Change between time points (4, 24 and 48 hr). **(E)** RTgutGC 4hr rIL-1β stimulation showing relative expression. **(F)** GALT leucocyte 4hr rIL-1β stimulation showing relative expression. For relative expression samples were compared between stimulated and unstimulated samples. [a] and [b] denote a significant difference where $p < 0.05$.

antiviral activity showed mixed responses between the cell types. The RTS11 cells responded strongly to Fibosel with the *MX* being significantly increased in expression at 24 and 48h, but not at 4h. The expression of *MX* was also found to be increased in the RTgutGC cells but decreased in expression in the GALT cells. *IFN-1α* showed a minimal change in the RTS11 cell line at all time points with downregulation seen in RTgutGC. *IL-4/13* was found to increase in expression in both the RTgutGC and the GALT cells but not in the RTS11, with *IFN-γ*not responding to the Fibosel in any cell type examined.

3.5.3 Comparison between M-glucans and Fibosel

Figure 6 shows a heatmap of the β-glucan stimulated samples at the 4hr time point. Both Fibosel and M-glucans can drive a proinflammatory response shown by upregulation of *IL8, IL-1β, SAA, TNFα* and *HAMP* in RTS11 cells, this response is also observed to a lesser extent in RTgutGC. This response is also seen in GALT but to a much lower extent in terms of fold change, potentially due to different cell types being present so a single response is not seen and is more subtle. Fibosel can drive the

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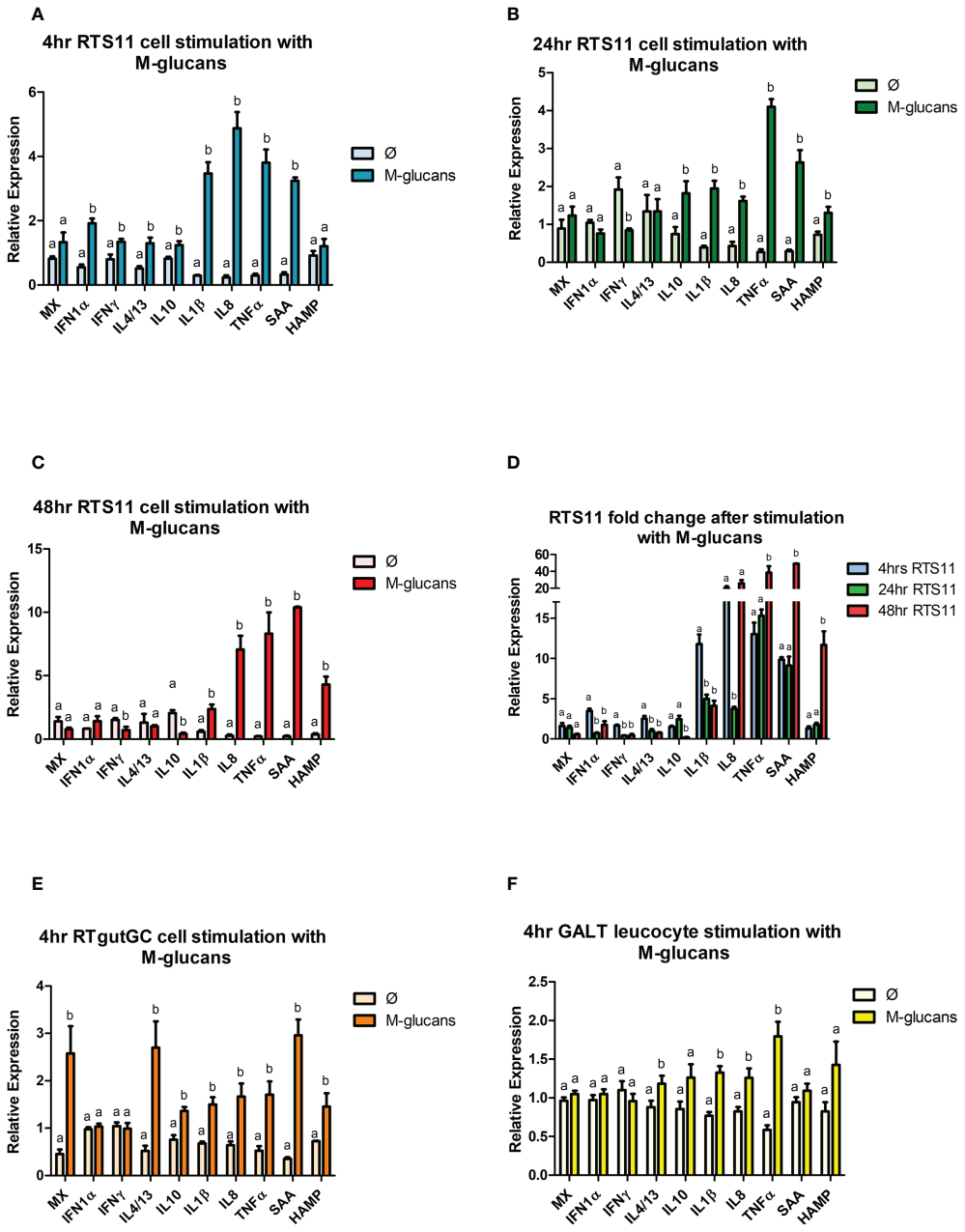

FIGURE 4
RTS11, RTgut and GALT leucocyte response to M-glucans. **(A)** RTS11 4hr M-glucans stimulation showing relative expression. **(B)** RTS11 24hr M-glucans stimulation showing relative expression. **(C)** RTS11 48hr M-glucans stimulation showing relative expression. **(D)** RTS11 Fold Change between time points (4, 24 and 48 hr). **(E)** RTgutGC 4hr M-glucans stimulation showing relative expression. **(F)** GALT leucocyte 4hr M-glucans stimulation showing relative expression. For relative expression samples were compared between stimulated and unstimulated samples. [a] and [b] denote a significant difference where $p < 0.05$.

proinflammatory response to a higher extent compared to M-glucans in RTS11 cells and in GALT cells however in RTgutGC it is the other way around. The secondary response gene HAMP also shows unique differences between cell types and stimulants. Both β-glucans can modulate the *HAMP* response with M-glucans driving a larger response in M-glucans in RTgutGC and GALT whilst Fibosel drives a larger response in RTS11. Viral genes show very little change across all cell lines with only *MX* seeming to be upregulated in RTgutGC to a similar extent between both M-glucans and Fibosel. These responses seen may offer an indication into the pathways that are driven by β-glucans, with the proinflammatory response and antibacterial genes seemingly

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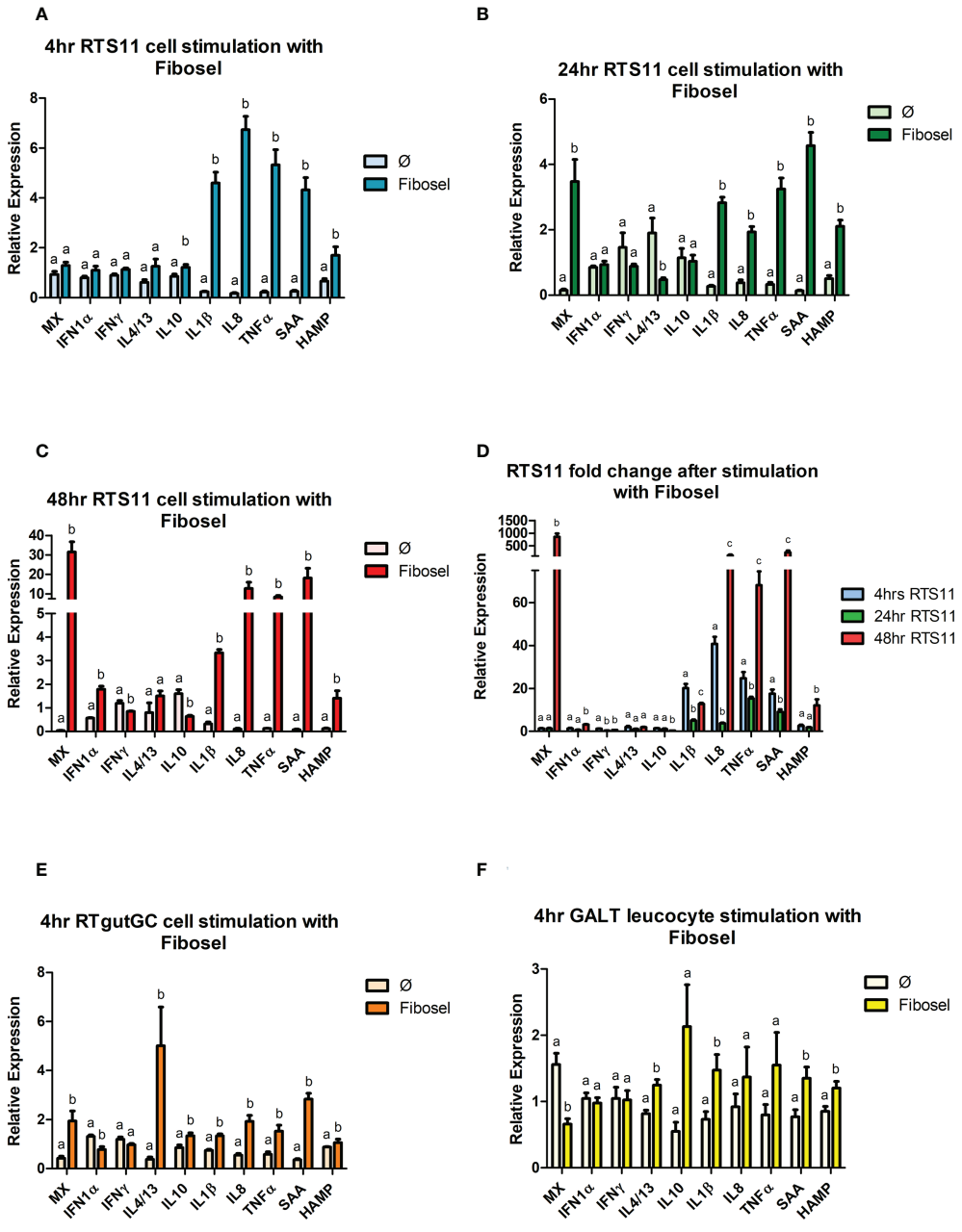

FIGURE 5
RTS11, RTgut and GALT leucocyte response to Fibosel. **(A)** RTS11 4hr Fibosel stimulation showing relative expression. **(B)** RTS11 24hr rIL1 β stimulation showing relative expression. **(C)** RTS11 48hr Fibosel stimulation showing relative expression. **(D)** RTS11 Fold Change between time points (4, 24 and 48 hr). **(E)** RTgutGC 4hr Fibosel stimulation showing relative expression. **(F)** GALT leucocyte 4hr Fibosel stimulation showing relative expression. For relative expression samples were compared between stimulated and unstimulated samples. ^{a,b,}and ^{c} denote a significant difference where $p < 0.05$.

affected more by β-glucan supplementation. Whilst the GALT response is more subtle as previously mentioned levels of background expression were much higher in this cell line in comparison to both RTS11 and RTgutGC so may demonstrate a lower fold change compared to a single cell type's response and as such, may not be seen as effectively as a cell line.

4 Discussion

Both *in-vivo* experiments, and cellular models can be used to identify molecular responses to PAMPs or functional ingredients through the advancements in *'omic'* technologies (32, 33). Many of the important economic aquaculture species

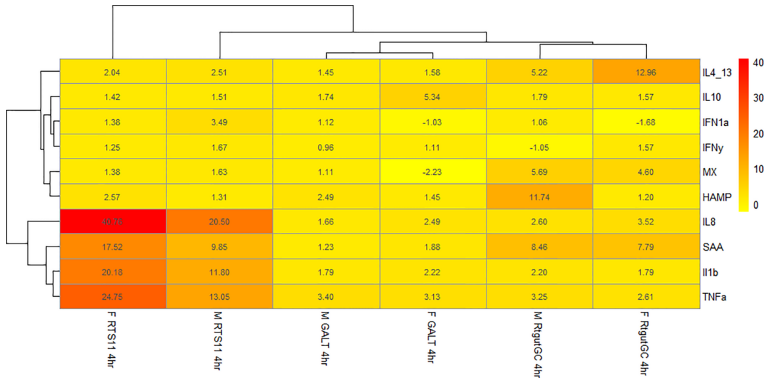

FIGURE 6
Heatmap of fold change for samples stimulated with either M-glucan or Fibosel across all three cell culture models.

have genomes which are now fully characterized and annotated allowing for in-depth immuno-transcriptomic experiments to illustrate the molecular machinery involved in the immune response (34). *In-vivo* studies have been used to identify the molecular responses to prebiotics and subsequent pathogenic challenges offer an insight into the methods in which prebiotics may modulate the immune response to protect against pathogens. The use of *in-vivo* models highlights the overall response where there is likely to be both direct and in-direct interaction of prebiotics with the GALT tissue. However, the extent to which these responses are being caused by the direct or in-direct interactions of prebiotics are not fully known. On the other hand, cell lines and other cellular models can be used to identify the direct effects of PAMPs and functional ingredients on specific cell types and their subsequent immune response as such these models can be used either as stand-alone models or to complement *in-vivo* studies to offer greater understanding and knowledge of the cellular mechanisms. For this study both the RTS11 and RTgutGC cell lines were used in combination with GALT primary leucocytes to determine how the cells direct immune responses to both characterized PAMPs and two β-glucans that are used as functional feed compounds. These cell culture models have been previously established as viable assays to identify immune responses; RTS11 (35), RTgutGC (24) and GALT leucocytes (20).

The initial experiments performed to assess the different responses of the cell types to PAMPS, secondly the cells were exposed to the two forms of β-glucan to determine the extent of direct cellular stimulation, and if so what type of response these molecules induced. The RTS11 cell line has been extensively characterized for gene expression responses to immunostimulants (24, 35, 36) especially for proinflammatory and antiviral responses (37) and is the go-to cell line for many immunological studies. RTS11 cells are derived from the spleen and are believed to have phenotype representing macrophages (23). RTgutGC was developed by 22, these cells represent epithelial cells. The role of these epithelial cells in immunological responses is relatively unknown. Recent studies by Alkie et al. (38) have reported immunological responses in the RTgutGC cells in response to Poly I:C showing an increase in several genes involved in antiviral activity including *IFN-1α*, *MX-1* and *VHSV-induced gene 3 (Vig3)* at both 6 and 12 h post stimulation. Both cell lines are believed to represent a single cellular phenotype, whereas the GALT primary cells may represent a diverse collection of leucocytes as previously described (20). Some cell lines (RTgutGC and RTgutF) have been used in conjunction with one another to offer a cell line model that represent a closer representation of the gut model and barrier function (39).

Primary cell cultures in contrast to permanent cell lines have the advantage of retaining the diversity of immune cell types from the organ they are extracted from, but there are the added complications of extraction time, and the phenotypic and genetic variability of the animal from which these cells are obtained. In this experiment, we assess how GALT cells respond in comparison to the responses seen in the characterized cell lines of rainbow trout. Primary leucocytes have been isolated from many tissues including isolated head kidney (40), gill (41), blood (42) and have been used to identify immune responses with these tissues being classically regarded as primary and secondary immune organs. However, it is becoming clearer that the intestine and GALT plays a very important and immediate role in immune responses as a consequence of dietary manipulations. Currently there is a limited amount of literature on the isolation of leucocytes from the gut in salmonids (20, 28, 43). GALT leucocytes from the whole gut have been tested against several different PAMPs (20), in this study our aim was to use just the distal intestine due to its theoretical importance as an immunological organ and is the basis of many feeding trial experiments on the gut. Cell

viabilities for GALT from the hind gut in this experiment matched previously reported viabilities from GALT cells extracted from the whole gut as shown by 20. Whilst there is good viability at 4hrs the GALT leucocytes show a significant drop after 24hrs probably due to the length of the extraction method used to isolate cells from both the lamina propria and intraepithelial layers of the gut. To our knowledge, this is the first comparison between GALT leucocytes and cell line models. The major difference between cell line models and primary cultures are the number of cellular phenotypes present. Cell line models typically only have one cell type present and as such may only display one or two phenotypes. GALT leucocytes have been demonstrated to contain multiple cell types with key cell markers seen for several different T-, B-cells, macrophages, neutrophils, and dendritic cell populations (20) and display a much larger repertoire of cellular phenotypes. This increase in cell types may offer a larger number of potential cellular responses to be identified in these models.

All cell types responded to the viral mimic Poly I:C and there was a clear increase in expression of both *IFN-1α* and *MX* following 4 hours of stimulation. At 4hrs the magnitude of response was highest in the RTgutGC cells, also of interest the GALT leucocytes had higher background expression so may not have as large a magnitude of response. RTS11 cells were also able to respond to Poly I:C stimulation. This confirms the GALT cells can respond to this viral mimic.

PHA has properties as a strong T-cell stimulant, as well as being able to stimulate proinflammatory mediators. PHA has the capacity to stimulate RTS11 cells with studies showing an increase in expression of *Interferon-gamma inducible protein (γIP)* (37), an IFN-γ responsive gene, suggesting these cells have receptors that enable PHA responses. The T-cell marker genes we chose to explore for PHA responses were IFN-γ and IL-4/13, neither cell lines responded with an increase in expression of these two markers in our experiments. Interestingly, the GALT cells, which are expected to have a diverse repertoire of cell types did respond to PHA with these genes both being significantly increased in expression at 4-hour post stimulation. PHA can also act as a proinflammatory mediator (44) as seen in our study with RTS11 and GALT cells demonstrating a significant upregulation of both *SAA* and *HAMP*. This change was not observed in the RTgutGC cell line suggesting that this epithelial cell line can only respond to certain proinflammatory triggers due to the constrained cell type and receptor repertoire.

IL-1β is a key proinflammatory cytokine involved in many different cell signaling pathways and is often upregulated during the early proinflammatory response. Recombinant IL-1β can trigger the immune response in RTS11 cells with over 20% of genes within this enriched biological process, including genes such as *Hepcidin* and immune response protein 1, being differentially expressed (35). For the rIL-1β response we decided to assess two early phase cytokines *IL-1β* and *IL-8* and two secondary mediators *SAA* and *HAMP*. All four genes assessed showed an increase in response to rIL-1β after 4 hours of stimulation in all cell culture models. Interestingly, the response at 4 hours between RTS11 and GALT were similar for all four genes potentially suggesting the macrophage cells were driving the response to rIL-1β. GALT leucocytes demonstrated higher background levels of expression compared to both RTS11 and RTgutGC samples. This data validates the need for further primary cell culture models to be developed. Gut specific cell culture models are needed to provide a unique insight into the mechanisms behind the interface between nutrition and the immune system (33).

The use of the PAMPS established the potential for the different cell lines and GALT cells to respond to the characterized stimulants, The next question was how these cells respond to the β-glucans. β-glucans can act as an immunostimulant causing the upregulation of the proinflammatory response as demonstrated by previous cell line models using both RTS11 and RTgutGC (24, 45, 46). In the current work, we have used two forms of β-glucans were used to identify if the refinement of the compound has an impact on the immune response to these molecules. Stimulation of RTS11, RTgutGC and GALT leucocytes, showed an increase in the proinflammatory markers tested with *IL-1β, IL-8, TNFα* and *SAA* commonly being significantly upregulated across both M-glucan and Fibosel stimulated samples. This data suggests that β-glucans can modulate the proinflammatory response directly and not just as a prebiotic (*i.e.*, only having effect *via* changes in microbiome activity) (47). Of the two β-glucans tested, M-glucan was able to induce a greater upregulation of the proinflammatory response. Previous research using the M-form (45) are consistent with the results of the present study. This indicates that the particulate β-glucan form acts as a stronger modulator of the proinflammatory response. Both β-glucans were able to significantly increase the *IL-4/13* gene in GALT leucocytes and RTgutGC, however only M-glucans were able to modulate RTS11 cells at 4hrs. Fibosel stimulation resulted in a much larger upregulation of the *IL-4/13* gene after 4 hours compared to M-glucans. These results further validate the idea that β-glucans can act as an immunostimulant interacting directly with immune cells.

Previous *in-vivo* studies have shown β-glucan administration generates a short-lived immune stimulatory effect resulting in enhanced resistance to both viral and bacterial infections (17, 48). Noticeably, short term responses on viral genes on GALT leucocytes had no effect on viral response. A viral response was seen in RTS11 cells treated with fibosel at the 48hr time point with *MX and IFN-1α* being upregulated, this response was not seen in cells treated with M-glucans. Further studies are needed to quantify whether other type 1 interferons are involved. Our data suggests that β-glucans may have immunostimulatory properties more aligned with antibacterial activity than antiviral activity in the short term innate immune response.

There is emerging evidence that β-glucans may be involved in immunotolerance as demonstrated using cell line approaches in trout (45) and primary cell cultures using carp macrophages (17). However, more research is needed to identify the extent to which this immunomodulation offers protection to pathogenic stimulants in both *in-vitro* and *in-vivo* trials and against bacterial or viral pathogens.

In summary, we demonstrate the importance of cellular models that contain multiple phenotypes present to ensure multiple immune cell types' responses are captured. We have demonstrated the effectiveness of RTS11 and RTgutGC in combination with GALT leucocytes as potential screening methods for functional feed analysis. With RTS11 and GALT leucocytes showing a clear indication of the immune pathways modulated. RTS11 and GALT leucocytes showed good responses against all PAMPs and could act to show relevance against bacterial pathogens and tolerance studies in future studies. Whilst GALT leucocytes showed clear responses to PAMPs and β-glucans, cell viability is still an issue after 24hrs being at 53%, so studies may be limited to 4-8hrs. However, using GALT leucocytes alongside RTS11 cells or an *in-vivo* bacterial challenge would enable tolerance studies to be carried out in conjunction with GALT immune modulation. This study further highlights the action of β-glucans as an immunostimulant with both β-glucans being able to modulate key proinflammatory cytokines and T-cell. Future studies using cell line models or *in-vivo* experiments will further demonstrate if supplementation with β-glucans can offer protection against pathogenic challenges.

Data availability statement

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

Ethics statement

Fish were looked after in accordance to UK Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. All animal procedures were carried out under UK project licence PFF8CC5BE. The study was reviewed and approved by the University of Aberdeen Animal Ethics Committee.

Author contributions

DPo contributed to the design of the experiment, sample acquisition, data analysis, and wrote the manuscript. SM contributed to the design of the experiment, supervision of the project, and reviewed the manuscript. DPe and CM reviewed the manuscript and provided B-glucans. All authors contributed to the article and approved the submitted version.

Funding

This work was funded by the University of Aberdeen.

Acknowledgments

This work was funded by Skretting AI and the University of Aberdeen. Technical support by Dr Dawn Shewring was greatly appreciated. We also thank Dr Tiehui Wang, Scottish Fish Immunology Research Centre for providing the rIL-1β.

Conflict of interest

CM and DPe are employees of Skretting AI.

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.

The authors declare that this study received funding from Skretting AI. The funder had the following involvement in the study: review of manuscript and provision of β-glucan samples.

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 17 November 2022
DOI 10.3389/fimmu.2022.1066936

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Comparative Immunology, a section of the journal Frontiers in Immunology

RECEIVED 11 October 2022
ACCEPTED 01 November 2022
PUBLISHED 17 November 2022

CITATION
Liu J-X, Zhu K-C, Guo H-Y, Liu B-S, Zhang N and Zhang D-C (2022) Effects of cysteine addition to low-fishmeal diets on the growth, anti-oxidative stress, intestine immunity, and *Streptococcus agalactiae* resistance in juvenile golden pompano (*Trachinotus ovatus*). *Front. Immunol.* 13:1066936. doi: 10.3389/fimmu.2022.1066936

Effects of cysteine addition to low-fishmeal diets on the growth, anti-oxidative stress, intestine immunity, and *Streptococcus agalactiae* resistance in juvenile golden pompano (*Trachinotus ovatus*)

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As the precursor of taurine, cysteine serves physiological functions, such as anti-oxidative stress and immune improvement. Investigation of cysteine and its derivatives has made positive progress in avian and mammalian species, yet the study and application of cysteine in aquatic animals are relatively rare. Therefore, we evaluated the effects of supplementing a low-fishmeal diet with various levels of cysteine on the growth, antioxidant capacity, intestine immunity, and resistance against *Streptococcus agalactiae* of the juvenile golden pompano (*Trachinotus ovatus*). According to our study, exogenous supplementation with 0.6-1.2% cysteine greatly increased the final body weight (FBW) and specific growth rate (SGR) of golden pompano compared to the control group. Under the present conditions, the optimum dietary cysteine supplementation level for golden pompano was 0.91% based on the polynomial regression analysis of SGR. Meanwhile, we found that the Nrf2/Keap1/HO-1 signaling pathway was notably upregulated with the increase of exogenous cysteine, which increased antioxidant enzyme activity in serum and gene expression in the intestine and reduced the level of reactive oxygen species (ROS) in the serum of golden pompano. In addition, morphological analysis of the midgut demonstrated that exogenous cysteine improved muscle thickness and villi length, which suggested that the physical barrier of the intestine was greatly strengthened by cysteine. Moreover, cysteine increased the diversity and relative abundance of the intestinal flora of golden pompano. Cysteine suppressed intestinal NF-κB/IKK/IκB signaling and pro-inflammatory cytokine mRNA levels. Conversely, intestinal anti-inflammatory cytokine gene expression and serum immune parameters were upregulated with the supplementary volume of cysteine and improved

intestine immunity. Further, exogenous cysteine supplementation greatly reduced the mortality rate of golden pompano challenged with *S. agalactiae*. In general, our findings provide more valuable information and new insights into the rational use of cysteine in the culture of healthy aquatic animals.

KEYWORDS

Trachinotus ovatus*, cysteine, intestine immunity, growth, antioxidant capacity, *Streptococcus agalactiae

Introduction

As a major concern of the global aquaculture industry, the price of fishmeal has markedly increased over the past years, making the identification of fishmeal substitutes an urgent matter (1–3). However, previous studies indicate that neither plant protein, such as soybean meal (1, 2), rapeseed protein (3), and corn gluten meal (1, 4), nor animal proteins, such as insect meal (5), poultry by-product meal (1), and chicken meal (6), which are closer to fish meal in terms of nutrient composition, can completely replace fish meal in the diet of aquatic animals. As good sources of protein, these alternatives may have advantages in terms of their price, and small quantities required, however, the role of fishmeal as a bottleneck in aquafeeds seems impenetrable (7–9). Studies have shown that if the proportion of fishmeal is markedly reduced, it can cause a change in the intestinal microbiota of aquatic animals (10, 11), leading to a decline in growth performance (11, 12). The presence of abundant anti-nutritional factors in their diet can elicit oxidative stress in aquatic animals (9, 11). This typically results in structural damage to the intestinal tract (12) and ultimately leads to a decline in intestine immunity (8, 12–15), reducing the ability of aquatic animals to cope with external pathogen infection and, thus, leading to death. Therefore, considering fishmeal scarcity and the lack of an adequate substitution, it is important to explore novel ways to reduce the proportion of fishmeal in aquatic animal diets while maintaining growth, reducing oxidative stress, and enhancing intestine immunity.

Previous studies have shown that appropriate inclusion of functional supplements in aquatic diets can effectively mitigate the adverse effects of low-fishmeal diets on fish (11, 16). Taurine is a functional amino acid that promotes fish growth and immunity, and its levels are correlated with fish health (17–19). The primary source of dietary taurine is fishmeal, thus reducing the proportion of fishmeal in the diet of fish will inevitably decrease dietary taurine content and reduce growth performance and intestine immunity (16, 19).

Cysteine, the precursor of taurine in living organisms (20, 21), has important biological functions, including antioxidative (22) and immunity (23) enhancing effects. Research on the functions of cysteine and its derivatives in mammals, such as rat (22, 24), pig (25–27), and sheep (28, 29), has produced considerable advances; however, respective research and applications in aquatic animals are relatively scarce. Studies in rats have indicated that cysteine as a dietary additive improves resistance to oxidative stress associated with high sucrose levels (22). Moreover, the potential of cysteine to enhance intestine immunity may be related to its resistance to oxidative stress and defense against inflammatory responses (24). For example, cysteine may protect the physical barrier of piglet intestines through the Nrf2 and NF-κB pathways, increasing the length of intestinal villi, and protecting intestinal structure and functioning (25).

The intestine immunity system, consisting of physical barriers, intestinal flora, and immune barriers, is essential for organismic health (30), and cysteine has been shown to improve intestine immunity. For example, dietary supplementation with 0.4% cysteine can increase the quantity and diversity of intestinal microorganisms in gestating sows (31). In addition, cysteine can revive the intestine immunity system in pigs by downregulating the mRNA levels of pro-inflammatory cytokines and improving the local inflammatory response in the intestine (27). Moreover, cysteine supplementation may, to some extent, protect broiler chickens infected with Eimeria from intestinal lesions (32).

The golden pompano (*Trachinotus ovatus*) is one of the predominant aquaculture fish in China, however, the effects and

Abbreviations: T-AOC, total antioxidant capacity; CAT, catalase; GSH-PX, glutathione peroxidase; SOD, superoxide dismutase; MDA, malondialdehyde; ROS, reactive oxygen species; LZM, lysozyme; IgA, immunoglobulins A; IgG, immunoglobulins G; IgM, immunoglobulins M; Nrf2, nf-e2-related nuclear factor 2; Keap-1, Kelch-like ECH-associated protein-1; HO-1, heme oxygenase-1; NF-κB, nuclear factor kappa B; IκB, inhibitor protein-κB; IKK, IκB kinase; TNF-α, tumor necrosis factor-α; IL-1β, interleukin 1β; IL-8, interleukin 8; IL-10, interleukin 10; WGR, weight gain rate; SGR, specific growth rate; FCR, feed conversion ratio; CF, condition factor; SR, survival rate; HSI, hepatosomatic index; VSI, viscerasomatic index; FI, feed intake; IBW, initial body weight; FBW, final body weight; FBL, final body length; DDI, dry diet intake; NOD, number of days; VW, viscera weight; LW, liver weight; NWG, net weight gain; FFN, final fish numbers.

mechanisms of cysteine on the intestine immunity of golden pompano are not entirely clear. Theoretically, satisfying the 42% dietary protein level of golden pompano with Peruvian fishmeal, containing 67% protein, would require a diet with a minimum of 60% fishmeal, which would inevitably increase the cost of farming given the current shortage of fishmeal resources (33). Nevertheless, diets with lower fishmeal content tend to reduce the immune capacity of fish (8, 9). Infectious diseases caused by *Streptococcus agalactiae* frequently broke out in recent years in large-scale cultures of marine fish due to excessive farming density and increasing environmental pollution (34). Therefore, we explored the effects of cysteine-supplemented low-fishmeal diets (containing 20% fishmeal) on growth performance, intestine immunity, and resistance against *S. agalactiae* in juvenile golden pompano in order to provide further insights into the mechanism of cysteine regulation of the intestine immunity of fish and to identify a solution for future usage of low-fishmeal diets.

Materials and methods

Ethical statement

All experiments in this study were conducted in accordance with the regulations and guidelines established and approved by the Animal Care and Use Committee of the South China Sea Fisheries Research Institute of the Chinese Academy of Fishery Sciences (No. SCSFRI96-253).

Experimental diets

Experimental diets of golden pompano were supplemented with food-grade cysteine (99.99% purity, Zhejiang Yi Nuo Biotechnology Co. Ltd., Wenzhou, China). The remaining ingredients were supplied by Guangzhou Nutriera Biotechnology Co. Ltd. (Guangzhou, China); Table 1 shows dietary formulations and nutritional levels. We produced five diets with equal nitrogen and lipid, based on the nutritional requirement of 42% protein for golden pompano (35). We mixed protein sources to avoid one of them reducing palatability; animal protein (fish meal and chicken meal) and plant protein (soybean protein concentrate, fermented soybean meal, and corn protein meal) were used as the base protein sources for golden pompano diets. Fish oil and soybean oil were used as sources of lipids. High gluten flour provided relatively little protein and fat and was used as a gamete to achieve the same quality in all diets (33). We set the amount of cysteine added to the diet at 0.00%, 0.30%, 0.60%, 0.90%, and 1.20% for groups C0 (control), C1, C2, C3, and C4 respectively, with reference to the amount of cysteine added to the *Paralichthys olivaceus* diet (36). In brief, the first step was to grind all solid ingredients separately until they could pass through a 40-mesh screen. Secondly, the ground ingredients were mixed at the proportions shown in Table 1; oil and water were added in batches during mixing, and each batch of diets was mixed for 30 min to achieve homogenization. The mixture was then placed in an extruder (Valva Machinery Co., Ltd., Guangzhou, China), and the diets were transformed into three sizes of spherical pellets (1, 2, and 3 mm diameter) to suit the growth state of the fish (37). Finally, the diets were placed in a drying oven at 45°C until the moisture content was reduced to approximately 10% (38); thereafter, the pellets were stored 4°C. Table 2 shows the amino acid composition of each diet.

Experimental procedure

To reproduce the large-scale culture environment of golden pompano, we used offshore cages (1.00 × 1.00 × 1.50 m) in Longgang District, Shenzhen, China. *S. agalactiae* was extracted from golden pompano and tested for its pathogenicity. The fish used for this experiment were selected from juvenile golden pompano bred in our laboratory throughout the year. Before the experiment, we separated 1,050 fish (10.05 ± 0.05 g) into 15 cages (five diets, three cages per diet), with 70 fish per cage. Since fish mortality is inevitable during the feeding process, we set the number of fish per cage to 70 to ensure that there would also be enough fish for subsequent challenge experiments. The fish were then fed the C0 diet without exogenous cysteine for one week to allow the fish to adjust to the experimental conditions. During the experiment, we fed the fish their respective diet at 8:00, 10:00, 14:00, and 16:00 each day, until the fish stopped eating. During the eight-week feeding period, feed intake status and hydrographic information at sea were observed and recorded daily. During the test period, the water conditions were maintained as follows: temperature at 28–32°C, pH at 7.4–8.3, salinity at 34–36 ‰ and dissolved oxygen > 6.0 mg/L.

Sample collection

At the end of the feeding experiment, the fish were fasted for 24 h, after which the fish were weighed and counted in each cage. Nine fish per cage were randomly chosen, and anesthetized with eugenol (100–200 mg/L; Shanghai Medical Devices Co., Ltd., Shanghai, China). Three fish per cage were transferred to -20°C after rapid freezing with liquid nitrogen for organism composition analysis. From three other individuals per cage, we collected blood and centrifuged it to obtain serum for measuring anti-oxidative stress and immunological parameters. After serum collection, the fish were dissected, and the intestines were collected for RNA extraction and gene expression analysis. The intestinal contents were removed and stored at -80°C for intestinal flora analysis. The midguts of the

TABLE 1 Formulation and nutrition level of the experimental diets (% dry matter basis).

Parameters	Group				
	C0	C1	C2	C3	C4
Ingredients (%)					
Fishmeal [a]	20.00	20.00	20.00	20.00	20.00
Chicken meal [a]	10.00	10.00	10.00	10.00	10.00
Soy protein concentrate [a]	10.00	10.00	10.00	10.00	10.00
Squid paste	5.00	5.00	5.00	5.00	5.00
Soybean meal [a]	12.00	12.00	12.00	12.00	12.00
Fermented soybean meal [a]	5.00	5.00	5.00	5.00	5.00
Corn gluten meal [a]	6.00	6.00	6.00	6.00	6.00
High gluten flour [a]	18.37	18.07	17.77	17.47	17.17
Fish oil [a]	6.00	6.00	6.00	6.00	6.00
Soybean oil [a]	3.00	3.00	3.00	3.00	3.00
$Ca(H_2PO_4)_2$ [a]	1.50	1.50	1.50	1.50	1.50
Choline chloride [a]	0.30	0.30	0.30	0.30	0.30
Vitamin mix [a,b]	1.00	1.00	1.00	1.00	1.00
Mineral mix [a,c]	1.00	1.00	1.00	1.00	1.00
L-lysine monohydrochloride [a]	0.50	0.50	0.50	0.50	0.50
DL-Methionine [a]	0.20	0.20	0.20	0.20	0.20
Threonine [a]	0.10	0.10	0.10	0.10	0.10
Ethoxyquin [a]	0.03	0.03	0.03	0.03	0.03
Cysteine [a]	0.00	0.30	0.60	0.90	1.20
Nutrition level [d]					
Crude Protein (% dry matter)	42.79	42.74	42.69	42.63	42.58
Crude Lipid (% dry matter)	13.42	13.40	13.38	13.37	13.35
Moisture (% dry matter)	10.15	10.76	11.24	10.98	11.32
Ash (% dry matter)	8.53	8.65	8.33	8.71	8.39
Cysteine	0.52	0.82	1.13	1.45	1.84

[a]Ingredients are provided by Guangzhou Nutriera Biotechnology Co., Ltd. and Zhejiang Yi Nuo Biotechnology Co. Ltd.
[b]Vitamin mix provides the following (Per kilogram content): vitamin A (8×106 IU), vitamin D3 (2×106 IU), vitamin E 40 000 mg, vitamin B 17 000 mg, vitamin B6 12 000 mg, vitamin B12 100 mg, vitamin K3 10 000 mg, D-pantothenic acid 35 000 mg, folic acid 1 000 mg, nicotinamide 90 000 mg, Biotin 200 mg, inositol 80 000 mg.
[c]Mineral provides the following (Per kilogram content): Fe 10 000 mg, Cu 1 200 mg, Zn 7 000 mg, Mn 5 500 mg, Co 250 mg, I2 250 mg, Se 50 mg, K 60 000 mg, Na 24 000 mg, Mg 60 000 mg
[d]Nutrition level is measured.

remaining three fish per cage were collected and preserved in 4% paraformaldehyde solution for histological analysis.

Growth performance

Growth performance-related parameters were calculated according to the following equations:

$$WGR\ (\%) = 100 \times (FBW - IBW) / IBW$$

$$SGR\ (\%/day) = 100 \times (\ln FBW - \ln IBW) / NOD$$

$$FCR\ (\%) = 100 \times DDI / NWG$$

$$CF\ (g/cm^3) = 100 \times FBW / FBL^3$$

$$SR\ (\%) = 100 \times FFN / 30$$

$$HSI\ (\%) = 100 \times LW / FBW$$

$$VSI\ (\%) = 100 \times VW / FBW$$

$$FI\ (\%/day) = 100 \times DDI / [(IBW + FBW)/2] / NOD$$

Serum biochemical and immunological parameter

To investigate the effects of exogenous dietary cysteine on the anti-oxidative stress capacity and immunity in juvenile golden pompano, we examined the relevant parameters in serum. For antioxidant indices, we determined the levels of MDA, ROS, and the activities of antioxidant enzymes, such as T-AOC, CAT, GSH-PX, and SOD. Serum immunological

10.3389/fimmu.2022.1066936

TABLE 2 Amino acid composition of the experimental diets(g·100g^{-1}).

Parameters	Group				
	C0	C1	C2	C3	C4
Aspartic acid	4.35	4.39	4.24	4.31	4.45
Threonine	1.90	2.02	2.11	1.91	2.02
Serine	2.04	2.05	2.04	2.12	2.05
Glutamic acid	8.41	8.66	8.69	8.36	8.62
Glycine	3.04	3.1	3.02	2.98	3.08
Alanine	3.06	3.09	3.06	2.95	3.07
Proline	3.21	3.13	3.04	3.16	3.13
Valine	2.13	2.11	2.07	2.12	2.11
Methionine	1.18	1.15	1.12	1.31	1.12
Isoleucine	1.77	1.84	1.84	1.82	1.80
Leucine	4.42	4.50	4.42	4.41	4.39
Tyrosine	1.27	1.30	1.31	1.29	1.22
Phenylalanine	2.24	2.32	2.25	2.37	2.25
Lysine	3.54	3.43	3.50	3.54	3.50
Histidine	1.11	1.13	1.12	1.07	1.15
Arginine	3.15	3.10	3.14	3.15	3.11
Cysteine	0.52	0.82	1.13	1.45	1.84

indicators included LZM, complement 3, complement 4, and immunoglobulins (IgA, IgG, and IgM).

Midgut histological examination

In accordance with a previous study (39), we stained the fish midgut with hematoxylin and eosin to observe effects of exogenous cysteine on the intestinal physical barrier. Briefly, the midguts were stored in a 4% paraformaldehyde solution for 24 h. We used ethanol to gradually eliminate the moisture. The midguts were then transferred to paraffin. When the paraffin was solidified, it was cut into 5-μm-thick slices using a microtome. Next, the slices were placed on slides and were stained with hematoxylin and eosin for nucleus and cytoplasm staining. Finally, we completely scanned the midgut sections using a light microscope (Leica, Wetzlar, Germany) with 200-fold magnification, divided the scans into eight equal parts, and randomly measured the length of intact intestinal villi, muscle thickness, and the number of goblet cells per villus in each part using Image-Pro Plus 6.0 software (National Institutes of Health, Bethesda, USA). The data were imported into GraphPad Prism 8 software (San Diego, California, USA) to examine differences and to draw graphs.

16S rDNA high-throughput sequencing of the intestinal flora

According to previous studies (39, 40), total DNA of intestinal bacteria was obtained using a DNA extraction kit (TIANGEN BIOTECH Co., Ltd., Beijing, China), DNA integrity was assessed using 1% agarose gel electrophoresis, and concentration was measured using a Nanodrop 2000 device (Thermo Fisher Scientific, Waltham, MA, USA). The DNA samples were diluted to 1 ng·μL^{-1} using sterile water, and PCR amplification of the bacterial 16S rDNA V3–V4 variable region was performed using specific primers. The primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTAC NNGGGTATCTAAT-3′) were used to amplify the V3–V4 variable region. The reaction system and procedure for PCR amplification were used as described previously (39). PCR products were recovered using an AxyPrepDNA Gel Recovery Kit (AXYGEN Inc., California, USA) for gel cutting, Tris HCl elution, and 2% agarose electrophoresis for detection. The PCR products were quantified using a QuantiFluor™-ST Blue Fluorescence Quantification System (Promega Corporation, Madison, Wisconsin, USA), based on the preliminary quantification results of electrophoresis. Paired-end Illumina libraries were then constructed by mixing the corresponding ratios according to the sequencing volume requirement of each sample. Paired-end reads obtained using an Illumina sequencing platform (Illumina, San Diego, CA, USA) were first spliced according to the overlapping relationship, the sequences were quality-controlled and filtered, and the samples were differentiated and then subjected to operational taxonomic unit (OTU) clustering and clustering-based taxonomy analyses. Various diversity indices were produced based on the OTU clustering analysis. Abundance, alpha diversity (Chao1 index, Shannon index, and Simpson index), beta diversity

(principal component analysis [PCA], UniFrac-based principal coordinate analysis [Pcoa], and UniFrac-based non-metric multi-dimensional scaling [NMDS] analysis), and linear discriminatory analysis were performed on OTUs to obtain information on species richness and evenness within the samples. Dilution curves were plotted using R software (V3.6.3, University of Auckland, New Zealand.) (36).

Quantitative real-time PCR

To further examine the modulatory effects of dietary cysteine on the growth, anti-oxidative stress, and intestine immunity of golden pompano, we assayed the expression of several genes. For the antioxidant stress capacity, we measured mRNA levels of antioxidant enzymes (*CAT*, *GSH-PX*, and *SOD*), critical factors of the Nrf2 pathway (*Nrf2* and *Keap-1*), and *HO-1* in the intestine of golden pompano. *EF-1α* was used as the internal reference gene (41). All primer sources are listed in Table 3.

The RNA extraction, cDNA production, and qPCR methods were based on a previous study (34). In brief, total RNA was extracted from the intestine of golden pompano using a HiPure Universal RNA Mini kit (Magen Biotech Co., Ltd., Guangzhou, China) according to the manufacturer's instructions. The integrity of RNA was assessed using 1% agarose gel electrophoresis, and the concentration was assayed using a Nanodrop 2000 (Thermo Fisher Scientific, MA, USA). We used a PrimeScript™ RT kit (Accurate Biotechnology Co., Ltd., Hunan, China) for reverse-transcription. The gDNA Eraser in the kit eliminated adverse effects. The SYBR® Green Premix Pro Taq HS qPCR Kit (Accurate Biotechnology Co., Ltd., Hunan, China) was used to perform qPCR. qPCR parameters were used as described previously (35, 43). To minimize the impact of incidental factors, each gene was repeated four times and three of the results were selected to calculate target gene mRNA levels using the $2^{-\Delta\Delta CT}$ method (45).

Streptococcus agalactiae challenge

To complement the impact of cysteine on golden pompano immunity, we conducted an *S. agalactiae* challenge experiment following the feeding trial. The *S. agalactiae* concentration of 2.0×10^7 CFU/fish was the LD50 of golden pompano challenged by *S. agalactiae* for 120 h, as determined previously by our lab (34). After sample collection, we stochastically selected 20 fish per cage with similar size and healthy condition, injected 200 μL of the bacterial suspension at a concentration of 2.0×10^7 CFU/fish into the peritoneal cavity of each fish using a sterile syringe and returned the fish to their respective cages for continued feeding for 120 h. The remaining number of fish in each group was recorded every 12 h, and dead fish were removed. At the end of the challenge trial, all fish were rendered harmless using the alcohol. The same hydrological conditions as those used in the feeding experiment were maintained throughout the challenge. After the challenge experiment, we imported the data into GraphPad Prism 8 software and used the Kaplan-Meier algorithm to calculate the survival curves. A log-rank test was used to compare variances among groups.

Statistical analyses

Gene expression and serum parameters were analyzed by one-way analysis of variance (ANOVA) using GraphPad Prism 8 and Origin Pro 2021 (OriginLab Corporation, Northampton, MA, USA), respectively. The results of the analysis are presented as mean ± standard deviation (mean ± SD). Tukey's test was

TABLE 3 qPCR primer sequences.

Primers	Forward primer sequences (5′-3′)	Reverse primer sequences (5′-3′)	Source
CAT	GGATGGACAGCCTTCAAGTTCTCG	TGGACCGTTACAACAGTGCAGATG	Liu et al. (41)
SOD	CCTCATCCCCCTGCTTGGTA	CCAGGGAGGGATGAGAGGTG	Liu et al. (41)
GSH-PX	GCTGAGAGGCTGGTGCAAGTG	TTCAAGCGTTACAGCAGGAGGTTC	Liu et al. (41)
HO-1	AGAAGATTCAGACAGCAGCAGAACAG	TCATACAGCGAGCACAGGAGGAG	Xie et al. (42)
Nrf2	TTGCCTGGACACAACTGCTGTTAC	TCTGTGACGGTGGCAGTGGAC	Liu et al. (43)
Keap-1	CAGATAGACAGCGTGGTGAAGGC	GACAGTGAGACAGGTTGAAGAACTCC	Liu et al. (43)
IL-1β	CGGACTCGAACGTGGTCACATTC	AATATGGAAGGCAACCGTGCTCAG	Liu et al. (41)
IL-8	CCGATCAACAGGGACTTCAA	GAGGACCGAGGGTTCAGACAG	Zhang et al. (44)
IL-10	AGTCAGTCTCCACCCCCATCTT	GCCCACTGGAGTTCAGATGCT	Zhang et al. (44)
TNF-α	GCTCCTCACCCACACCATCA	CCAAAGTAGACCTGCCCAGACT	Liu et al. (41)
NF-κB	CGTGAGGTCAGCGAGCCAATG	ATGTGCCGTCTATCTTGTGGAATGG	Liu et al. (41)
IKK	CCTGGAGAACTGCTGTGGAATGAG	ATGGAGGTAGGTCAGAGCCGAAG	Liu et al. (41)
IκB	GCTGGTCCATTGCCTCCTGAAC	GTGCCGTCTTCTCGTACAACTGG	Liu et al. (41)
EF-1α	AAGCCAGGTATGGTTGTCAACTTT	CGTGGTGCATCTCCACAGACT	Ma et al. (35)

used for multiple comparisons when there was a significant difference ($P< 0.05$).

Results

Growth performance

As shown in Table 4, the levels of SR, FI, and CF in C0 fish were not significantly different from those in the other groups ($P > 0.05$). However, growth performance was proportional to cysteine content in the diets. Cysteine markedly increased the levels of FBW, WGR, and SGR in C2, C3, and C4 fish compared to C0 ($P< 0.05$). In addition, dietary cysteine addition of 0.6% was responsible for a significant downregulation of FCR, HSI, and VSI in fish compared with the C0 group ($P< 0.05$). Under these experimental conditions, the optimal cysteine supplementation level in golden pompano diet was 0.91%, according to the polynomial regression results of SGR (Figure 1).

Serum antioxidant capacity and non-specific immune parameters

To explore the resistance of fish supplied with exogenous cysteine to oxidative stress, we determined the activity of several antioxidant enzymes in the serum of golden pompano (Figures 2A–D). The activity of T-AOC in serum was remarkably increased in C1, C3, and C4 fish compared to C0 fish ($P< 0.05$), but there was no remarkable difference between C2 and C0 fish ($P >0.05$, Figure 2A). With higher dietary cysteine levels, CAT activity was significantly higher in all treatment fish than in the C0 fish ($P< 0.05$, Figure 2B). Exogenous supplementation with 0.6-1.2% cysteine remarkably upregulated the activities of GSH-PX compared to the C0 fish ($P< 0.05$, Figure 2D). Even though 0.3%–0.6% dietary cysteine supplementation decreased the activity of SOD, with higher cysteine supplementation of 0.9%–1.2%, the SOD activity of C3 and C4 fish was considerably higher than in the C0 group ($P< 0.05$, Figure 2C). This suggests that the antioxidant effect can only be achieved at a specific dietary cysteine level. This phenomenon was also observed for the levels of MDA and ROS (Figures 2E, F). The MDA and ROS levels in the serum of golden pompano decreased considerably when the diet was supplemented with 0.9%–1.2% cysteine, and they were markedly lower than in the C0 group.

The serum immunological parameters were also affected by exogenous cysteine (Figure 3). Exogenous supplementation with 0.3% cysteine did not increase the immunoglobulin (IgM, IgA, and IgG) content and LZM activity in serum of golden pompano, compared to the C0 group ($P > 0.05$, Figures 3A–D); however, at 0.6%–1.2% cysteine, the immunoglobulin content and LZM activity were markedly higher than those in the C0 group ($P< 0.05$). The levels of complement 3 and complement 4, important parameters of serum immunology increased significantly with increasing cysteine supplementation ($P< 0.05$), reaching a maximum at 0.9%–1.2% cysteine supplementation (Figures 3E, F).

Midgut histological observation

The histological parameters of the golden pompano intestine are shown in Figure 4. With higher cysteine supplementation, intestinal villus length and muscular thickness increased markedly (Figure 4F). Villus length was significantly higher in C2, C3, and C4 fish than in C0 and C1 fish ($P< 0.05$). In addition, the statistical results indicated that exogenous supplementation with 0.3% and 1.2% cysteine substantially increased muscular thickness, compared to C0 fish ($P< 0.05$, Figure 4G).

TABLE 4 Growth performance of *T. ovatus* fed diets with different dose cysteine supplementation after 8 weeks.

Parameters	Group					*P* valve
	C0	C1	C2	C3	C4	
SR (%)	94.67 ± 1.15^{ab}	98.00 ± 2.00^{b}	88.67 ± 3.06^{a}	89.33 ± 5.03^{a}	90.67 ± 2.31^{ab}	0.016
IBW (g)	10.17 ± 0.02	10.13 ± 0.05	10.11 ± 0.05	10.15 ± 0.03	10.18 ± 0.04	0.259
FBW (g)	76.00 ± 10.32^{a}	85.06 ± 9.87^{a}	114.88 ± 14.48^{b}	121.36 ± 10.15^{b}	107.51 ± 10.11^{b}	<0.001
WGR (%)	656.22 ± 34.22^{a}	746.32 ± 36.55^{a}	1043.06 ± 119.62^{b}	1107.58 ± 46.59^{b}	969.76 ± 38.55^{b}	<0.001
SGR (%/day)	3.60 ± 0.07^{a}	3.80 ± 0.07^{a}	4.34 ± 0.18^{b}	4.44 ± 0.07^{b}	4.22 ± 0.07^{b}	<0.001
FI (%/day)	1.35 ± 0.04	1.28 ± 0.04	1.27 ± 0.11	1.27 ± 0.05	1.34 ± 0.05	<0.001
FCR (%)	1.98 ± 0.07^{b}	1.83 ± 0.07^{ab}	1.70 ± 0.17^{a}	1.68 ± 0.07^{a}	1.82 ± 0.08^{ab}	<0.001
HSI (%)	1.04 ± 0.04^{b}	0.95 ± 0.03^{ab}	0.88 ± 0.04^{a}	0.95 ± 0.03^{ab}	0.89 ± 0.04^{a}	0.017
VSI (%)	6.11 ± 0.26^{b}	4.97 ± 0.11^{a}	5.19 ± 0.15^{a}	5.20 ± 0.21^{a}	5.55 ± 0.22^{ab}	<0.001
CF (g/cm ^{3})	2.90 ± 0.14^{ab}	2.67 ± 0.17^{a}	3.15 ± 0.24^{ab}	3.52 ± 0.12^{b}	2.79 ± 0.12^{a}	0.008

values in the same row with different superscripts are significantly different (P< 0.05).

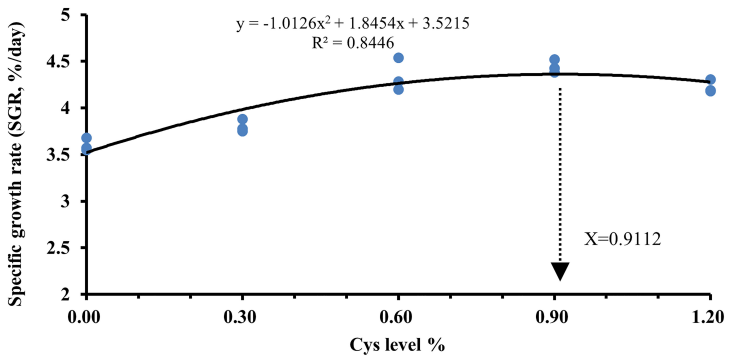

FIGURE 1
Estimation of the optimal dietary cysteine supplementation level for *T. ovatus* by means of polynomial regression analysis using the SGR.

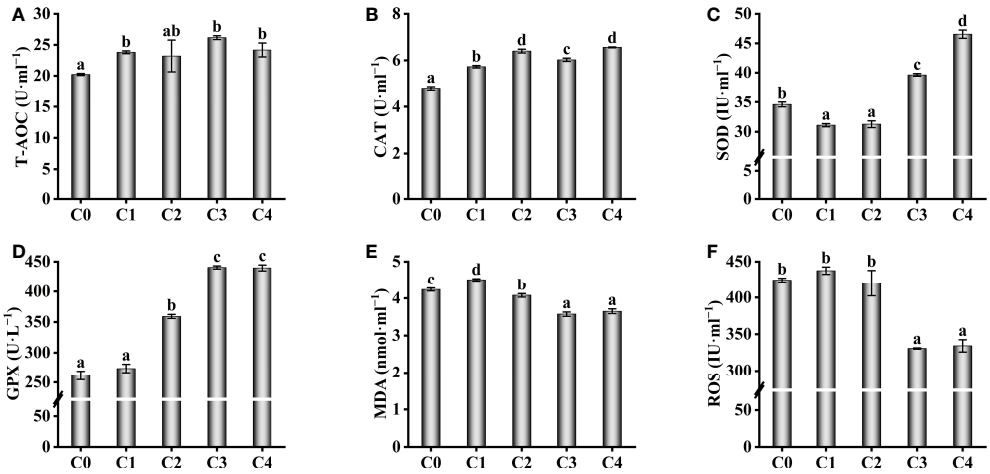

FIGURE 2
Effect of antioxidant capability such as T-AOC **(A)**, CAT **(B)**, SOD **(C)**, GSH-PX **(D)**, MDA **(E)** and ROS **(F)** in the serum of *T. ovatus* fed diets with different dose cysteine supplementation after 8 weeks. Mean values (n = 9) within values in the picture above with different superscripts are significantly different ($P< 0.05$).

Nevertheless, cysteine supplementation decreased the number of goblet cells per intestinal villus (Figure 4H). The abundance of goblet cells was considerably lower in C3 and C4 fish compared to that in the controls ($P< 0.05$).

16S rDNA high-throughput sequencing of the intestinal flora

We obtained 678,797 clean reads and 2,807 OTUs by high-throughput sequencing of the intestinal microbes of the golden pompano. A Venn diagram was produced to visualize that 15 OTUs were identical in all groups, and dietary cysteine increased the amount of specific OTUs in the gut flora (Figure 5A). Rarefaction curves (Figure 5B), OTU rank-abundance curves (Figure 5C), and species accumulation curves (Figure 5D) converged to saturation, allowing assessment of sequencing depth, species evenness, and species richness. The dominant phyla included Proteobacteria, Firmicutes, and Bacteroidetes (Figure 5E). Firmicutes showed a decreasing trend (0%–0.9%) and then increased (0.9%–1.2%) with increasing dietary cysteine content. At the genus level, *Ralstonia*, *Saccharibacterianorank*, and *[Ruminococcus] gnavus* were dominant (Figure 5F). A sample clustering tree (Figure 5G) and abundance similarity clustering (Figure 5H) of intestinal flora among the groups showed that cysteine supplementation enriched the composition of the intestinal flora of golden pompano. With increasing dietary cysteine content, the abundance of the intestinal flora of golden pompano gradually increased.

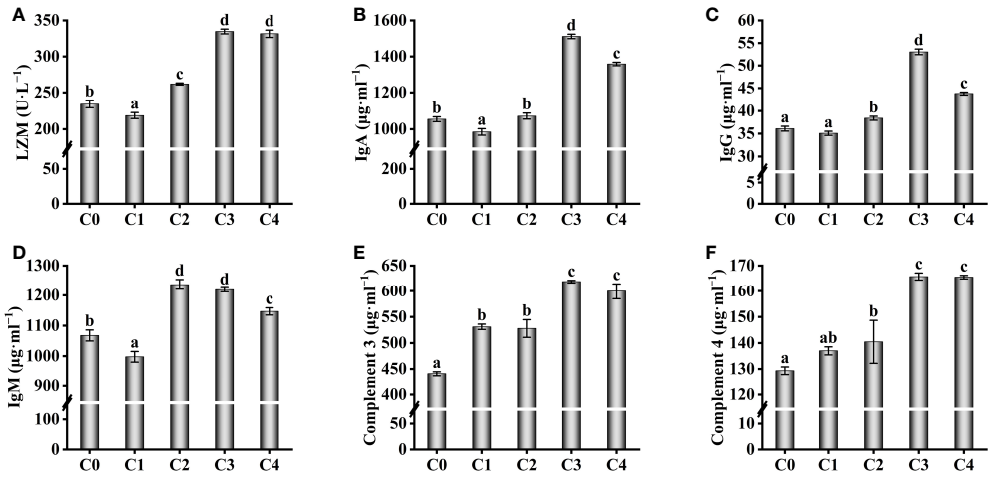

FIGURE 3
Effect of immunological parameters such as LZM **(A)**, IgA **(B)**, IgG **(C)**, IgM **(D)**, C3 **(E)**, C4 **(F)** in the serum of *T. ovatus* fed diets with different dose cysteine supplementation after 8 weeks. Mean values (n = 9) within values in the picture above with different superscripts are significantly different ($P< 0.05$).

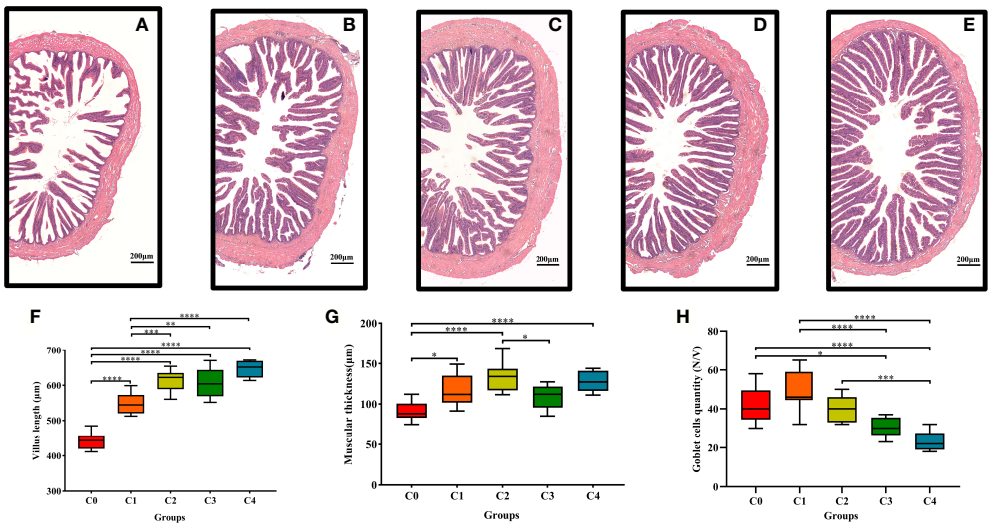

FIGURE 4
Effects of dietary cysteine on mid-gut morphology of *T. ovatus*. **(A)** 0% cysteine; **(B)**: 0.40% cysteine; **(C)**: 0.80% cysteine; **(D)**: 1.20% cysteine; **(E)**: 1.60% cysteine. Scale bar: 200 μm. The villus length **(F)**, muscular thickness **(G)**, and goblet cells quantity **(H)** of mid-gut in *T. ovatus*. data are presented as mean ± SD (n = 9). Asterisks *, **, ***, and **** indicate statistically significant difference between treated group and control group at $P< 0.05$, $P< 0.01$ $P< 0.001$, and $P< 0.0001$, respectively.

To further investigate the effect of cysteine on the abundance and diversity of intestinal microbial populations, we used alpha diversity index analysis, which showed that supplementation with 0.6%–1.2% cysteine significantly increased the Chao1 index (Figure 5I) and reduced the Simpson index (Figure 5K), compared to the controls, except for the C0 and C4 fish in which the Simpson index did not differ significantly. Similarly, the Shannon index showed (Figure 5J) that the exogenous supplementation with 0.9%–1.2% cysteine increased the abundance and diversity of the flora.

We used PCA, UniFrac-based PCOA, and UniFrac-based NMDS for comparative analysis of the similarity between groups and samples (β diversity analysis). Exogenous supplementation with 0.6%–1.2% cysteine caused a similar gut microbial composition across experimental fish, which differed from that of the C0 fish (Figures 5L, N). The UniFrac-based PCOA

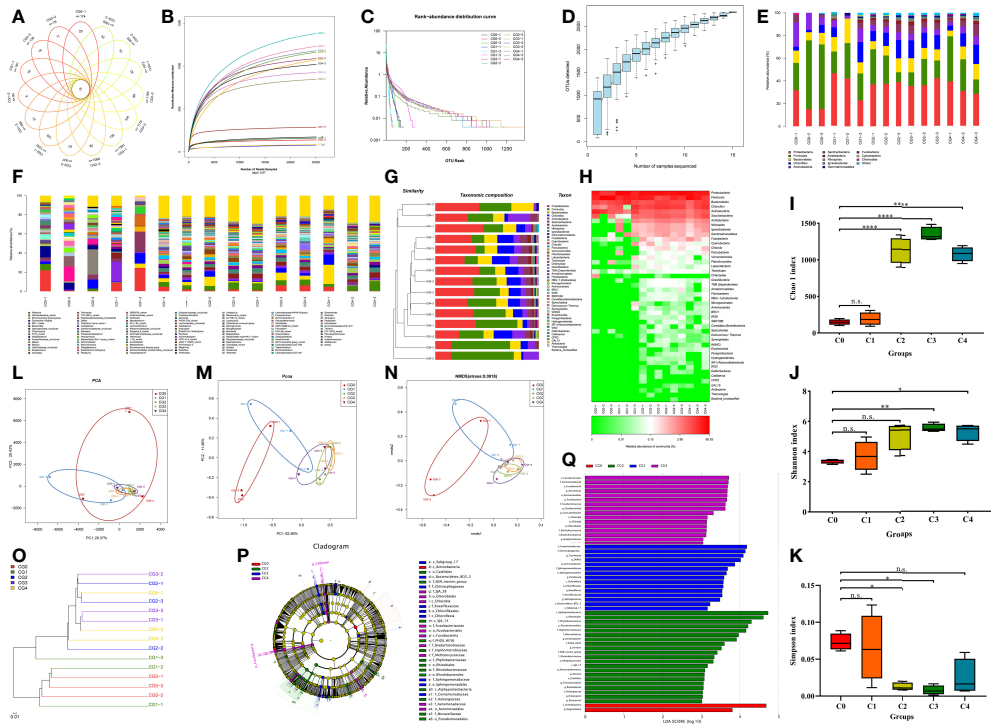

FIGURE 5
Cysteine supplementation regulated gut microbiota composition and abundance. Profile of the intestinal microbiota (n = 3). The basic structure of the gut microbiota was determined by Venn diagram **(A)**, Rarefaction curves **(B)**, Rank-Abundance curves **(C)**, species accumulation curves **(D)**, relative abundance histograms of phylum **(E)** and genus **(F)**, Mcrobial community barplot with cluster tree **(G)**, and heat map comparison **(H)** were depicted. The diversity and richness of the communities were compared by alpha diversity indices, including Chao 1 index **(I)**, Shannon index **(J)**, and Simpson index **(K)**. The community diversity and richness were compared by Principal Component Analysis **(L)**, UniFrac-based principal co-ordinates analysis **(M)**, and UniFrac-based non-metric multi-dimensional scaling analysis **(N)** were performed for β-diversity analysis. Communities or species with significantly different effects on sample delineation were identified by UniFrac-based cluster tree **(O)**, Evolutionary branching plots **(P)**, and Linear discriminant analysis **(Q)**. "n.s."indicate No significant difference. Asterisks *, **, and **** indicate statistically significant difference between treated group and control group at $P < 0.05$, $P < 0.01$, and $P < 0.0001$, respectively.

weighted results showed that differences in the PC1 axis and PC2 axis explained 62.56% and 11.46% of the variation, respectively (Figure 5M). UniFrac-based cluster tree analysis using the unweighted pair group method with arithmetic mean was used to visualize the evolutionary similarities and differences of microorganisms in different samples, and the results showed that the gut flora of C0 and C1 fish were more similar and clustered in one branch. In contrast, C2, C3, and C4 fish were similar and clustered on a different branch (Figure 5O).

To identify communities or species that had a significant differential effect on sample delineation, we used the non-parametric factorial Kruskal-Wallis sum-rank test method to detect characteristics with significant abundance differences and to identify taxa that differed significantly in abundance. Linear discriminant analysis was applied using LEfSe software to estimate the magnitude of the effect of abundance on the different effects for each component (species). Evolutionary branching plots (Figure 5P) and linear discriminant analysis (Figure 5Q) showed that only the class Actinobacteria and the genus *Megasphaera* played a significant role in C0 fish. In contrast, C2, C3, and C4 fish had more diverse gut flora. Six phyla (Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, Chloroflexi, and Ignavibacteriae), nine orders, eleven families, and eight genera were present more frequently in C2 fish. Four phyla (Proteobacteria, Acidobacteria, Chloroflexi, and Bacteroidetes), six orders, four families, and six genera were more abundant in the C3 fish. Four phyla (Fusobacteria, Proteobacteria, Firmicutes, and Chlorobi), five orders, five phyla, six families, and four genera were more abundant in C4 fish.

Antioxidant enzyme expression in the intestine

We examined the expression of antioxidant enzyme genes in the intestine of golden pompano to examine the effects of exogenous cysteine on antioxidant stress capacity (Figure 6A). Compared to the control group, *CAT* and *GSH-PX* expression

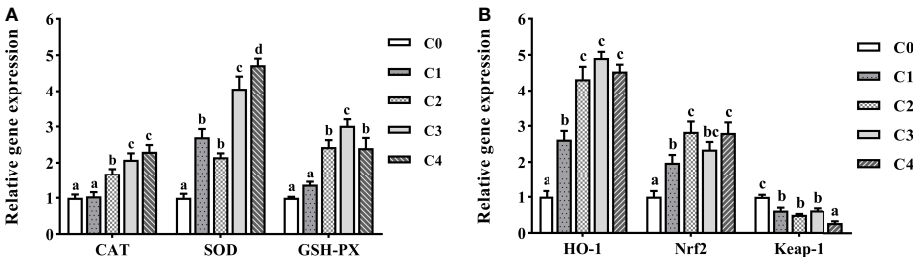

FIGURE 6
The expression profiles of antioxidant genes **(A)** and signaling pathway **(B)** in the intestine of *T. ovatus* fed diets with different dose cysteine supplementation after 8 weeks. Mean values (n = 9) within values in the picture above with different superscripts are significantly different (*P*< 0.05).

were substantially upregulated in C2, C3, and C4 fish (*P*< 0.05), and the *GSH-PX* expression was highest in the C3 group. The CAT expression levels between the C3 and C4 groups were not markedly different (*P* > 0.05), with both significantly higher than the other groups (*P*< 0.05). The effect of exogenous cysteine on *SOD* expression was considered significant. The expression of these three genes was upregulated owing to exogenous cysteine supplementation, compared to that in the control group, and it was considerably higher than that in the C0 group (*P*< 0.05). In addition, dietary cysteine reduced intestinal *Keap-1* mRNA levels and led to upregulation of *HO-1* and *Nrf2* (Figure 6B).

Intestine immunity-related gene expression analysis

To gain further information on the effect of exogenous cysteine on intestine immunity of golden pompano, we selected the essential genes of inflammatory response-related cytokines and the NF-κB pathway for qPCR (Figure 7). Exogenous supplementation with cysteine suppressed the expression of *TNF-α*, *IL-1β*, *IL-8* (Figure 7A), *NF-κB*, and *IKK* (Figure 7B) in the intestine of golden pompano. Dietary cysteine supplementation of 0.6%–1.2% led to lower expression levels of *TNF-α*, *IL-1β*, *NF-κB*, *IKK*, and *IL-8*, compared to C0 fish (*P*< 0.05). Meanwhile, expression of *IκB* (Figure 7B) and *IL-10* (Figure 7A) was significantly upregulated by exogenous cysteine, compared with that in the controls (*P*< 0.05). This demonstrated that cysteine could promote intestine immunity in golden pompano.

Streptococcus agalactiae challenge

The survival rates of juvenile golden pompano after *S. agalactiae* challenge is presented in Figure 8. The survival rates of C0 (0), C1 (0.3%), C2 (0.6%), C3 (0.9%), and C4 (1.2%) fish after the challenge for 120 h were 41.67%, 50.00%, 63.33%, 73.33%, and 63.33%, respectively. The survival of juvenile golden pompano gradually increased with an increase in dietary cysteine supplementation from 0% to 0.9%. Exogenous supplementation with 0.6%–1.2% cysteine markedly improved the resistance of golden pompano to *S. agalactiae*, compared to the C0 group (*P*< 0.05). Thus, appropriate cysteine supplementation can greatly promote immunity in golden pompano.

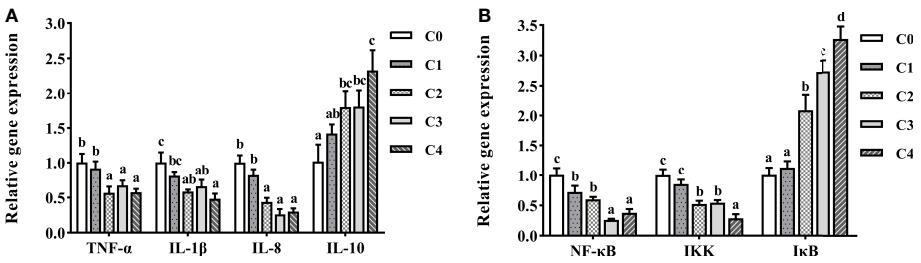

FIGURE 7
The expression profiles of inflammatory genes **(A)** and signaling pathway **(B)** in the intestine of *T. ovatus* fed diets with different dose cysteine supplementation after 8 weeks. Mean values (n = 9) within values in the picture above with different superscripts are significantly different (*P*< 0.05).

Discussion

Supplementation of low-fishmeal diets with cysteine promotes growth of juvenile golden pompano

In the present study, supplementation with 0.6%–1.2% cysteine remarkably increased the FBW, WGR, and SGR levels in golden pompano. Moreover, adding 0.6% cysteine to the diet decreased the FCR, HSI, and VSI significantly, compared to the C0 group. These indices indicated that dietary supplementation with cysteine greatly enhanced the growth performance of golden pompano. Similar findings were made in a previous study on *Psetta maxima*, which showed that a moderate amount of dietary cysteine (0.3%) enhanced the SGR levels and the mean final weight of *P. maxima* and improved its growth performance (20). Oxidative stress and immune capacity reduction caused by low fishmeal content are the main reasons for reduced growth performance (8, 9, 46). In the present study, there were many reasons that cysteine supplementation improved the growth performance of golden pompano in low fishmeal conditions, such as the increased abundance and diversity of gut microbes (31), increased antioxidant capacity (47, 48), and improved immune ability (27). Our results showed that dietary supplementation with 0.6%–1.2% cysteine could increase the activity of serum antioxidant enzymes (T-AOC, CAT, and GSH-PX) and decrease the serum MDA and ROS levels in juvenile golden pompano. Meanwhile, non-specific serum immune parameters were improved, and the levels of immunoglobulins (IgM, IgA, and IgG), complement 3, complement 4, and LZM activity were increased, which may help the fish cope with oxidative stress and immunity reduction caused by low-fishmeal diets, thus improving growth performance. Our findings are similar to those found in *Seriola lalandi* (47) and *Scophthalmus maximus* L (48), where cysteine enhanced growth performance by improving resistance to oxidative stress and intestine immunity.

Supplementation of low-fishmeal diets with cysteine improves the intestinal physical barrier in juvenile golden pompano

The intestinal physical barrier is a critical component of intestine immunity with the function of taking in nutrients and defending against harmful substances (49, 50). Low-fishmeal diets damage the intestinal physical barrier; for example, replacing fishmeal with soybean meal reduces the villi length of *Epinephelus coioides* (12), *Litopenaeus vannamei* (49), and *Penaeus monodon* (50) and damages the intestinal mucosal folds, leading to intestinal inflammation. Cysteine effectively protects the physical barrier of the intestine. For instance, cysteine protects the intestine of broiler chicks by increasing the length of the intestinal villi (51). In addition, cysteine has been found to improve intestinal integrity and permeability and reduce the intestinal inflammatory response in pigs (25, 27). Similarly, our results showed that exogenous cysteine supplementation at 0.6% and 1.2% greatly improved intestinal villus length and muscular thickness, thus effectively alleviating the weakening of the intestinal physical barrier associated with low-fishmeal diets. Alterations in the intestinal physical barrier typically accompany changes in the abundance of intestinal flora (52, 53) and expression of intestinal immune-related genes (54).

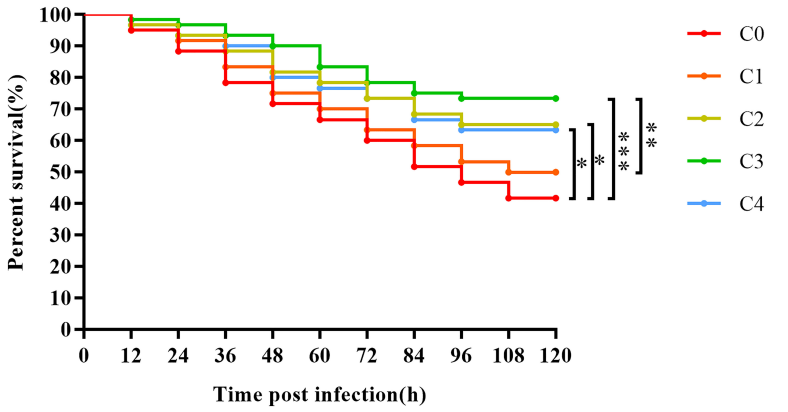

FIGURE 8
The Kaplan-Meier survival analysis of *T. ovatus* after *S. agalactiae* infection. Asterisks *, **, and *** indicate statistically significant difference between the two groups at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

Therefore, we propose that the protective effect of cysteine on the intestinal physical barrier of golden pompano may be correlated with increased abundance of intestinal flora and high expression of intestinal antioxidant stress- and immune-related genes.

Supplementation of low-fishmeal diets with cysteine improves the diversity of intestinal flora in juvenile golden pompano

Maintaining the diversity and abundance of intestinal flora is essential for the intestinal health of fish, and disturbances to the intestinal flora tend to damage the physical barrier of the intestine and elicit inflammatory responses (53, 55). In our study, alpha and beta diversity analysis of the intestinal flora showed that supplementation of the diet with cysteine regulated the abundance and diversity and improved the structure of intestinal flora in golden pompano. Alpha diversity results demonstrated that exogenous supplementation with 0.6%–1.2% cysteine remarkably increased the Chao 1 index and decreased the Simpson index, thus the diversity of the intestinal flora was markedly improved, and similar results were found regarding the Shannon index (C2 and C3 fish). At the phylum level, the dominant taxa included Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria, Chloroflexi, and Actinobacteria. Appropriate addition of cysteine to the diet increased the relative abundance of Proteobacteria and Bacteroidetes and decreased the relative abundance of Firmicutes. Bacteroidetes are essential players in polysaccharide (56–58) and cholesterol metabolism (59), and it is possible that altering the abundance of Bacteroidetes is one of the ways cysteine regulates nutrient metabolism. At the genus level, *Ralstonia*, *Saccharibacterianorank*, and *[Ruminococcus] gnavus group* were predominant in the intestinal tract of golden pompano. Adequate cysteine addition can thus increase the relative abundance of the *[Ruminococcus] gnavus* group. Similarly, previous studies have shown that dietary cysteine has a positive effect on intestinal flora richness and diversity in gestating sows (31) and *S. maximus* L (48).

Supplementation of low-fishmeal diets with cysteine enhances the expression of intestinal antioxidant and immune-related genes in juvenile golden pompano

The protective effect of cysteine on intestinal integrity may be related to signaling pathways associated with antioxidant and inflammatory responses. Numerous studies confirmed that excessive substitution of fishmeal in diets elicits oxidative stress in the fish intestine owing to excessive anti-nutrients, increasing ROS levels, and leading to structural damage in the intestine (8, 9). For instance, the addition of plant proteins to diets causes oxidative stress and structural damage in the intestine of *S. maximus* L (9)., *Monopterus albus* (11), and *Amurenser schrenckii* (60). ROS levels are predominantly regulated by the Nrf2/Keap-1 signaling pathway (43, 61). Nrf2 can reduce ROS levels and increase the expression of antioxidant enzyme genes in golden pompano by enabling the high expression of *HO-1* (42). Our results indicate that cysteine supplementation activated the Nrf2/Keap-1 signaling pathway in the intestine of golden pompano. Downregulation of *Keap-1* mRNA levels led to the upregulation of *HO-1* and *Nrf2* mRNA levels, increasing the mRNA levels of intestinal antioxidant enzymes (*CAT*, *SOD*, and *GSH-PX*) and the corresponding enzyme activities in serum.

Oxidative stress not only damages tissue integrity but may also elicit inflammatory responses and reduce the immune capacity of the body (41). Inflammatory responses are regulated by a combination of anti-inflammatory cytokines (IL-10, TGF-β, etc.) (30, 62) and pro-inflammatory cytokines (IL-1β, IL-8, TNF-α, etc.) (30, 48, 63), which in turn are typically modulated through the NF-κB/IκB/IKK signaling pathway (64, 65). A study showed that cysteine inhibited *NF-κB* expression and downregulated pro-inflammatory cytokines (*IL-6*, *TNF-α*, and *IL-8*) in the intestine of piglets, which improved the physical barrier of the intestine and promoted intestinal health (25). Similarly, we found that *NF-κB*, *IKK* (Figure 7B), *IL-1β*, *IL-8*, and *TNF-α* (Figure 7A) in the intestine of golden pompano were negatively correlated with dietary cysteine content, whereas the expression levels of *IκB* and *IL-10* showed contrasting patterns. These results suggest that dietary supplementation with 0.6%–1.2% cysteine (C2, C3, and C4 fish) decreases the expression level of pro-inflammatory cytokines by inhibiting the NF-κB signaling pathway. Thus, the inflammatory response was suppressed, and the intestine immunity of golden pompano was improved.

Supplementation of low-fishmeal diets with cysteine enhances the resilience of juvenile golden pompano to *Streptococcus agalactiae*

Infectious diseases caused by *S. agalactiae* have frequently erupted in recent years in large-scale aquacultures of marine fish because of high culture densities (34). Therefore, we also examined the protective effect of cysteine during an *S. agalactiae* challenge in golden pompano. Survival curves showed that cysteine had a positive effect on the survival rate of golden pompano. The survival rate was remarkably higher in C2, C3, and C4 (0.6%–1.2% cysteine) than in C0 fish. Bacterial challenge can reduce the diversity of intestinal flora in fish (66, 67), whereas our results showed that cysteine substantially increased the diversity of the intestinal flora. Therefore, considering the high agreement between

the survival rate of the present challenge test and intestinal immune parameters, such as histological results of the intestine, results of microbial diversity analysis, and intestinal immune gene expression, we infer that cysteine may have enhanced the resistance of golden pompano to *S. agalactiae* by improving its intestine immunity.

Conclusions

Overall, a moderate level of cysteine (0.6%–1.2%) improved the growth performance of golden pompano, and the optimum dietary cysteine supplementation level for juvenile golden pompano was 0.91%, based on polynomial regression analysis of SGR. Moderate dietary cysteine also improved the abundance and diversity of intestinal flora and enhanced the structural integrity of the intestine to maintain the stability of the intestinal physical barrier. Furthermore, dietary cysteine activated the Nrf2/Keap1/HO-1 signaling pathway and inhibited NF-κB signaling, increased intestinal antioxidant enzyme genes (*CAT*, *SOD*, and *GSH-PX*) and anti-inflammatory cytokine mRNA levels, increased serum antioxidant enzyme activity, and substantially improved intestine immunity, resulting in higher survival rates of golden pompano exposed to *S. agalactiae*. These findings provide new insights into the development and use of cysteine as a dietary supplement.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number: PRJNA889580. The data can be found below: https://www.ncbi.nlm.nih.gov/search/all/?term=PRJNA889580.

Ethics statement

The animal study was reviewed and approved by Animal Care and Use Committee of the South China Sea Fisheries Research Institute of the Chinese Academy of Fishery Sciences.

Author contributions

D-CZ designed the experiments and wrote the manuscript. J-XL conducted the experiments and wrote the manuscript. K-CZ: Methodology, Software. H-YG: Data curation, Methodology. B-SL: Supervision, Software. NZ: Visualization, Investigation. All authors contributed to the article and approved the submitted version.

Funding

This research was financially supported by Hainan Yazhou Bay Seed Lab (B21HJ0702), China Agriculture Research System of MOF and MARA (CARS-47), Central Public-Interest Scientific Institution Basal Research Fund of South China Sea Fisheries Research Institute CAFS (2021SD12), Central Public-interest Scientific Institution Basal Research Fund, CAFS (NO.2020TD29), Key Projects of Joint Fund for Regional Innovation and Development of NSFC (U20A2064), National Marine Genetic Resource Center, Guangdong Provincial Special Fund for Modern Agriculture Industry Technology Innovation Teams (2019KJ143).

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 17 November 2022
DOI 10.3389/fimmu.2022.1074399

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Comparative Immunology, a section of the journal Frontiers in Immunology

RECEIVED 19 October 2022
ACCEPTED 31 October 2022
PUBLISHED 17 November 2022

CITATION
Zheng X, Liu B, Wang N, Yang J, Zhou Q, Sun C and Zhao Y (2022) Low fish meal diet supplemented with probiotics ameliorates intestinal barrier and immunological function of *Macrobrachium rosenbergii via* the targeted modulation of gut microbes and derived secondary metabolites. *Front. Immunol.* 13:1074399. doi: 10.3389/fimmu.2022.1074399

Low fish meal diet supplemented with probiotics ameliorates intestinal barrier and immunological function of *Macrobrachium rosenbergii via* the targeted modulation of gut microbes and derived secondary metabolites

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The unsuitable substitution ratio of fish meal by plant protein will reshape the intestinal microbial composition and intestine immunity. However, previous studies were mostly limited to investigating how different feed or probiotics characterized the microbial composition but ignored the biological interactions between bacteria and host physiology through secondary metabolites. Therefore, this study integrates the apparent indicators monitoring, 16S rDNA sequencing, and metabonomics to systematically investigate the effects of cottonseed protein concentrate (CPC) substitution of fish meal and *Bacillus coagulans* intervention on gut microbes, secondary metabolites, and intestinal immunity of *Macrobrachium rosenbergii*. Prawns were fed with three diets for 70 days: HF diets contained 25% fish meal, CPC in LF diets were replaced with 10% fish meal, and LF diets supplemented with 2×10^8 CFU/g diet *B. coagulans* were designated as BC diets. Results showed that CPC substitution induced a significant decrease in digestive enzyme activities (trypsin and lipase) and gut barrier protein *PT-1* expression and a significant increase in γ-GT enzyme activity and inflammatory-related factors (*Relish* and *Toll*) expression. *B. coagulans* treatment mitigated the negative changes of the above indicators. Meanwhile, it significantly improved the expression levels of the barrier factor *PT-1*, the reparative cytokine *IL-22*, and *Cu/Zn-SOD*. CPC substitution resulted in a remarkable downregulated abundance of Firmicutes phyla, *Flavobacterium* spp., and *Bacillus* spp. *B. coagulans* treatment induced the callback of Firmicutes abundance and improved the relative abundance of *Sphingomonas*, *Bacillus*, and *Ralstonia*. Functional prediction indicated that CPC substitution resulted in elevated potential pathogenicity of microbial flora, and *B. coagulans* reduces the pathogenesis risk. Pearson's

correlation analysis established a significant positive correlation between differential genera (*Sphingomonas*, *Bacillus*, and *Ralstonia*) and secondary metabolites (including sphingosine, dehydrophytosphingosine, amino acid metabolites, etc.). Meanwhile, the latter were significantly associated with intestinal immunoregulation-related genes (*Cu/Zn-SOD*, *IL-22*, *PT-1*, *Toll*, and *Relish*). This study indicated that *B. coagulans* could mediate specific gut microbes and the combined action of multiple functional secondary metabolites to affect intestinal barrier function, digestion, and inflammation. Our study revealed the decisive role of gut microbes and derived secondary metabolites in the model of dietary composition-induced intestinal injury and probiotic treatment from a new perspective.

KEYWORDS

***Macrobrachium rosenbergii*, cottonseed protein concentrate, bacillus coagulans, fish meal, intestinal microbial, secondary metabolites**

Introduction

As a high-quality protein source, fish meal is widely used in aquafeeds, especially crustacean feeds, thanks to its good palatability and balanced nutritional composition (1). Due to the rapid development of aquaculture and the depletion of wild capture fisheries resources, the feed cost is gradually increasing, and the sustainable development of aquaculture is being seriously challenged (2). Therefore, one of the solutions is using more widely available and inexpensive plant proteins to partially or entirely replace fish meal in aquafeeds. In recent years, aquatic nutritionists have been conducting a series of studies on substituting fish meal with plant proteins (3).

However, with the in-depth study, plant protein substitution problems have gradually come to the fore. The excessive substitution of plant protein for fish meal leads to dysbacteriosis, impaired intestinal barrier function, reduced digestion and absorption of nutrients, and intestinal inflammation in aquatic animals. These side effects are attributed to the inherent anti-nutritional factors and unbalanced nutritional composition, which have received immense attention across aquaculture nutrition. For example, Miao et al. found that different dietary soybean meal substitutions for defatted fish meal resulted in a decrease in the relative abundance of beneficial intestinal bacteria and an increase in the abundance of conditionally pathogenic bacteria (4).

Persistent elevation of the substitutions proportion led to a significant upregulation of intestinal mucosal inflammatory factor expression (4). Zhang et al. reported that a 20% soybean meal substitution (basal fish meal level of 50%) induced intestinal mucosal barrier injury, downregulated immunization parameters, and enteritis of juvenile grouper (5). With the upgrading of the processing industry, the palatability and nutritional value of cottonseed protein concentrate obtained by low-temperature extraction, dephenolization, and moderate desugarization have significantly improved. Recent reports indicated that cottonseed protein concentrate could replace fish meal and soybean protein with a higher level, which is currently a preferable alternative (6). However, high levels of cottonseed protein concentrate (CPC) substitution will still cause oxidative damage and intestinal barrier damage in aquatic animals. Fu et al. (7) reported that substituting low-gossypol cottonseed meal for more than 20% fish meal gradually aggravated the intestinal barrier function damage of juvenile golden pompano (*Trachinotus ovatus*). The phenotypes appeared as a suppressed expression of tight junction and antioxidant-related genes and an intensified inflammation response (7).

Recently, probiotic bacteria alleviating the adverse effects of aquatic animals induced by plant protein substitution is arousing great interest. Studies on zebrafish (8) and bullfrog (*Lithobates catesbeianus*) (9) have shown that probiotics can improve the growth inhibition, metabolic disorder, intestinal microdysbiosis, and intestinal inflammation of aquatic animals caused by fish meal replacement. As such, increasingly comprehensive probiotic solutions for fish meal substitution to improve the host's gut health and stress resistance are being proposed in the aquaculture industry. The probiotic *Bacillus coagulans* is a Gram-positive lactic acid-producing, spore-forming bacterial species. Recent studies have shown that *B. coagulans* GBI-30, 6086 could enhance plant protein digestion, absorption, and amino acid bioavailability (10, 11). Moreover, *B. coagulans* could promote the intestinal health of aquatic animals by strengthening the gut barriers, relieving oxidative stress, and modulating the intestinal microflora (12). Given this, this study chose *B. coagulans* as a regulator for mitigating the side effects of plant protein replacement fish meal.

Notably, recent medical studies have provided us with a new perspective: the regulation essence of host metabolic diseases

and inflammatory diseases by probiotics, prebiotics, medicine, and other regulators is *via* the modulation of secondary metabolites, such as secondary bile acids (13), short-chain fatty acids (14), and branched-chain amino acids (15). These microbiota-derived metabolites signal to host immune and metabolism organs by ligand–receptor interaction, enabling the implementation of microbe–host communication (16). However, previous studies were mostly limited to investigating how different probiotics or feed types characterized intestinal microbial composition. Alternatively, it only evaluated the variations of growth performance and host physiological index but ignored the biological interactions between intestinal bacteria and host, i.e., the functions of bacterial exo-metabolites.

Therefore, our study evaluated the effects of *B. coagulans* on intestinal microbial composition and functional secondary metabolites related to the gut health of *Macrobrachium rosenbergii* based on dietary fish meal substitution treatment. Above all, we provided new complementary insights combining 16S flora sequencing profiling with metabolomics datasets of intestinal contents, aimed to reveal their interactions with host gut immune barrier function and interpret the mechanism of probiotics in alleviating the adverse effects of plant protein substitution.

Materials and methods

Ethics statement and prawn management

The use of prawns in this study was approved by the Animal Care and Use Committee of the Committee on the Ethics of Animal Experiments of Freshwater Fisheries Research Center. *M. rosenbergii* was obtained from the Freshwater Fisheries Research Center breeding base at the Chinese Academy of Fishery Science. After 15 days of domestication, 600 healthy prawns with a uniform initial weight (0.28 ± 0.02 g) were randomly assigned to three treatment groups with five replicates in each treatment and 40 individuals per replicate bucket (150-cm inner diameter, 45-cm water depth). The three experimental diets were fed at a rate of 5%–10% of body weight at 9:00, 13:00, and 18:00 daily. The feeding amount was observed and timely adjusted based on the amount of residual bait after 30 min, and the residual bait and feces were sucked out by siphoning prior to feeding. Prawns' mortality was recorded daily. Daily continuous aeration was performed to ensure adequate dissolved oxygen (≥6 mg/ml). Water quality was measured once a week, and the water temperature was 28°C–32°C, nitrite ≤ 0.02 mg/L, ammonia ≤ 0.2 mg/L, pH = 7.0–8.5, and the trial period was 70 days.

Experimental design, diet preparation, and growth assessment

The group containing 25% fish meal as the positive control group was designated as the HF group. The negative control group replaced 10% fish meal with CPC and was designated the LF group. Subsequently, *B. coagulans* JSSW-LA-07-1 was added to the LF diet at 2×10^8 CFU/g diet under sterilized conditions and was designated as the BC group. The additional amount of *B. coagulans* JSSW-LA-07-1 was referred to in previous reports (17, 18). The strain was kept by the China General Microbiological Culture Collection Center (CGMCC No. 10182). The ingredients and proximal composition of the control groups are shown in Table 1. All raw materials were finely ground and passed through a 60-mesh sieve. Premix additives and raw materials were weighed accurately according to the formula, mixed step by step, and then squeezed into strips by a twin-screw extruder with a 1-mm aperture.

After a 70-day feeding trial, the survival rate and the weight of prawns in each group were recorded. The weight gain rate, specific growth rate, and feed conversion ratio were calculated in the following formulas:

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

$$\text{Specific growth rate(SGR, \%/day)} = (\text{Ln final weight} - \text{Ln initial weight})/\text{days} \times 100$$

$$\text{Feed conversion ratio(FCR)} = \text{Dry feed intake(g)}/\text{weight gain(g)}$$

Sample collection, RNA extraction, and DNA extraction

Hemolymph was collected using sterile 1-ml syringes from the pericardial cavity, mixing 1:1 with precooling anticoagulant solution, and immediately centrifuged at 8,000 r/min, 4°C for 10 min. The supernatant was collected and stored at −20°C before measuring antioxidant indicators. Part of the intestine was dissected aseptically and stored at −20°C for subsequent biochemical analysis. The remaining intestine was dissected aseptically, quick-frozen in liquid nitrogen, and stored at −80°C for RNA isolation and PCR analysis. The total RNA of intestine tissues was isolated using TRIzol (TaKaRa Biomedical Technology Co., Ltd., Beijing, China). RNA integrity was tested using 1% agarose gel electrophoresis. RNA concentration and purity were determined based on OD 260/280 readings (ratio > 1.8) using a NanoDrop ND-1000 UV Spectrophotometer (NanoDrop Technologies Co., Ltd., Wilmington, DE, USA). In addition, the adequate intestinal contents were collected aseptically and stored in a −80°C freezer for subsequent 16S rDNA sequencing and metabolomics analysis. Microbial DNA was extracted from the samples following the manufacturer's protocol using the E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA). The integrity of the extracted DNA was measured using a NanoDrop ND2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and

10.3389/fimmu.2022.1074399

TABLE 1 Ingredients and proximate analysis of HF diets and LF diets.

Ingredients	HF	LF
	Concentration (% dry matter)	
Fish meal	25	15
Cottonseed protein concentrate		10
Chellocken meal	3	3
Poultry by-product powder	2	2
Squid paste	3	3
Shrimp meal	4	4
Soybean meal	19	19
Rapeseed meal	15.5	15.5
α-Starch	20.5	19.9
Fish oil:soybean oil	2	2.6
Lecithin powder	1	1
Cholesterol	0.3	0.3
Ecdysterone	0.01	0.01
Choline chloride	1	1
Premix[a]	1	1
Bentonite	0.69	0.69
Monocalcium phosphate	2	2
Proximate analysis		
Moisture	10.46	10.61
Crude protein	40.14	40.3
Ether extracts	5.98	5.42
Ash	11.19	12.1

[a]Premix supplied the following vitamins (IU or mg/kg) and minerals (g/kg): vitamin A, 25,000 IU; vitamin D3, 20,000 IU; vitamin E, 200 mg; vitamin K3, 20 mg; thiamin, 40 mg; riboflavin, 50 mg; calcium pantothenate, 100 mg; pyridoxine HCl, 40 mg; cyanocobalamin, 0.2 mg; biotin, 6 mg; folic acid, 20 mg; niacin, 200 mg; inositol, 1,000 mg; vitamin C, 2,000 mg; choline, 2,000 mg; calcium biphosphate, 20 g; sodium chloride, 2.6 g; potassium chloride, 5 g; magnesium sulfate, 2 g; ferrous sulfate, 0.9 g; zinc sulfate, 0.06 g; cupric sulfate, 0.02 g; manganese sulfate, 0.03 g; sodium selenate, 0.02 g; cobalt chloride, 0.05 g; potassium iodide, 0.004 g.

1% agarose gel electrophoresis. Concentrations of isolated DNA were measured using a Quant-iT PicoGreen dsDNA Assay kit (Invitrogen, Carlsbad, CA, USA) and a fluorometer and diluted to 20 ng/μl. Samples were stored at −20°C until sequencing.

Detection of biochemical indicators in hemolymph and intestine

The content of malondialdehyde (MDA) (A003-1-2) and the antioxidant enzymatic activities of catalase (CAT) (A00-1-1) and total superoxide dismutase (T-SOD) (A001-3-2) in hemolymph were detected and quantitated by using the commercial kits purchased from Nanjing Jiancheng Institute of Bioengineering (Nanjing, China), according to the manufacturer's instructions. Approximately 0.1 g of intestinal tissue was minced and homogenized in ice-cold 0.86% stroke-physiological saline solution (w/v, 1:9) using an Ultra-Turrax homogenizer (Tekmar Co., Cincinnati, OH, USA) and centrifuged at 4,000 r/min at 4°C for 10 min to obtain the supernatant for further analysis. The digestive enzymes and brush border enzyme indicators including trypsin, lipase, amylase, and γ-GT were determined using commercial assay kits (A080-2-2, A054-2-1, C016-1-1, and C017-2-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocols.

Quantitative real-time PCR

The extracted total RNA from the intestine was synthesized using a Perfect Real Time SYBR Prime Script TM RT Reagent Kit (TaKaRa Biotechnology, Dalian, China) as per the manufacturer's instructions. The reaction parameters of RTqPCR are as follows: pre-run at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 15 s, and a 60°C annealing step for 34 s. The reaction mixture comprised 2 μl of cDNA, 0.4 μl of forward primer, 0.4 μl of reverse primer, 10 μl of SYBR Premix ExTaqTM (TaKaRa, Dalian, Liaoning, China), 0.4 μl of ROX Reference Dye (TaKaRa, Dalian, Liaoning, China), and 6.8 μl of double-distilled water. *β-Actin* gene was selected as a reference gene (19), and each sample was tested in triplicate. Primers were designed online (NCBI, Bethesda, MA, USA) (Table 2). Notably, the CDS sequences used in the primer

design of some genes were obtained from our laboratory's database of the hepatopancreas transcriptome sequencing of *M. rosenbergii*. The results were calculated by the $2^{-\Delta\Delta Ct}$ method, and the gene-specific primers were synthesized by Shanghai Generay Biotech Co., Ltd. (Shanghai, China).

16S rRNA sequencing of intestinal microorganisms

The target fragment library was prepared by PCR amplification of the V4–V5 region of the 16S rRNA gene. The universal primers 515 F (5′-GTGCCAGCMGCCGCGG-3′) and 907 R (5′-CCGTCAATTCMTTTRAGTTT-3′) were used to amplify the V4–V5 region of the 16S rRNA gene. PCR products were detected by 2% agarose gel electrophoresis, and target fragments were collected using the AxyPrep DNA Gel Extraction Kit. Amplicons of every sample were combined, purified, and quantified. Library construction and double-ended sequencing were conducted using the Illumina HiSeq platform to produce 2 × 250-bp paired-end reads. Raw fastq files were de-multiplexed and quality-filtered by removing the reads' barcode and linker sequences and merging with the paired-end reads into a longer fragment. Then, reads that were truncated at any site from the beginning of the window to the 3′ terminus and received an average quality score<20 over a 6-bp sliding window, and truncated reads<100 bp were discarded. Mismatching primer sequences or ambiguous bases (Ns) exceeding 5% were removed from the downstream analyses, and reads that could not be assembled were discarded. FLASH (fast length adjustment of short reads, v1.2.8, http://ccb.jhu.edu/software/FLASH) was used for spliced paired-end sequences, and chimeric sequences were removed using VSEARCH (https://github.com/torognes/vsearch, v2.3.4). Finally, the operational taxonomic units (OTUs) were generated after denoising. Quality-filtered reads were clustered into OTUs following the criterion of more than or equal to 97% similarity. For detailed steps and parameters of 16S rRNA, refer to our previous description (20).

Metabolomics analysis of intestinal contents

Intestinal contents samples were prepared from the prawns of the LF and BC groups. Each sample measuring 150 mg was accurately weighed and placed into the EP tube, added with 800 µl of pre-cooled 50% methanol–water (1:1, V:V), and grinded thoroughly in a tissue grinder (50 Hz, 5 min). After three centrifugations at 4°C, 2,500 *g*, 550 µl of supernatant was extracted and dried in the freeze dryer. Then, 600 µl methanol–water (1:9, V:V) was added to re-dissolve and vortex the residue and perform ultrasonic treatment, and finally, the supernatant was obtained. Meanwhile, 50 µl of supernatant from each sample was mixed to be the quality control (QC) sample, and all the specimens were stored at −80°C until liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis. The ACQUITY UPLC BEH C18 column (100 mm * 2.1 mm, 1.7 µm, Waters, Cheshire, UK, column temperature 50°C) was used for chromatographic separation, and the injection volume was 10 µl. The high-resolution tandem mass spectrometry Xevo G2-XS QTOF (Waters, UK) was used to collect the small molecules eluted from the column, under the positive (ES+) and negative ion modes (ES−). The raw data were imported into commercial software Progenesis QI (version 3.0.3) for peak alignment, peak extraction, and peak identification and data correction, and the mass charge ratio, retention time, and ion area of metabolites were obtained. Then, the metabolomics R software package-Metax was used to remove low-quality ions. The univariate and multivariate analyses were used to identify the differential metabolites with different abundances under different treatment

TABLE 2 Primer sequences for real-time PCR.

Gene	Primers	Sequence 5′–3′	Product length (bp)	Reference
Toll	F	TTCGTGACTTGTCGGCTCTC	145	KX610955.1
	R	GCAGTTGTTGAAGGCATCGG		
Relish	F	GATGAGCCTTCAGTGCCAGA	149	KR827675.1
	R	CCAGGTGACGCCATGTATCA		
IL-22	F	ACGAGCTGCGATCCAGTAAG	101	Cluster-21039.10593
	R	GCAACGCACTGCTCCTTAAC		
Cu-Zn-SOD	F	AGAGCAGTTGTAGGCCGAAG	116	EU077527.1
	R	GTGCAGCAAGCCAATCTAGC		
PT-1	F	TTGCTTGGTCAGTCTCCTGC	106	Cluster-1871.0
	R	CTCTAAGGTCTGGGCCTGTC		
β-Actin	F	TCCGTAAGGACCTGTATGCC	101	AY651918.2
	R	TCGGGAGGTGCGATGATTTT		

10.3389/fimmu.2022.1074399

conditions. The variable importance in projection (VIP) was calculated to assist in the screening of metabolic markers. This study used multivariate analysis of VIP values of the first two principal components of the partial least squares discriminant analysis (PLS-DA) model, combined with univariate analysis of fold change and *p*-value to screen the differential ions. The differential ions should simultaneously satisfy the following three conditions: 1) VIP ≥ 1, (2) fold change ≥1.2 or ≤0.8333, and 3) *p*-value< 0.05. The identification databases of metabolites included the Kyoto Encyclopedia of Genes and Genomes (KEGG), Human Metabolome Database (HMDB), and LIPID MAPS commercial databases. The differential metabolites were identified and screened out based on the fragment score, isotope similarity, mass error, and the pathway annotation results of the differential ions. Finally, the KEGG pathway analysis of different metabolites was performed. For detailed steps and parameters of metabolomics, refer to our previous description (21).

Statistical analysis

The differences in growth performance parameters, biochemical indicators, and gene expression parameters were analyzed using one-way ANOVA and Tukey's honestly significant difference (Tukey's HSD) (SPSS 22.0 software, Chicago, IL, USA). Statistically significant was considered when $p< 0.05$. The above data were expressed as means ± SEMs, and before statistical analysis, all data were tested for the normality of distribution and homogeneity of variances with Levene's test. The alpha diversity was calculated using QIIME software, including Chao1, observed species, Shannon index, and Simpson index. An independent-samples t-test was performed for all diversity indices and presented as mean ± standard deviation (SD). Statistically significant was considered when $p< 0.05$. The R package drew the principal coordinates analysis (PCoA) diagram. The microbial community and relative abundance values analysis were conducted using STAMP software. Mean differences were considered significant at $p< 0.05$. Furthermore, intestinal differential metabolite clusters were conducted using a Pearson's correlation test with differential microbial genera abundance and intestinal gene richness. Tables were generated using Excel 2016, while figures were made using GraphPad Prism 8.

Results

Growth performance indicator determination

As shown in Table 3, the levels of growth performance indicators were not significantly different among the three groups; however, WGR, SGR, and SR clearly presented a trend of decreasing followed by increasing, and FCR showed contrary trends.

Analysis of hemolymph antioxidant indicators

Figure 1 shows no significant difference in hemolymph MDA contents and CAT levels of prawns among these three groups. The hemolymph SOD activities of prawns were significantly increased in the BC group compared with the HF group.

Intestinal digestive enzymes and brush border enzyme activity determination

As shown in Figure 2, the trypsin activities of prawns were significantly decreased in the LF group compared with the HF group, and the lowest level was found in the BC group. No significant difference in lipase activity was found between the HF and BC groups; the activity in LF was significantly decreased compared with these two groups. There was no significant difference in amylase activities among these three groups. The γ-GT activities of prawns were considerably increased in the LF and BC groups compared with the HF group.

Analysis of intestinal gene expression profile

As shown in Figure 3, the *Toll* and *Relish* expression levels of prawns were significantly upregulated in the LF group compared with the HF group. BC intervention downregulated the *Toll* and *Relish* expression levels compared to the LF group. BC intervention significantly downregulated the expression of *Toll* to a level close to the HF group; however, the *Relish* expression level in the BC group was significantly higher than in the HF group. The *IL-22* and *Cu/Zn-SOD* expression levels of prawns were significantly upregulated

TABLE 3 Effect of cottonseed protein concentrate as a substitute for fish meal and *Bacillus coagulans* supplementation on the growth performance of *Macrobrachium rosenbergii*.

Group	FW (g)	WGR (%)	SGR (%/day)	FCR	SR (%)
HF	4.22 ± 0.28	1,521.85 ± 71.92	3.98 ± 0.06	1.63 ± 0.12	84.17 ± 3.63
LF	4.29 ± 0.16	1,489.40 ± 37.13	3.95 ± 0.03	1.69 ± 0.04	80.83 ± 1.67
LF+BC	4.93 ± 0.47	1,582.71 ± 134.00	4.02 ± 0.11	1.42 ± 0.18	86.67 ± 4.17

FW, final weight; WGR, weight gain rate; SGR, specific growth rate; FCR, feed conversion ratio; SR, survival rate.

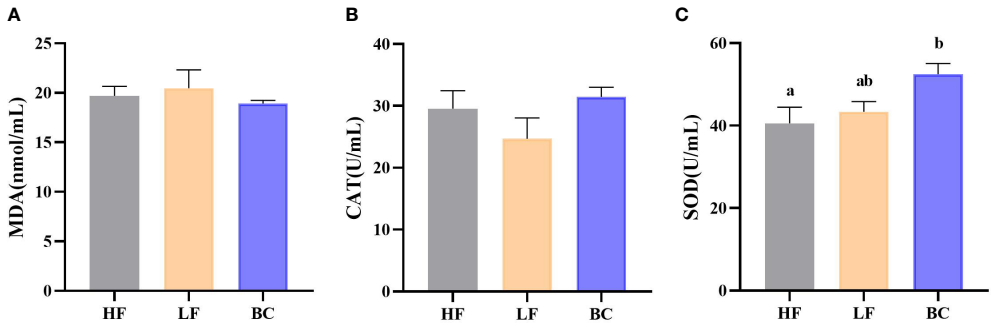

FIGURE 1
(A–C) Hemolymph antioxidant indicators. **(A)** Hemolymph MDA content; **(B)** Hemolymph CAT activity; **(C)** Hemolymph SOD activity.

in the BC group than the HF and LF groups, and no significant difference was found between the HF and LF groups. The *PT-1* expression in the BC group was significantly upregulated compared with the LF group, and there was no significant difference between the HF and LF groups.

Community diversity analysis of prawns' intestinal microflora

This study obtained 2,682 OTUs from all samples. The Chao1, observed species, Shannon, and Simpson indices were calculated to

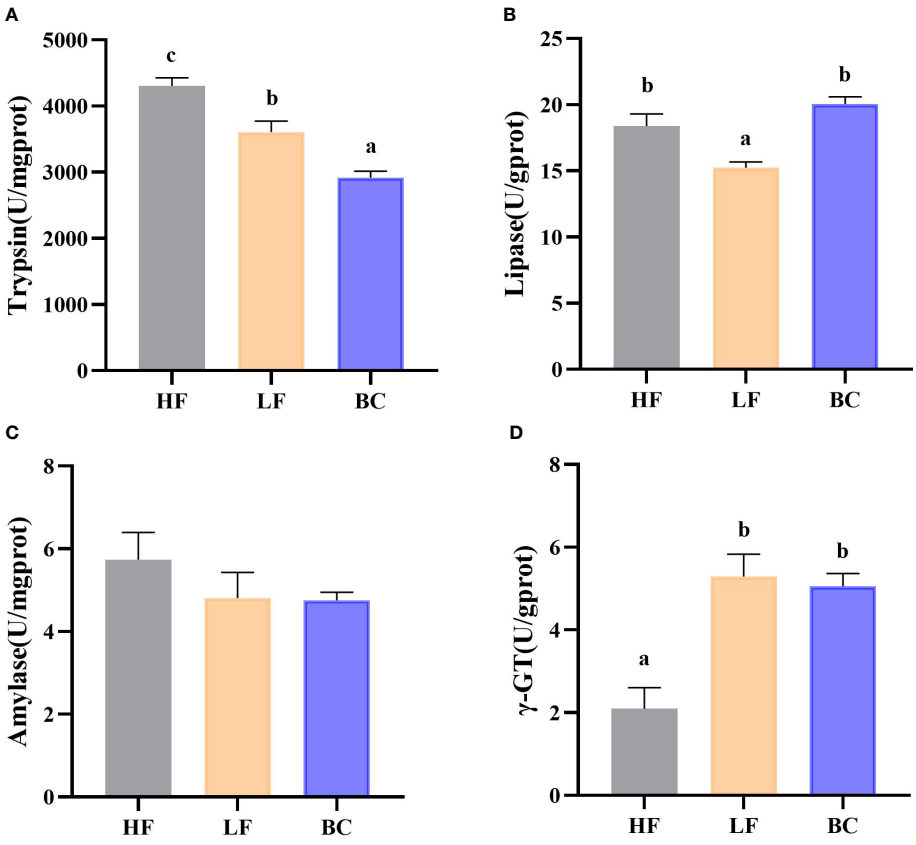

FIGURE 2
(A–D) Intestinal digestive enzymes and brush border enzyme activity. **(A)** Intestinal trypsin activities; **(B)** Intestinal lipase activities; **(C)** Intestinal amylase activities; **(D)** Intestinal γ-GT activities.

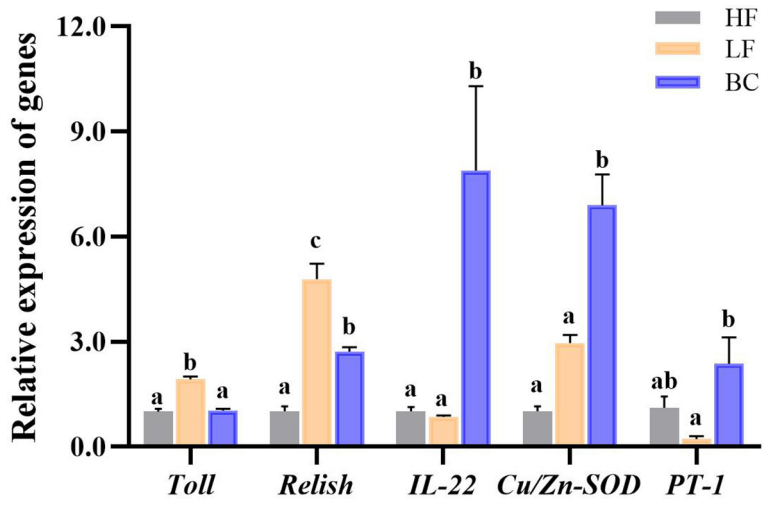

FIGURE 3
Intestinal gene expression profile. Different lowercase letters indicate significant differences ($P < 0.05$) between the three groups.

evaluate the microbial community richness and diversity of prawns' intestines. As shown in Figure 4, the Chao 1 and observed_species index of prawns' intestinal microflora in HF were significantly higher than in LF prawns. There was no significant difference in Chao 1 and observed_species index between the BC group and the other two groups. The Shannon index of prawns' intestinal microflora in the BC group was significantly lower than in the HF group. No significant difference was found in the Simpson index among these three groups. PCoA of bacterial communities of *M. rosenbergii* fed with three different diets showed that the LF group was distant from the HF group samples. In contrast, the BC group sample cluster intersected the LF and HF groups, shortening the distance from the HF group samples.

Gut microbiota function prediction

The predicted_phenotypes of potentially_pathogenic by BugBase software are shown in Figure 5. The potentially_pathogenic of prawns in the LF group was higher than in the HF group, and the BC group's predicted_phenotypes were decreased compared with the LF group. There was no significant difference among these three groups.

Microbial community structure of *Macrobrachium rosenbergii* treated with different diets

As shown in Figure 6, four predominant phyla (mean relative abundance >1%) were detected in *M. rosenbergii* samples, including Proteobacteria (81.33%), Firmicutes (11.84%), Tenericutes (3.85%), and Bacteroidetes (1.11%). Compared with the HF group, the mean relative abundance of Proteobacteria, Firmicutes and Bacteroidetes decreased in the LF group, while the Tenericutes increased. *B. coagulans* treatment adjusted the relative abundance of these changed genera to a level similar to that of the HF group, except for Bacteroidetes. At the genus level, compared with the HF group, the main differential bacteria in the LF group were *Flavobacterium*, *Bacillus*, *Muricauda*, *Nocardioides*, *Hyphomicrobium*, *Nitrospira*, and *Acinetobacter*, all of which showed decreased abundance. Compared with the LF group, the different main bacteria in the BC group were *Sphingomonas*, *Bacillus*, *Ralstonia*, *Demequina*, and *Sphingopyxis*, of which the abundance of the former was significantly upregulated, and the latter two were significantly decreased. Compared with the BC group, a higher abundance of *Nocardioides*, *Hyphomicrobium*, *Sphingopyxis*, *Nitrospira*, and *Acinetobacter* was found in the HF group.

Analysis of the differential metabolites in fecal between LF and BC groups

The differential metabolites of intestinal flora between the LF and BC groups are listed in Table 4. Among them, 89 metabolites were significantly upregulated, and one metabolite was significantly downregulated in the BC group. The differential metabolites mainly included amino acids and amino acid derivatives, sphingolipids and other lipids, aromatic compounds, energy metabolism-related metabolites, secondary metabolism of plants, nucleotides, and nucleotide derivatives.

Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of differential metabolites between LF and BC groups

As shown in Figure 7, the top 20 KEGG pathways with significant enrichment are as follows: Secondary metabolite

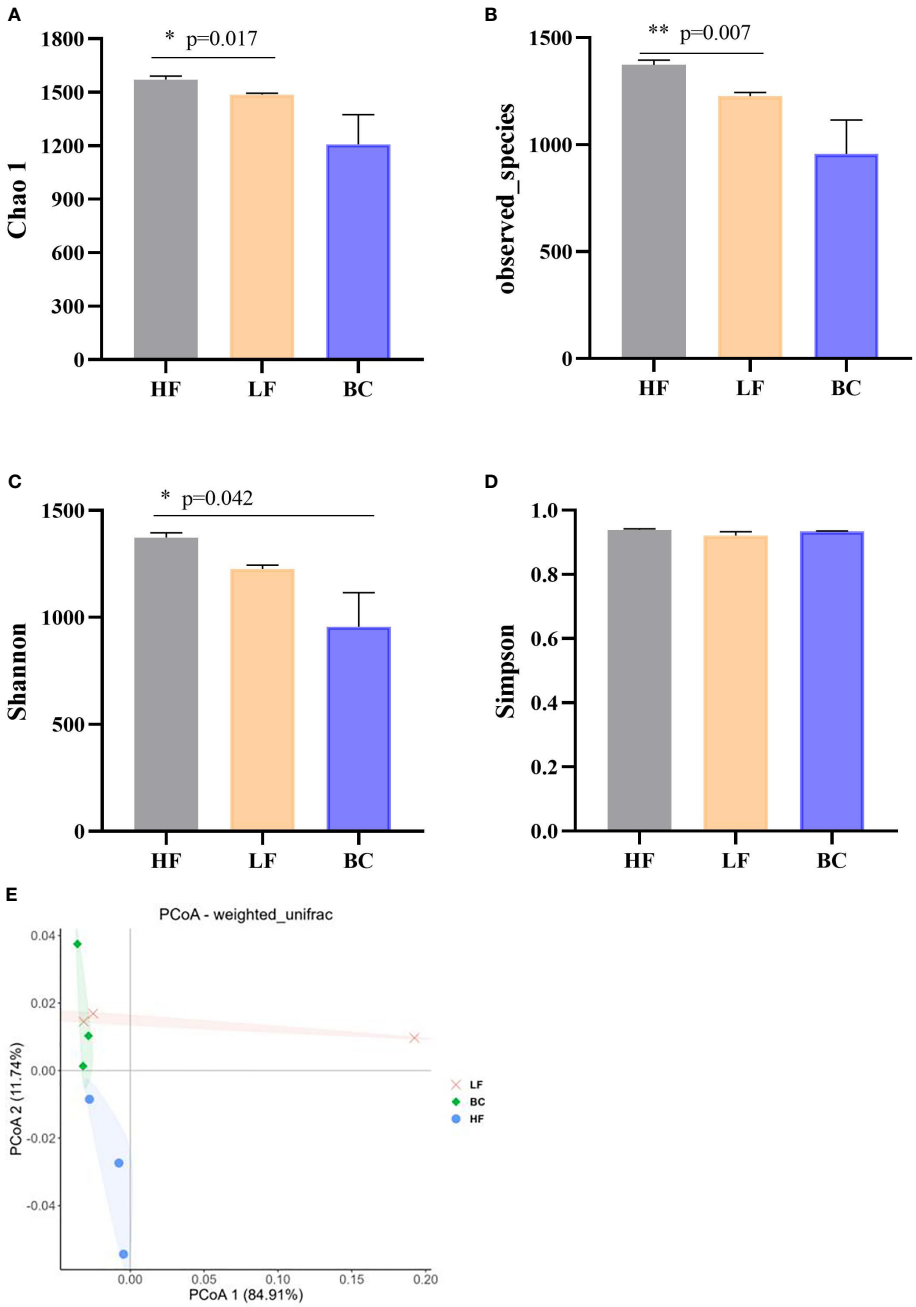

FIGURE 4
(A–E) Community diversity analysis and principal coordinates analysis (PCoA) of bacterial communities of *Macrobrachium rosenbergii* fed with different diets. * P < 0.05, ** P < 0.01.

synthesis-related (Biosynthesis of secondary metabolites, Biosynthesis of plant secondary metabolites, Glucosinolate biosynthesis, Biosynthesis of alkaloids derived from shikimate pathway, Biosynthesis of various secondary metabolites-part 3, Biosynthesis of various secondary metabolites-part, Sphingolipids metabolism-related, Sphingolipid signaling pathway, and Sphingolipid metabolism), Amino acid synthesis, metabolism and transfer-related (Biosynthesis of amino acids; Aminoacyl-tRNA biosynthesis; Cyanoamino acid metabolism; Phenylalanine metabolism; Alanine, aspartate, and glutamate metabolism; Phenylalanine, tyrosine, and tryptophan biosynthesis), Microbial metabolism in diverse environments, Protein digestion and absorption, Central carbon metabolism in cancer, 2-Oxocarboxylic acid metabolism, Necroptosis, and Mineral absorption.

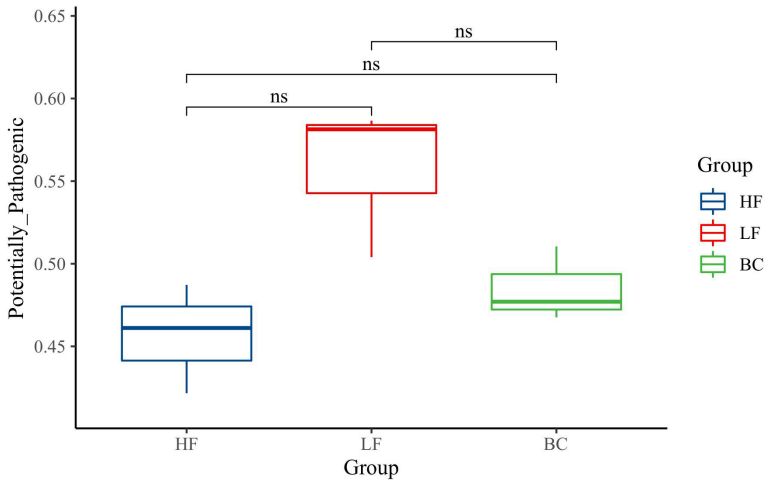

FIGURE 5
Gut microbiota function prediction. ns, P > 0.05.

Correlation analysis of intestinal microbes and metabolites

Figure 8 shows significant correlations between 63 differential metabolites and five differential microbial genera found through Pearson's correlation analysis. The correlation analysis heat map showed that *Bacillus*, *Ralstonia*, and *Sphingomonas* were positively correlated with differential metabolites and that *Demequina* and *Sphingopyxis* negatively correlated with differential metabolites. Among them, 32 metabolites were significantly positively correlated with the *Bacillus* genus, 36 with the *Ralstonia* genus, 46 with the *Sphingomonas* genus, and 57 with the *Sphingopyxis* genus, and seven were negatively associated with the *Demequina* genus.

Correlation analysis of selected differential metabolites and intestinal genes

As shown in Figure 9, differential metabolites with significant microbial correlation were selected for further correlation analysis with intestinal genes. The results showed that immune and intestinal barrier-related genes (*Cu/Zn-SOD*, *IL-22*, and *PT-1*) were positively correlated with differential metabolites. Inflammation-related factor (*Toll* and *Relish*) expression was negatively correlated with differential metabolite levels. Pearson's correlation analysis revealed that the primary differential metabolites (significant correlation with more than three genes) associated with intestinal immune genes were as follows: L-Lysine, Glycitein, Pipericine, Isoquinoline, 7-Hydroxy-2-phenyl-4*H*-chromen-4-one, PC(16:1(9*Z*)/P-18:1(11*Z*)), Quinone, PC(15:0/15:0), Vinylacetylglycine, Vitamin D3, N^6-Acetyl-L-lysine, Sphinganine, LysoPC(16:0), *N*-Acetyldopamine, Lysyl-Lysine, SM(d18:1/16:0), SM(d18:0/18:1(9*Z*)), 1*H*-Indole-2,3-dione, 5-Hydroxy-L-tryptophan, Glutamyllysine, Dehydrophytosphingosine, L-Glutamic acid, and PC(16:1(9*Z*)/14:0).

Discussion

Aquaculture relies heavily on fish meal to produce aquafeeds, leading to its continually inflated price. As a cost-effective and sustainable alternative to fish meal, various plant proteins are used to partially or entirely replace fish meal. However, an inappropriate plant protein substitution ratio could adversely affect aquatic animals' growth due to limiting amino acids and anti-nutritional factors (6, 22). Using probiotic bacteria to alleviate the adverse effects of growth suppression in aquatic animals induced by plant protein substitution is becoming an effective auxiliary strategy (23). Similarly, in our study, 10% fish meal substitution (basal fish meal level of 25%) by CPC depressed the growth to some extent, and *B. coagulans* supplementation alleviated the downregulated levels of WGR and SGR. However, there were no significant differences in growth and survivability among these three groups. Meanwhile, the phenotypic differences in hemolymph antioxidant indicators (MDA and CAT) coincided with the growth indicators variation and trend among the groups. The significantly upregulated SOD activity reflected the improvement of the overall antioxidant capacity of *M. rosenbergii* after treatment with *B. coagulans*, which echoed the upregulated growth performance following *B. coagulans* fortification, indicating the growth promotion function of *B. coagulans* in the model of fish meal replacement by plant

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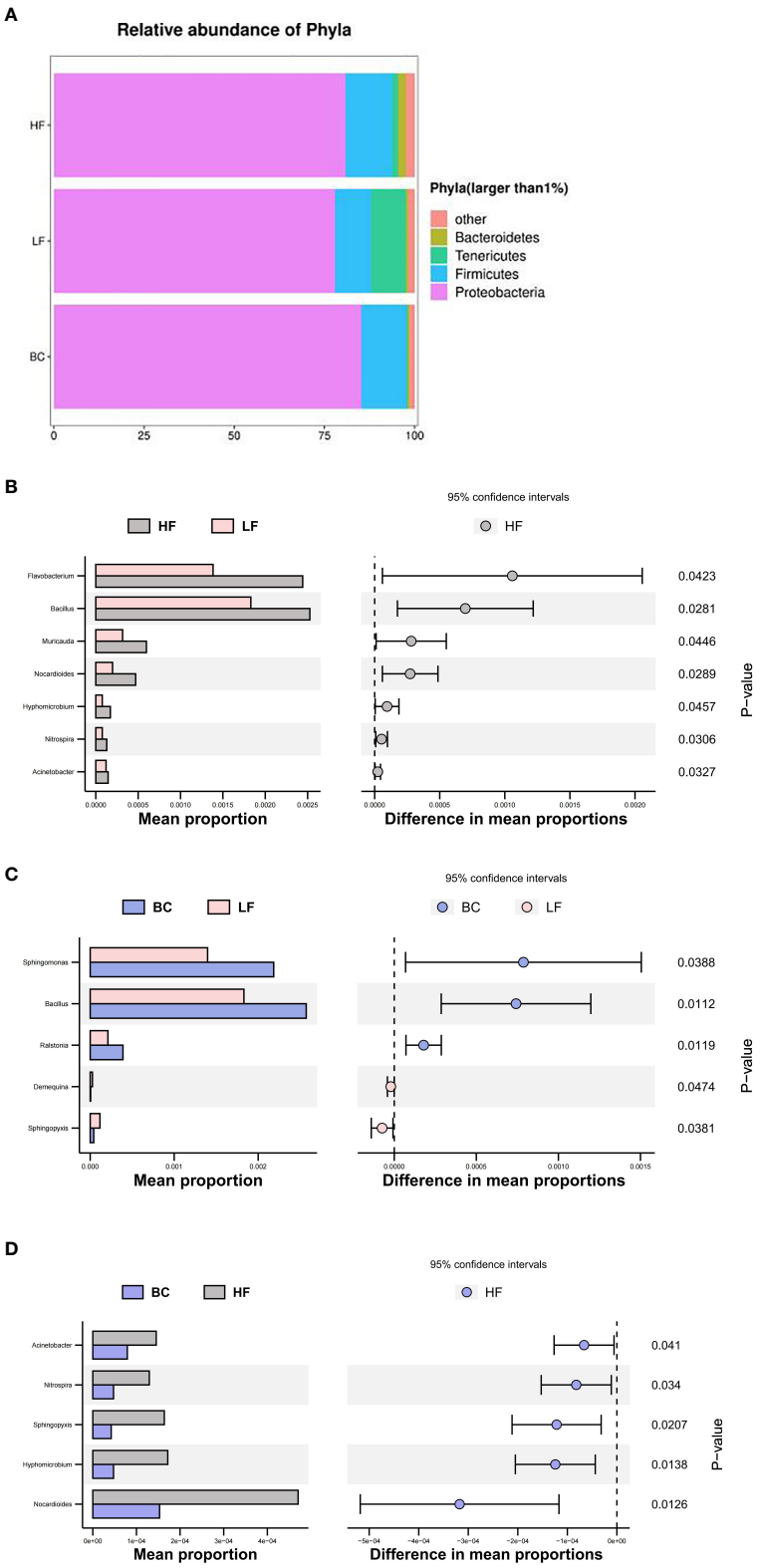

FIGURE 6
(A–D) Microbial community structure of *Macrobrachium rosenbergii* treated with different diets.

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TABLE 4 The differential metabolites in fecal between LF and BC groups.

Metabolites	rt	Mass	MeanLF	MeanBC	Trend	VIP	*p*-Value	FC
Amino acids and amino acid derivatives								
L-Serine	389.699	106.050	1.437	3.838	Up	1.235	0.047	2.671
L-Lysine	532.865	147.112	3.324	19.025	Up	1.318	0.006	5.723
beta-Alanine	359.246	90.055	10.680	24.322	Up	1.243	0.024	2.277
D-Ornithine	547.123	133.097	0.386	1.065	Up	1.229	0.036	2.760
D-Proline	514.857	116.070	1.293	4.484	Up	1.316	0.010	3.467
Tryptophan	0.876	205.096	4.911	16.538	Up	1.242	0.018	1.717
5-Hydroxy-L-tryptophan	297.080	221.091	0.051	0.190	Up	1.217	0.039	3.701
L-Norleucine	279.448	130.086	255.886	427.238	Up	1.283	0.045	1.670
L-Valine	313.992	118.086	24.588	58.664	Up	1.250	0.037	2.386
L-Phenylalanine	273.762	166.085	35.455	102.182	Up	1.275	0.029	2.882
L-Tyrosine	316.869	182.080	8.767	33.059	Up	1.225	0.027	3.771
Prolylglycine	311.634	173.091	0.045	0.160	Up	1.236	0.019	3.531
L-Glutamic acid	411.255	148.059	10.094	24.526	Up	1.204	0.035	2.430
L-Asparagine	389.770	133.060	3.387	9.566	Up	1.228	0.029	2.825
Argininosuccinic acid	484.113	291.127	0.052	0.179	Up	1.230	0.028	3.430
Glutamyllysine	506.193	276.153	0.014	0.089	Up	1.331	0.001	6.526
Gamma-glutamyl ornithine	465.060	262.138	0.116	0.419	Up	1.316	0.005	3.601
Lysyl-Glycine	470.018	204.133	0.543	1.578	Up	1.251	0.021	2.906
Leucyl-Glycine	120.022	189.122	1.042	2.057	Up	1.174	0.043	1.974
Lysyl-Arginine	572.540	303.211	0.080	0.462	Up	1.332	0.003	5.748
Lysyl-Aspartate	482.117	262.138	0.022	0.093	Up	1.276	0.028	4.164
Aspartyl-Arginine	456.445	290.143	0.254	0.816	Up	1.282	0.021	3.215
Lysyl-Lysine	574.436	275.206	0.043	0.237	Up	1.344	0.000	5.507
Prolyl-Lysine	485.670	244.164	0.412	1.618	Up	1.247	0.020	3.924
Hydroxyprolyl-Arginine	380.695	288.165	0.094	0.160	Up	1.171	0.048	1.706
N^6-Acetyl-L-lysine	403.155	189.122	0.108	0.230	Up	1.195	0.036	2.125
Epsilon-(gamma-glutamyl)-lysine	342.282	114.091	0.426	0.164	down	1.294	0.002	0.385
Vinylacetylglycine	445.764	144.065	0.948	1.564	Up	1.215	0.035	1.650
N-Acetyldopamine	389.971	196.096	0.052	0.139	Up	1.206	0.035	2.658
5-Aminopentanal	268.421	102.091	0.855	1.795	Up	1.146	0.050	2.100
5-Aminopentanoic acid	285.672	118.086	153.482	232.481	Up	1.193	0.037	1.515
(3*S*,5*S*)-3,5-Diaminohexanoate	358.914	147.112	0.082	0.127	Up	1.221	0.031	1.543
Sphingolipids								
Sphinganine	126.829	302.303	0.150	0.483	Up	1.245	0.030	3.213
Dehydrophytosphingosine	150.057	316.283	2.173	4.968	Up	1.266	0.018	2.286
Phospholipids								
PC(15:0/15:0)	165.938	706.534	0.876	2.051	Up	1.219	0.044	2.341
PC(16:0/16:0)	143.618	734.567	0.113	0.247	Up	1.166	0.049	2.182
PC(16:1(9Z)/14:0)	165.938	704.517	0.871	1.784	Up	1.224	0.027	2.048
PC(16:1(9Z)/15:0)	165.148	718.532	0.430	1.172	Up	1.282	0.006	2.726
PC(18:1(11Z)/14:0)	163.671	732.548	7.850	16.581	Up	1.262	0.017	2.112
PC(16:1(9Z)/16:1(9Z))	164.080	730.535	2.463	5.134	Up	1.203	0.042	2.085
PC(18:2(9Z,12Z)/18:0)	158.697	786.596	11.337	23.771	Up	1.263	0.008	2.097
PC(15:0/18:2(9Z,12Z))	163.281	744.549	0.774	1.979	Up	1.248	0.015	2.557
PC(24:1(15Z)/14:1(9Z))	61.254	814.623	0.138	0.500	Up	1.257	0.012	3.630
PC(20:2(11Z,14Z)/15:0)	160.063	772.580	0.970	2.096	Up	1.263	0.013	2.162
PC(16:1(9Z)/P-18:1(11Z))	157.795	742.570	0.117	0.261	Up	1.303	0.009	2.221

(Continued)

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TABLE 4 Continued

Metabolites	rt	Mass	MeanLF	MeanBC	Trend	VIP	*p*-Value	FC
PC(18:3(6*Z*,9*Z*,12*Z*)/15:0)	163.278	742.532	0.383	0.964	Up	1.277	0.010	2.517
PC(18:3(9*Z*,12*Z*,15*Z*)/14:0)	164.912	728.519	0.237	0.600	Up	1.246	0.041	2.536
PC(22:2(13*Z*,16*Z*)/16:1(9*Z*))	157.827	812.611	1.076	1.904	Up	1.194	0.042	1.769
PC(18:3(6*Z*,9*Z*,12*Z*)/18:1(11*Z*))	59.786	782.561	4.189	14.056	Up	1.185	0.028	3.355
PC(18:3(6*Z*,9*Z*,12*Z*)/P-18:1(11*Z*))	156.085	766.568	2.699	4.163	Up	1.169	0.046	1.542
PC(18:4(6*Z*,9*Z*,12*Z*,15*Z*)/18:1(11*Z*))	60.817	780.549	5.307	23.397	Up	1.270	0.010	4.409
PC(18:4(6*Z*,9*Z*,12*Z*,15*Z*)/P-18:1(11*Z*))	154.689	764.555	1.898	3.151	Up	1.242	0.013	1.661
PC(20:2(11*Z*,14*Z*)/20:4(5*Z*,8*Z*,11*Z*,14*Z*))	153.256	834.593	4.658	9.579	Up	1.230	0.044	2.056
PC(22:5(7*Z*,10*Z*,13*Z*,16*Z*,19*Z*)/20:1(11*Z*))	150.997	862.623	0.162	0.494	Up	1.290	0.009	3.048
PC(P-18:1(11*Z*)/22:5(4*Z*,7*Z*,10*Z*,13*Z*,16*Z*))	149.122	818.601	0.802	1.068	Up	1.237	0.042	1.332
PE(P-16:0/20:5(5*Z*,8*Z*,11*Z*,14*Z*,17*Z*))	155.152	722.508	1.542	2.651	Up	1.181	0.048	1.719
LysoPE(0:0/14:0)	224.850	426.258	0.013	0.048	Up	1.235	0.022	3.755
LysoPC(16:0)	212.374	496.336	6.094	25.253	Up	1.253	0.021	4.144
LysoPC(P-16:0)	205.967	480.341	0.194	0.479	Up	1.250	0.020	2.470
LysoPC(18:1(9*Z*))	209.520	522.353	14.907	66.989	Up	1.226	0.034	4.494
SM(d18:1/16:0)	200.851	703.569	0.195	0.937	Up	1.332	0.001	4.798
SM(d18:1/20:0)	197.867	759.630	0.029	0.118	Up	1.267	0.018	4.072
SM(d18:0/18:1(9*Z*))	198.900	731.601	0.113	0.611	Up	1.319	0.002	5.425
Other lipids								
(2*E*)-Decenoyl-ACP	532.921	130.085	1.731	8.939	Up	1.317	0.007	5.164
Phosphodimethylethanolamine	460.647	170.057	0.123	0.561	Up	1.312	0.003	4.561
5α-Cholesta-8,24-dien-3-one	31.273	383.328	0.201	0.341	Up	1.225	0.026	1.702
Aromatic compounds								
Quinone	223.123	109.028	0.106	0.386	Up	1.220	0.026	3.653
Phenol	316.869	95.049	0.194	0.763	Up	1.212	0.037	3.929
Parabanic acid	16.343	112.998	0.825	0.976	Up	1.495	0.012	1.183
Benzaldehyde	273.701	107.049	0.358	1.088	Up	1.291	0.022	3.037
4-Hydroxybenzaldehyde	316.993	123.044	1.072	4.121	Up	1.221	0.031	3.844
2-Phenylacetamide	184.356	128.143	11.688	20.587	Up	1.280	0.018	1.761
1*H*-Indole-3-carboxaldehyde	276.993	146.059	0.518	1.876	Up	1.190	0.028	3.619
1*H*-Indole-2,3-dione	47.215	148.038	0.163	0.464	Up	1.209	0.029	2.855
Energy metabolism-related								
O-Phosphotyrosine	492.769	262.049	0.271	1.058	Up	1.308	0.002	3.902
Isocitral	183.881	153.127	0.087	0.177	Up	1.147	0.041	2.034
3-Dehydroxycarnitine	390.707	146.117	31.835	78.692	Up	1.260	0.018	2.472
Secondary metabolites of plants								
7-Hydroxy-2-phenyl-4*H*-chromen-4-one	202.998	237.054	0.326	0.640	Up	1.569	0.011	1.961
Glycitein	145.862	307.060	0.154	0.317	Up	1.327	0.001	2.061
Luteolin 4′-sulfate	26.207	367.009	0.032	0.100	Up	1.318	0.001	3.154
Momordicinin	30.867	439.355	0.026	0.045	Up	1.311	0.002	1.708
Isoquinoline	137.025	130.064	0.099	0.269	Up	1.279	0.018	2.713
2-Propylpiperidine	184.356	128.143	11.688	20.587	Up	1.280	0.018	1.761
Pipericine	1.954	336.324	0.007	0.016	Up	1.139	0.044	2.296
Nucleotides and nucleotide derivatives								
8-Hydroxyguanine	309.359	168.051	0.253	0.403	Up	1.189	0.045	1.593
Pyrimidine	172.823	81.045	0.134	0.585	Up	1.259	0.043	4.361
3-Aminoisobutanoic acid	350.392	102.055	0.655	1.177	Up	1.500	0.027	1.796
Cyclic AMP	281.191	328.044	0.319	1.564	Up	1.461	0.041	4.903
3′-AMP	420.136	346.055	0.424	0.863	Up	1.330	0.041	0.662
Vitamin D3	31.910	385.343	0.164	0.326	Up	1.204	0.039	1.988

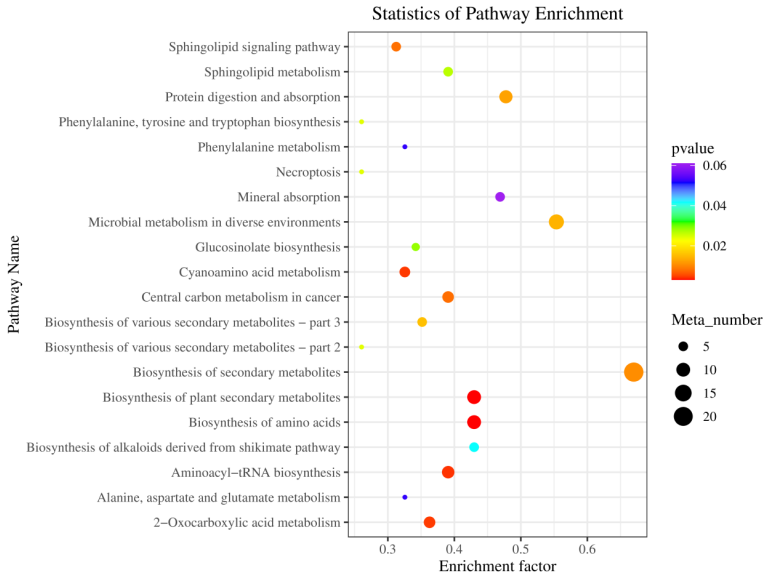

FIGURE 7
KEGG pathway enrichment of differential metabolites in fecal between LF and BC groups. Colors indicate enriched Q-value; the redder color represents a smaller Q-value. The size of the bubble indicates the number of metabolites mapped within a KEGG pathway. KEGG, Kyoto Encyclopedia of Genes and Genomes.

protein. The growth performance of animals is closely related to intestinal physiological function. The activity of digestive enzymes, the integrity of barrier structure, and the immune function of the intestine directly determine the utilization efficiency of nutrients in the feeds and the body's defense against environmental pathogens. Also, the primary mechanism by which dietary probiotics augment the growth of cultured species is by improving intestinal digestive and absorptive activity, and health (24, 25). Given this, we further explored the effects of fish meal replacement and probiotic supplementation on intestinal digestive enzyme activity and health status.

In this study, substituting 40% fish meal with CPC considerably decreased intestinal digestive enzyme activities (trypsin and lipase). This result is consistent with previous studies that fish meal replaced with excessive plant proteins elicited intestinal villus morphology destruction and suppressed the digestive and absorptive-related enzyme activities in aquatic animals (26). The inherent anti-nutritional factors or unbalanced amino acid composition in high-plant-protein diets may be intrinsically responsible for this (27). Gamma-glutamyl transferase (γ-GT) is located on the surface of the brush border epithelium in the intestine. It is essential in the epithelial cells' amino acid uptake and dipeptide transport (28). In addition, gene expression of γ-GT is highly sensitive to oxidative stress as part of the cellular antioxidant defensive system (29), as γ-GT is involved in the degradation and resynthesis of glutathione *in vivo* and regulates GSH levels. There is a relationship between the intestine structural damage and the upregulated level of γ-GT in the intestine. In this study, the intestine γ-GT enzyme activity was significantly upregulated following the replacement of fish meal with CPC. On the one hand, the differentiation in amino acid composition between the two diets may result in the intestinal epithelial cells' differential amino acid absorption strategy. On the other hand, reflecting that plant protein substitution may stimulate the onset of intestinal oxidative stress. Contrary to other research results, the dietary supplementation with *B. coagulans* significantly increased the activities of the digestive enzymes, including trypsin. In this study, *B. coagulans* supplementation with high-protein plant diets significantly downregulated or somewhat did not improve to a higher level of the trypsin activities, speculating for a reason related to the timing and tissue region of sampling. The spatial and temporal orders of the digestive enzymes involved in protein and peptide digestion in aquatic animals differ (30). Our sampling node may miss the active phase of trypsin action or *B. coagulans* altered the strategy for protein digestion and absorption in the intestine. However, the exact reasons need to be further explored.

In addition to digestive and absorptive capacity, the barrier and immune functions of the gut are also crucial to the health of aquatic animals. To investigate the effects on intestinal barrier function and inflammatory response in *M. rosenbergii*, we evaluated the variations in gene expression levels of the gut barrier protein *PT-1*, inflammatory-related factors (*Relish*, *Toll*, and *IL-22*), and antioxidant factor (*Cu/Zn-SOD*). In invertebrates, the peritrophic membrane (PM) protects the epithelium cells from mechanical abrasion and prevents pathogens from adhering to intestine

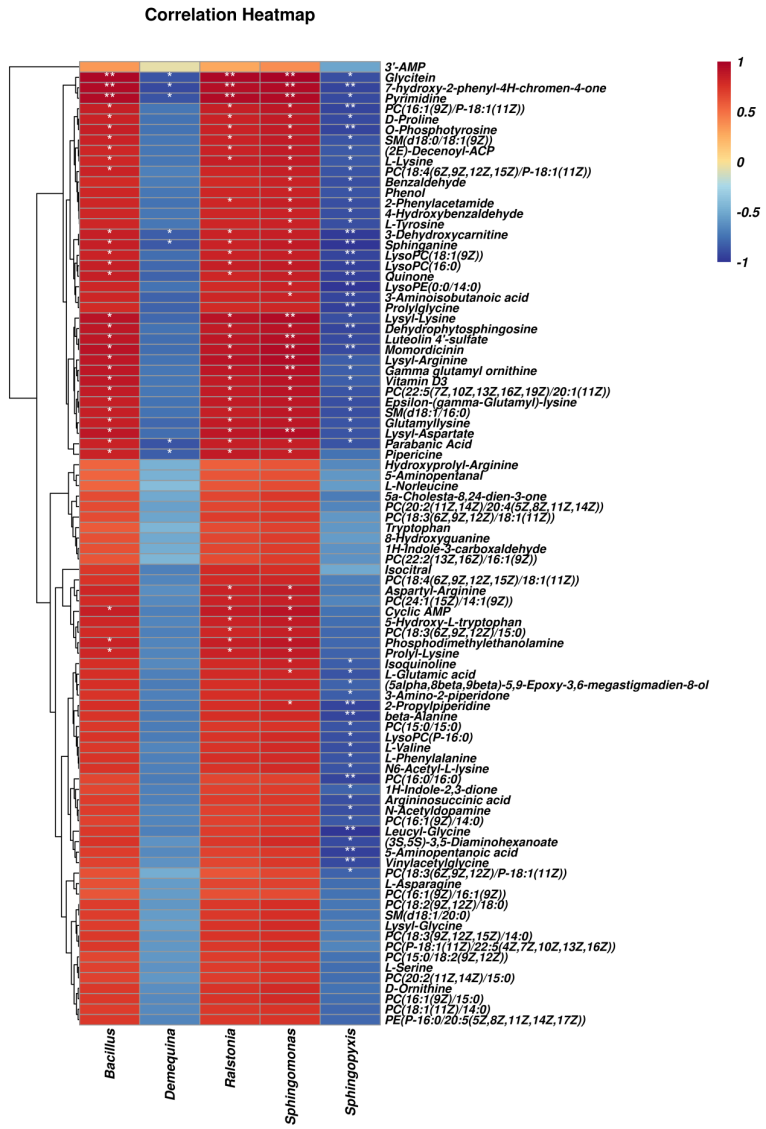

FIGURE 8
Correlation analysis of intestinal microbes and metabolites between LF and BC groups. Red represents a positive correlation; blue represents a negative–positive correlation. The darker the color, the stronger the correlation. *$p < 0.05$, **$p < 0.01$.

epithelial cells (31). Moreover, the dominant proteins of PMs were identified mainly including digestion-related, immune-related, antioxidant proteins, and structural proteins of PM in *Litopenaeus vannamei*, highlighting its essential role in the intestinal immune system (32). Peritrophins (PTs) are essential for the generation of PM, which directly affect the permeability, elasticity, porosity, and strength of PM that lines the crustacean midgut. Therefore, PT plays a role in the immune response against pathogenic bacteria by protecting the intestine from colonization, intrusion, and secondary tissue distribution of pathogenic bacteria in crustaceans (33). The expression of intestinal inflammatory transcription factors (*Toll* and *Relish*) was significantly upregulated, while the expression of barrier factor *PT-1* was significantly downregulated following the substitution of 40% fish meal by cottonseed protein concentrate, as determined. This indicates that plant protein substitution caused impairment of intestinal barrier function and secondary inflammatory responses. This may be attributed to anti-nutritional factors in plant proteins, which could impair the intestine peritrophic membrane's morphological structure and thus induce gut inflammation (significantly upregulated inflammation factor) (34). Interleukin (IL)-22 is a homeostatic cytokine that promotes the expression of a series of genes that enforce intestinal epithelial cell defense function (35). For example, IL-22 signaling promoted the upregulation of

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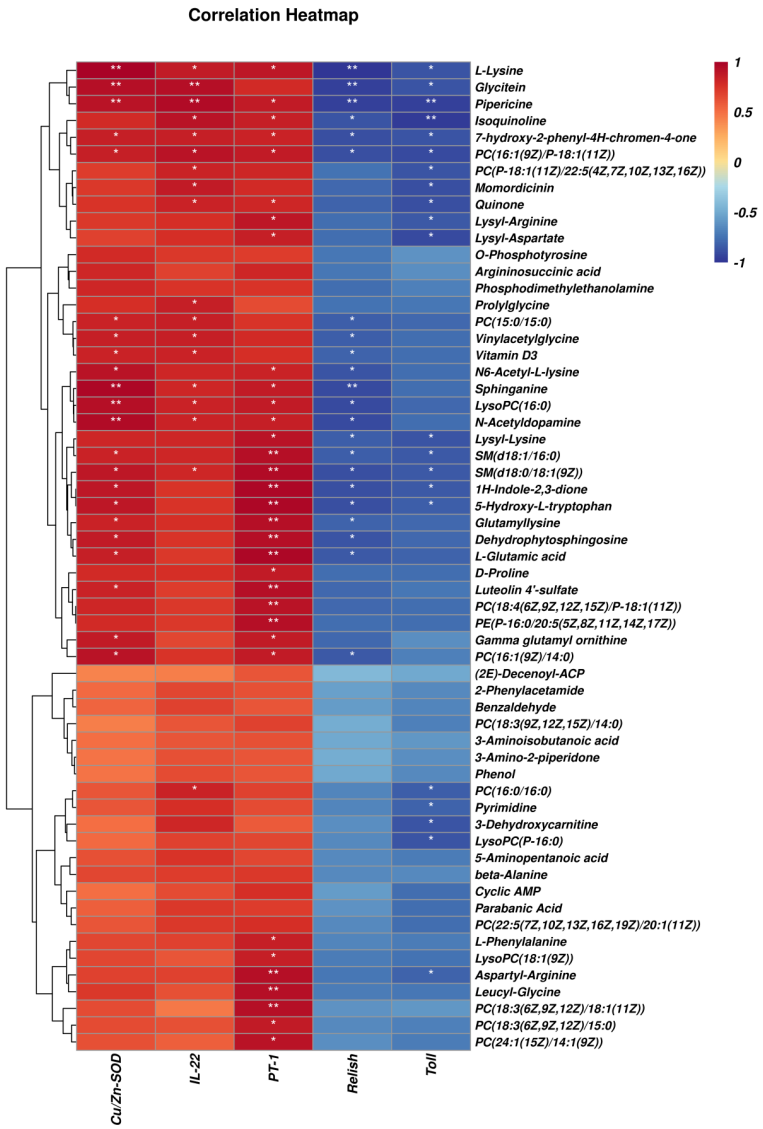

FIGURE 9
Correlation analysis of selected differential metabolites and intestinal genes between LF and BC groups. Red represents a positive correlation; blue represents a negative–positive correlation. The darker the color, the stronger the correlation. *$p < 0.05$, **$p < 0.01$.

intestine membrane mucin Muc17 in neonatal mice, suggesting its critical role in the epithelial barrier function of the intestine (36). Moreover, the reparative cytokine IL-22 was confirmed to drive repair in the intestinal epithelium through decreasing inflammation response in the colitis model (37). Additional supplementation with *B. coagulans* significantly alleviated the upregulated expression of inflammatory factors. Meanwhile, it significantly upregulated the expression levels of the barrier factor *PT-1*, the reparative cytokine *IL-22*, and the antioxidant factor *Cu/Zn-SOD*. The above results indicate that *B. coagulans* can alleviate the side effects of plant protein substitution and that the critical target regulatory molecules were *PT-1* and *IL-22*. Similar studies have shown that probiotic *Lactobacillus casei* treatment strengthened the function of the gut mucosal epithelium by boosting *IL-22* and tight junction protein (*Zonulin-1* and *Claudin-1*) expression levels in the intestinal inflammatory model of chicks infected with *Salmonella pullorum* (38).

From the gut microbiota perspective, partial substitution of fish meal with plant protein sources affected the composition and diversity of gut microbes, and inappropriate substitution ratio induced host intestine mucosa lesions and hypoimmunity (26, 39). At the phylum level, consistent with the previous study, Proteobacteria, Firmicutes, Bacteroidetes, and Tenericutes were determined as the predominant phyla in *M. rosenbergii* intestine

(40). We noted a remarkable downregulation of Firmicutes abundance after plant protein substitution. Speculating that the fiber content in the CPC substitution group diet is higher than the HF group, a recent report suggested that dietary fiber intake decreases the proportion of Firmicutes (41). The Firmicutes phylum includes the vast majority of lactic acid bacteria genera, such as *Streptococcus*, *Lactobacillus*, *Leuconostoc*, and *Carnobacterium*, which are generally considered to be probiotics for intestinal health (42). The phylum Firmicutes produces short-chain fatty acids (SCFAs) through collaboration with oligosaccharide fermentation bacteria such as Bifidobacteria (43). Thus, the callback of Firmicutes abundance after *B. coagulans* intervention suggests that *B. coagulans* has the potential to improve the structure of the intestinal flora positively. In addition, substituting 40% fish meal with CPC significantly decreased the richness and diversity of the intestinal flora, as well as downregulated the abundance of *Flavobacterium* and *Bacillus*, which were regarded as antagonistic probiotics in aquaculture (44). Furthermore, the potential pathogenicity in the CPC substitution group apparently increased through the phenotype prediction of potentially_pathogenic by BugBase software. This indicated that this substitution ratio led to the unbalance of a game between probiotics and conditionally pathogenic bacteria, disrupting intestinal health. This is consistent with previous studies showing that appropriate levels of CPC substitution can increase the richness and diversity of gut flora, while excessive substitution induces enteritis and disrupt the homeostasis and part of the functions of the gut microbiota (45, 46). In recent years, *Bacillus* spp. has been reported as a potential probiotic candidate in aquatic farming sectors due to its high antagonistic activities and extracellular enzyme synthesis (47). Dietary *Bacillus* supplementation benefits the host's growth, intestinal microbial homeostasis, stress resistance, and immune and anti-inflammatory response (48–51). *Sphingomonas* is also considered to be a potential probiotic for aquaculture. The prebiotic effects mainly include the following ways: 1) regulates nutrient metabolism by secreting cellulase, protease, and amylase (52); 2) degrades toxic compounds, ammonia nitrogen, and nitrite (53); 3) produces oligosaccharide prebiotics (54); 4) inhibit *Vibrio* (53, 55)). In this study, we noted that *B. coagulans* treatment significantly improved the relative abundance of *Sphingomonas*, *Bacillus*, and *Ralstonia* while reducing the potential pathogenic flora after CPC substitution, echoing the trend in gene expression of intestinal inflammatory and barrier factors. This indicates that *B. coagulans* treatment is conducive to the colonization of *Bacillus* spp. in the gut and has a positive regulatory effect on intestinal immunity. This is similar to a recent study on turbot that different species of *Bacillus* treatment induced a significant positive correlation between the immune and antioxidant indexes and intestinal probiotics such as *Bacillus*, *Ralstonia*, and *Bifidobacterium* and a negative correlation with pathogenic bacteria (50).

Strikingly, a new research perspective has been condensed through integrating clinical monitoring, 16S rDNA and metagenomic sequencing, metabonomics analysis, and whole-genome sequencing. Accumulating medical reports have revealed the decisive role of specific gut microbes and gut microbes-derived secondary metabolites in metabolic and gastrointestinal disease (insulin resistance, non-alcoholic fatty liver, and chronic enteritis) (56–58). Studies likewise highlighted that the secondary metabolites of intestinal microorganisms, for instance, secondary bile acids, short-chain fatty acids, and branched-chain amino acids, play a substantial role in the process of medicine and diet treatment-induced remission (13, 59). These findings raised our focus on gut microbiota-derived metabolites. In this study, we identified several differential metabolites through metabonomics analysis, mainly sphingolipids, amino acids, amino acid derivatives, phospholipids, etc. Sphingolipids are a class of lipids with a long chain of amino alcohol sphingolipid backbones and amide-bound fatty acyl chains. As signaling molecules, sphingolipids mediated microbial-host interactions, such as immunity and inflammation. Sphingolipids have been identified as the most differential biomarker metabolites in the stool of inflammatory bowel disease patients (60). Known sphingolipid-producing bacteria include most of the Bacteroidetes phylum, a few members of the Chlorobi phylum, and a subset of α-Proteobacteria (*Sphingomonas*, *Acetobacter*, and *Neospora*). Sphingolipid metabolites are essential signaling molecules mediating host cell processes, such as ceramide, sphingosine, and sphingosine-1-phosphate (S1P) (61). Sphingosine and dehydrophytosphingosine are the precursors of S1P, which play critical immunological functions in intestinal tissue (62). Our study identified the increased sphingosine and dihydrosphingosine, and the upregulated abundance of sphingosine-producing bacteria *Sphingomonas* after *B. coagulans* intervention. Meanwhile, Sphingolipid metabolism-related pathways (Sphingolipid signaling pathway and Sphingolipid metabolism) were enriched. In addition, correlation analysis established a significant positive correlation between sphingosine, *Sphingomonas*, and immune-related genes (*Cu/Zn-SOD*, *IL-22*, and *PT-1*) and a significant negative correlation with inflammatory factor *Relish*. The above results indicated that the *Sphingomonas*–sphingosine axis is involved in the intestinal inflammatory repair and immune regulation of *M. rosenbergii* mediated by *B. coagulans*.

In terms of amino acids, studies have proved that intestinal microorganisms can synthesize, incorporate, and metabolize several available amino acids to maintain amino acid homeostasis through bidirectional switching with the host. In addition, the microbially derived metabolites mediate mucosal immunoregulation, epithelial cell barrier maintenance, and enteroendocrine regulation, such as serotonin, ammonia, hydrogen sulfide, branched-chain amino acids, polyamines, and phenolic and indolic compounds (63). In mammals, L-tryptophan, endogenous tryptophan metabolites, and bacterial tryptophan metabolites-indole derivatives could enhance intestinal barrier function by promoting the expression of tight junction protein of intestinal epithelial cells (63) and regulating the expression of inflammatory-related factors (such as anti-

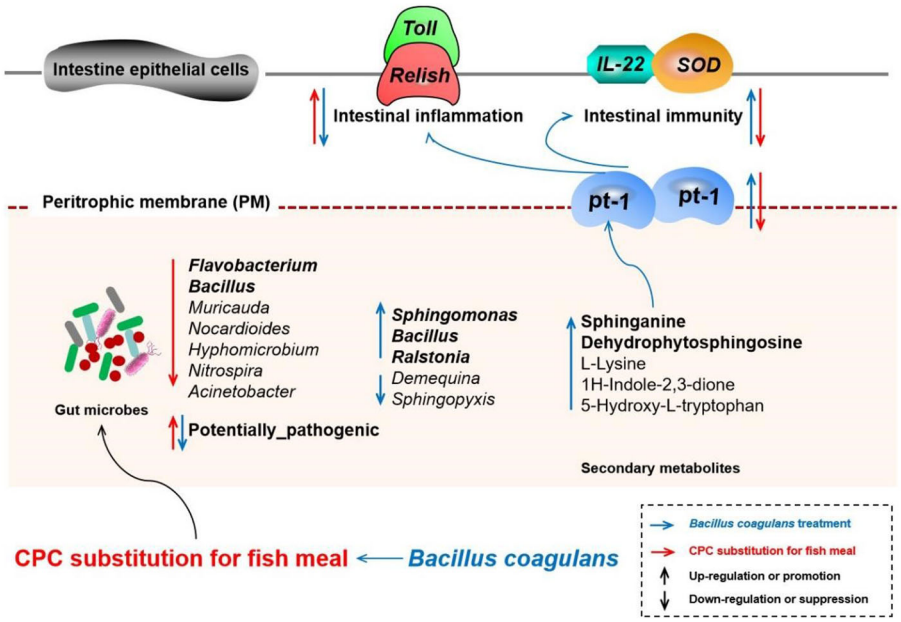

FIGURE 10
Schematic diagram depicting the effects and possible mechanisms of the effects of CPC substitution and *Bacillus coagulans* intervention on gut microbes, derived secondary metabolites, and intestinal physiological functions of *Macrobrachium rosenbergii*. The red arrow represents the action path of CPC substitution, and the blue arrow represents the action path of *Bacillus coagulans* intervention. The upward arrow represents upregulation or promotion, and the downward arrow represents downregulation or suppression. CPC, cottonseed protein concentrate.

inflammatory cytokine IL-22) (64). In addition, sufficient evidence suggested that lysine (65), glycine (66), arginine (67), etc., can shape the intestinal microbial composition and improve the host intestinal mucosal immunity. In the present study, we noted that *B. coagulans* treatment considerably affected the amino acid metabolic profile of the *M. rosenbergii* gut microbes, such as L-Lysine, Lysyl-Lysine, Epsilon-(gamma-glutamyl)-lysine, Tryptophan, 5-Hydroxy-L-tryptophan, 1*H*-Indole-2,3-dione, Lysyl-Arginine, Aspartyl-Arginine, and Leucyl-Glycine. Moreover, the correlation analysis also established a significant positive correlation between the above amino acid metabolites and the intestinal factors PT-1 and Cu/Zn-SOD. These results showed that changes in the amino acid metabolic spectrum of gut microorganisms induced by *B. coagulans* treatment are correlated with the intestinal immunity and barrier function of *M. rosenbergii*. However, to date, studies mainly focused on exogenous amino acid's contribution to hosting intestine immunity but rarely explored the effect and mechanism of bacterial amino acid intermediates themselves on the host physiology through the intestinal epithelial cells; their contribution degree and fate remain to be further explored. In addition, our study also established the correlation between other differential metabolites (glycitein, pipericine, isoquinoline, 7-hydroxy-2-phenyl-4*H*-chromen-4-one, PC(16:1(9*Z*)/P-18:1(11*Z*)), PC(15:0/15:0), PC(16:1(9*Z*)/14:0), LysoPC(16:0), SM(d18:1/16:0), SM(d18:0/18:1(9*Z*)), Vitamin D3, and *N*-acetyldopamine) and intestinal function genes (*Cu/Zn-SOD*, *IL-22*, *PT-1*, *Toll*, and *Relish*). Our results suggested that the effects of *B. coagulans* on gut microbe metabolism are multifaceted, and the positive regulation of intestinal immunity, barrier function, and inflammatory responses in *M. rosenbergii* depends on the combined action of multiple metabolites (Figure 10). However, the precise mechanisms require further research.

Data availability statement

The raw dataset for 16SrDNA sequencing have been deposited in the National Institutes of Health's Short Read Archive database (SRA accession no. PRJNA894136; BioSample accessions: SAMN31438493, SAMN31438494, SAMN31438495, SAMN31438496, SAMN31438497, SAMN31438498, SAMN31438499, SAMN31438500, SAMN3138501).

Author contributions

XZ contributed in the areas of experimental design, sampling, data analysis, and write-up. BL, YZ, QZ and XZ contributed to the experimental design and manuscript review. JY and NW contributed to feeding and cultivating experimental prawns, sampling, and statistics. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Project of National Key R&D Program of China (2019YFD0900200), China Agriculture Research System of MOF and MARA (CARS-48), Central Public-interest Scientific Institu-tion Basal Research Fund, CAFS (2020TD59).

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1074399/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 24 November 2022
DOI 10.3389/fimmu.2022.1074198

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Comparative Immunology, a section of the journal Frontiers in Immunology

RECEIVED 19 October 2022
ACCEPTED 11 November 2022
PUBLISHED 24 November 2022

CITATION
Yang G, Liang X, Hu J, Li C, Hu W, Li K, Chang X, Zhang Y, Zhang X, Shen Y and Meng X (2022) Feeding tea polysaccharides affects lipid metabolism, antioxidant capacity and immunity of common carp (*Cyprinus carpio* L.).
Front. Immunol. 13:1074198.
doi: 10.3389/fimmu.2022.1074198

Feeding tea polysaccharides affects lipid metabolism, antioxidant capacity and immunity of common carp (*Cyprinus carpio* L.)

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Tea polysaccharides plays a role in lipid metabolism, antioxidant capacity and immunity of mammals. To investigate the functions of tea polysaccharides on fish, the common carp (*Cyprinus carpio* L.) was selected as the animal model in this study. In our study, the common carp (45±0.71g) were randomly divided into four groups and were fed fodder with 50% carbohydrate. The common carp were orally administrated with 0 mg/kg BW (control group), 200 mg/kg BW (low-dose group), 400 mg/kg BW (medium-dose group) and 800 mg/kg BW (high-dose group) tea polysaccharide for two week. At the end of experiment, the serum glucose, TG, MDA contents and antioxidase activities were measured by commercial kits. The serum immune factors levels were tested by ELISA. The genes expression levels related to antioxidant capacity, metabolism and immunity were measured by real-time PCR. The results showed that the glucose, TG and MDA contents in serum were significantly decreased by tea polysaccharides treatment. The serum activities of SOD were significantly increased by low-dose tea polysaccharides treatment. The serum activities of GPX were significantly increased by medium-dose tea polysaccharides treatment. The serum levels of IL-1β and TNFα were significantly decreased in the tea polysaccharides treatment group. In the high-dose treatment group, the serum level of TGFβ was significantly increased, and the serum level of IL-12 was markedly decreased. In the hepatopancreas, the expression of acc1, fas, srebp1c, lpl, gys and pparγ were significantly reduced, and the expression of pygl, cat, mnsod, ho-1 and gr were significantly up-regulated in the tea polysaccharides group. In the intestine, the expression of zo-1, occ and gip was significantly up-regulated in the high-dose treatment group. Moreover, the expression of glut2 and sglt1 were significantly down regulated. In the spleen, the expression of il-12, tnfα and il-6 were significantly decreased, and the

10.3389/fimmu.2022.1074198

expression of il-10 and tgfβ was significantly increased by the tea polysaccharides. In the spleen cells, the tea polysaccharides could relieve the LPS-induced immune damage. In conclusion, tea polysaccharides can improve antioxidant capacity, lipid metabolism and immunity of common carp.

KEYWORDS

tea polysaccharide, metabolism, antioxidant, immunity, common carp

Introduction

Aquaculture provides an essential source of edible protein for humans. With the rapid development of aquaculture, the demand of fishmeal is increasing, which induces the escalating price of fishmeal (1). The study showed that dietary carbohydrate and lipid inclusion at optimal levels could promote protein utilization, prevent lipid oxidation, and save fishmeal protein in the aquatic animal feed (2). However, overloaded dietary carbohydrate induced low feed intake, poor growth performance (3), metabolism dysfunction, impairment of antioxidant capacity and sub-health status of fish (4, 5). Moreover, overloaded dietary carbohydrate will reduce the immunity, and increase the infection with disease of cultured fish (6). In addition, with the expansion of farming scale and environmental degradation, diseases in farming occur frequently, which causes a number of deaths of cultured fish (7). Although the antibiotic can relieve the morbidity of fish, antibiotic resistance has become a severe problem worldwide (8). The frequent occurrence of antibiotic-resistant bacteria in the aquaculture sites is due to the abuse of antibiotics in aquaculture (9). Therefore, the antibiotics were prohibited in aquaculture by the Chinese government because of the various negative effects after July 2020. For these reasons, it is an urgent problem that the new antibiotic substitute has been exploited for use in aquaculture. As the environmental friendly substance, plant extracts are low toxicity, safety, and minimal environmental impacts (10). In aquatic animals, plant extracts play important role in enhancing the immune function, and promote antibacterial, antiviral, antiparasitic activities of the immune system (10). In addition, plant extracts have been as immunostimulant to prevent diseases of aquatic animals in recent years (11).

As the important economic agricultural product, tea possesses multiple beneficial effects, including antioxidant capacity, reduction of cholesterol, protect against cardiovascular disease, anti-microbial, and anti-cancer (12–14). The beneficial effects of tea attribute to its variety of bioactive compounds, including polysaccharides, polyphenols, alkaloids, volatile oils, amino acids, etc (14–18). The tea polysaccharides attracted attention for its bioactivities, such as antioxidant, anti-cancer, anti-radiation, hypoglycemic activities and anti-HIV (14, 15, 19, 20). Tea polysaccharides were mostly heteropolysaccharides, in which a protein *via* N- or O- covalently linkages carries one or more carbohydrate chains attached to a polypeptide backbone (14, 15). The bioactivity study indicated that the tea polysaccharides suppressed the formation and accumulation of fat, and promoted its decomposition to prevent obesity of rats (*Rattus norvegicus*) (21). For example, a report of polysaccharides from green tea of *Huangshan Maofeng* (HMTP) showed that HMTP could protect against liver injury by CCl_4-induced, and inhibit lipid peroxidation and the increase antioxidant activity in mice (*Mus musculus*) (22). Furthermore, the tea polysaccharides could significantly reduce blood glucose levels, and increase the capacity of glucose tolerance in mice (23, 24).

Common carp is one of the most widely cultivated freshwater fish species all over the world, which is the fourth most cultured fish species in aquaculture (25). The production of common carp accounts for 7.7% of the total fish production in 2018 in the world (26). In addition, the production of common carp reaches 4,411,900 metric tons in 2019 (25). To meet the needs of human for fish, the intensive high-density and high nutrition farming model was rapidly developed. With the farming model and environmental degradation, diseases in fish farming occur frequently, which causes huge losses to the aquaculture industry. As a safe and environmental friendly plant extracts, tea polysaccharides have antioxidant capacity, anti-microbial actions and increases immunity in mammals. But the beneficial effects of tea polysaccharides in fish have never been reported. To assess the biological functions of tea polysaccharides on fish, the common carp was employed as a model in this study. The effects of tea polysaccharides on the immunity, metabolism and antioxidant capacity were evaluated in common carp in our study.

Materials and methods

Materials and chemicals

Tea polysaccharides was produced in meilunbio (Dalian, China). Glucose test kit was purchased from Rsbio (Shanghai, China). Triglyceride (TG) test kit was purchased from Dongou (Zhejiang, China). Superoxide dismutase (SOD), glutathione oxidase (GPX), total antioxidant capacity (T-AOC) and methane

10.3389/fimmu.2022.1074198

dicarboxylic aldehyde (MDA) test kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Animals experiment

The experiment process was referred to the previous studies (27, 28). The common carp with an initial body weight (45 ± 0.71g) were obtained from Yanjin Fishery (Yanjin, Henan). Approximately 120 healthy individuals were randomly divided into four groups (30 fish in each group). Before the experiment, fish were acclimated to indoor tanks (diameter: 52 cm, water high: 62cm) at room temperature with recirculating water under a cyclical light-dark photoperiod (12 h: 12 h) for two weeks. Then, the fish were fed with a high carbohydrate diet or the same diet with 200 mg/kg BW/day (low-dose group), 400 mg/kg BW/day (medium-dose group) and 800 mg/kg BW/day (high-dose group) tea polysaccharides by gavage for two weeks. The formulations and compositions of each diet were listed in Table 1. All ingredients were weighted individually before mixed thoroughly in a feed drum mixer for 30 min. Then dissolved water was added and mixed to form a loosely shaped dough. The mixture was transformed into pellets using a single screw extruder (Fishery Machinery and Instrument Research Institute, China Academy of Fishery Science, Shanghai, China); the pellets were then air-dried at room temperature and stored at − 20°C.

TABLE 1 Ingredients and proximate composition (% dry matter) of experimental diet.

Ingredients	Percent of total
Casein	33
Fish meal	10
Fish oil	2.5
soybean oil	1.5
Mineral premix[a]	1
Vitamin premix[b]	1
Sodium carboxymethylcellulose	0.5
Glucose	50
Choline chloride	0.5
Total	100
Proximate composition	
Crude protein (%)	34.75
Crude lipid (%)	4.28
Ash (%)	2.46

[a]Vitamin premix (mg or IU/kg diet): vitamin A, 6000 IU; vitamin D3, 2000 IU; vitamin E, 50 mg; ascorbic acid, 200 mg; pantothenic acid, 35 mg; nicotinic acid, 30 mg; thiamine, 15 mg; riboflavin, 15 mg; pyridoxine Hcl, 6 mg; cyanocobalamin, 0.03 mg; menadione, 5 mg; inositol, 200 mg; folic acid, 3 mg; biotin, 0.2 mg.
[b]Mineral premix (mg or g/kg diet): magnesium, 100 mg; iron, 150 mg; zinc, 80 mg; manganese, 20 mg; copper, 4 mg; iodine, 0.4 mg; cobalt, 0.1 mg; selenium, 0.1 mg.

Biochemical analysis

At the end of the experiment, all fish were anesthetized by MS222 (Sigma, USA), and the blood samples were collected from the caudal vein. After still standing at 4°C at least 30 min, the serum was isolated by centrifugation at 7500 g for 10 min. The serum was stored at −80°C for the detection of immune factors and biochemical analysis. And then, the fish were decapitated. The hepatopancreas, foregut and spleen samples were immediately collected, and snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction. Parts of the hepatopancreas tissue was removed for glycogen contents measure *via* the commercial kit (Jiancheng, China). All animal experiments were approved by the Animal Care Committee of Henan Normal University.

Serum samples analysis

In the serum, the content of glucose, TG and MDA of all groups was determined by commercial kits (Jiancheng, China). The enzyme activities of SOD, GPX and T-AOC were determined by commercial kits (Jiancheng, China). The experiments were performed according to the manufacturer's protocol. The levels of IL-1β, IL-6, IL-10, IL-12, tumor necrosis factor α (TNFα) and transforming growth factor β (TGFβ) were measured by ELISA assay referred to pervious study (28).

Common carp spleen cells isolation and treatment

The common carp spleen cells were isolated by collagenase IV/DNase II digestion method. The experimental method of isolation was referred to previous study (29). The isolated spleen cells were cultured in the 24 wells plate with 1 mL DMEM/F12 medium contained 10% fetal bovine serum (FBS) with the density of 1×10^6 cells/well. After overnight cultured, the cell medium was replaced to fresh DMEM/F12 without FBS. Before treatment, the cells were cultured for 1 h in the DMEM/F12 without FBS. Then, the cells were treated with LPS (25 μg/mL), tea polysaccharides (400 μg/mL), LPS (25 μg/mL) + tea polysaccharides (400 μg/mL) for 12 h. By the end of the study, the cells were lysed by RNAiso Plus for RNA extraction.

RNA extract, cDNA synthesis and real-time PCR

The total RNA of the hepatopancreas, gut and spleen were extracted by RNAiso Plus (Takara, Janpa). The total RNA concentration was measured by UV spectrophotometer (Nanodrop 2000, Thermo). 1 μg of total RNA was digested

with gDNA Eraser at 42°C for 2 min to eliminate genomic DNA. Then, the first-strand cDNA was synthesized using PrimeScript RT reagent kit (PrimeScript RT reagent kit with gDNA Eraser, Takara). The synthesized first-strand cDNA was used as template for real-time PCR and the primers were shown in Table 2.

The genes expression levels were evaluated by real-time PCR. Real-time PCR was performed using SYBR green qPCR mix (Bimake, China) on the LightCycler 480 II Sequence Detection System (Roche, Switzerland) according to the manufacturer's instructions. The real-time PCR reaction was in a total volume of 10 µl and the following conditions were used: 95°C for 5 min; and 40 cycles of 95°C for 15 s, 56°C for 15 s, and 72°C for 30 s. *18S* rRNA are used as the internal reference, and remained stable in various treatments throughout the study. The genes relative expression levels were normalized to *18S* rRNA. The results were calculated by the comparative Ct method (30).

Statistical analysis

All data are shown as mean ± standard error of the mean (S.E.M). Statistical analysis was performed with SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA followed by Fisher's Least Significance Difference (LSD) test was used to identify the significant difference. A probability value of $P < 0.05$ was considered significant.

Results

Serum content of glucose and triglyceride, the activity of antioxidant enzyme and glycogen content in hepatopancreas

In our present study, the result showed that the content of glucose and TG in serum was significantly decreased in the tea polysaccharides treatment groups compared to that of control group (Table 3). Moreover, the activity of SOD enzyme significantly increase by low-dose tea polysaccharides treatment (Table 3). The activity of GPX enzyme significantly increase by medium-dose tea polysaccharides treatment (Table 3). However, the contents of MDA were dramatically reduced in the tea polysaccharides groups compared to that of control group (Table 3). As shown in the Figure 1A, the contents of glycogen in hepatopancreas were markedly decreased in the high-dose group compared to that in the control group.

TABLE 2 Primers used in this study.

Gene	Accession no.	Forward (5'→3')	Reverse (5'→3')
occ1	KF975606	ATGTTGTCCTTCCCGTGATAAG	TCCGTAAGAACCTCCGTAAGA
zo-1	KY290394	AGGAAGTTCTCCCTCGTACTC	CCTCTGTTGTGGTTGAGTGTAG
sglt-1	JN867793.1	CTAAAGAAGAGGAGGCAGAGTTG	ACAGACGGTGAGGAGGATAATA
mnsod	XM_019111527.1	CGCACTTCAACCCTCAT	CATTGCCTCCTTTACCC
cat	JF411604.1	TTCCTGTGGGACGCCTTGT	TCCGAGCCGATGCCTATGT
gr	XM_019102099.1	TGGCTGGTATCCTTTCC	TGTCGTCAGGGTCTTTT
tnfα	XM_019088899.1	AGCCAGGTGTCTTTCCACAT	ATGTAGCCGCCATAGGAATCG
IL-6	XM_019073058.1	CATCTGGGGACGAGGTTCAG	AGGGTTTGAGGAGAGGGGTT
il-1β	AB010701.1	CAAACTGGAGCTGTCTTCGC	CTTCACCAGACGCTCTTCGAT
il-10	JX524550.1	TTGCTCATTTGTGGAGGGCT	TGTTGCACGTTTTCGTCCAG
il-12	AJ480354	TGCTTCTCTGTCTCTGTGATGGA	CACAGCTGCAGTCGTTCTTGA
tgfβ	AF136947.1	TGCCTGTTGGGATTTGTGC	AGCCGCCTGCTCTTCATTT
acc1	XM_019096370.1	TTCACTGGCGTATGAGGATATC	TCCACCTGTATGGTTCTTTGG
fas	GQ466045.1	GACAGGCCGCTATTGCTATT	TGCCGTAAGCTGAGGAAATC
srebp1c	KY763985	GACGCCGCTGAACAACTT	TCCTCGGGCTTCTCCACA
glut2	XM_019072653.1	GAGGGTCTTTGTGGGAACTATG	GTTTCAGGTACACGCAAGTAGA
pygl	XM_019125106.1	TGGTTGACGACGATGCTTTC	ACTGCGCAAACTTCAGCTTG
gip	GFWU01032553.1	AGTTTAGCCGCCGTTAC	TTTCTTCTCCCTCTGATTG
gys	XM_019090903.1	TTTTGGCCGCTGGTTGATTG	ATAGGGTAGTCCAATGCTGCAC
lpl	FJ716101.1	CGCTCCATTCACCTGTTCAT	GCTGAGACACATGCCCTTATT
pparγ	XM_019096045.1	TGCAAGGGATTCTTCCGCAG	AACGAATGGCGTTGTGTGAC
ho-1	JX257180.1	TCAGCCCATCTACTTCCCTCA	GGCAGGCACTGTTACTCTCT
18s	FJ710827.1	GAGACTCCGGCTTGCTAAAT	CAGACCTGTTATTGCTCCATCT
β-actin	M25013	CGTGACATCAAGGAGAAG	GAGTTGAAGGTGGTCTCAT

10.3389/fimmu.2022.1074198

The contents of immune factors in serum

In Table 4, the results showed that the contents of IL-1β and TNFα in serum were significantly reduced in the tea polysaccharides treatment groups compared to that of the control group. The content of IL-12 in serum was markedly decreased in the high-dose tea polysaccharides treatment group. However, the content of TGFβ in serum was dramatically elevated in the high-dose tea polysaccharides treatment group compared to that of the control group.

Effect of tea polysaccharides on expression of the genes related to metabolism in hepatopancreas

The results showed that the expression of fatty acid synthesis gene *acc1*, *fas* and *pparγ* in hepatopancreas was significantly inhibited in the tea polysaccharides treatment groups compared to that of control group (Figures 1B, C). The *srebp1c* expression level was markedly decreased by the low- and medium-dose tea polysaccharides treatment (Figure 1D). The *lpl* expression level was markedly decreased by the medium- and high-dose tea polysaccharides treatment (Figure 1F). Moreover, the *pygl* expression level was significantly increased in the high-dose tea polysaccharides treatment group (Figure 1G). However, the *gys* expression level was significantly decreased in the high-dose tea polysaccharides treatment group (Figure 1H).

Effect of tea polysaccharides on expression of the genes related to glucose intake and intestinal barrier in foregut

In the foregut, the expression of *sglt1* and *glut2* was dramatically inhibited by the tea polysaccharides treatment (Figures 2A, B). Moreover, the *gip* expression level was significantly promoted by the high-dose tea polysaccharides treatment (Figure 2C). In the foregut, the mRNA levels of *occ1* and *zo-1* were dramatically increased in the medium- and high-dose tea polysaccharides treatment groups (Figures 2D, E).

Effect of tea polysaccharides on expression of the genes related to antioxidant in hepatopancreas

In the Figure 3, the results showed that the *ho-1* expression level in hepatopancreas was significantly increased in the high-dose tea polysaccharides treatment group compared to that of control group (Figure 3A). In the low and medium-dose tea polysaccharides treatment groups, the expression of *gr* and *mnsod* were markedly promoted compared to that of control group (Figures 3B, C). In addition, the *cat* expression level was significantly up-regulated in hepatopancreas by the medium-dose tea polysaccharides treatment (Figure 3D).

Effect of tea polysaccharides on expression of the genes related to immunity in spleen

The effect of tea polysaccharides on expression of immune-related genes was detected in the spleen tissue of common carp. The results showed that the mRNA levels of *tnfα* and *il-12* were significantly inhibited in the medium- and high-dose tea polysaccharides treatment groups (Figures 4A, B). The *il-6* expression level was significantly decreased in the tea polysaccharides treatment groups (Figure 4C). Moreover, the expression of *il-10* was markedly promoted in the low- and medium-dose tea polysaccharides treatment groups (Figure 4E). The expression of anti-inflammatory factor *tgfβ* was significantly increased by the high-dose tea polysaccharides treatment (Figure 4F).

Effect of tea polysaccharides and LPS on expression of the genes related to immunity in common carp spleen cells

The genes expression of *tnfα*, *il-1β*, *il-6* and *il-12* were significantly increased in common carp spleen cells by

TABLE 3 Effects of tea polysaccharides on the plasma components of common carp.

	0mg/kg BW	200mg/kg BW	400mg/kg BW	800mg/kg BW
Glucose (mM)	2.81 ± 0.22^{a}	1.44 ± 0.20^{c}	1.96 ± 0.16^{bc}	2.15 ± 0.27^{b}
TG (mM)	3.13 ± 0.33^{a}	2.28 ± 0.18^{b}	2.10 ± 0.18^{b}	2.31 ± 0.29^{b}
SOD (U/mL)	57.52 ± 3.02^{b}	110.56 ± 5.37^{a}	80.53 ± 13.86^{b}	65.19 ± 9.26^{b}
T-AOC (U/mL)	4.07 ± 0.42	4.90 ± 0.44	4.17 ± 0.38	4.75 ± 0.45
MDA (nM)	10.72 ± 1.27^{a}	7.42 ± 0.66^{b}	7.61 ± 0.44^{b}	7.47 ± 0.34^{b}
GPX (U/mL)	342.74 ± 37.47^{b}	382.64 ± 34.15^{ab}	431.60 ± 18.98^{a}	324.34 ± 24.84^{b}

All data are shown as mean ± S.E.M. (n = 7-8). Significant differences (P<0.05) were indicated by different letters.

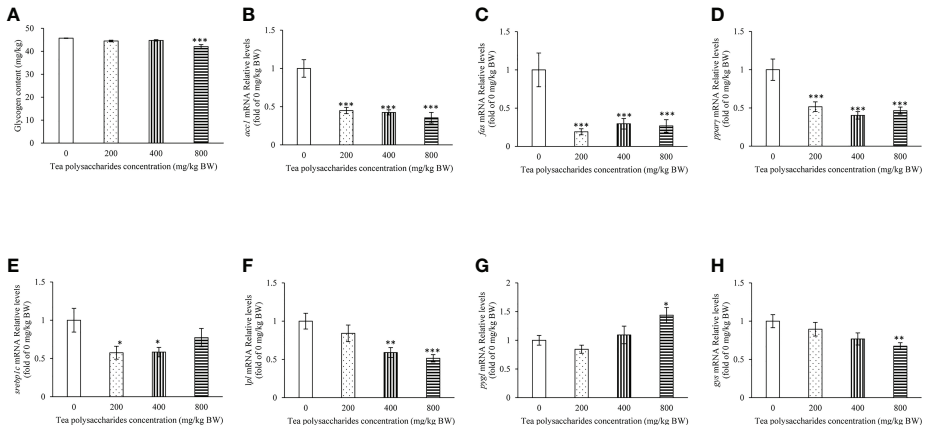

FIGURE 1
Effects of tea polysaccharides on hepatopancreas glycogen content and genes expression related to lipid metabolism in hepatopancreas. **(A)** Hepatopancreas glycogen content. At end of the experiment, fish were killed and the hepatopancreas was collected for glycogen content detected. **(B–H)** genes expression related to lipid metabolism in hepatopancreas. **(B)** *acc1*; **(C)** *fas*; **(D)** *pparγ*; **(E)** *srebp1c*; **(F)** *lpl*; **(G)** *pygl*; **(H)** *gys*. At end of the experiment, fish were killed and the hepatopancreas was collected for RNA extraction and real-time PCR. All data are shown as mean ± S.E.M. (n = 10-12). Significant differences were indicated by asterisks, *,$P < 0.05$; **,$P < 0.01$; ***,$P < 0.001$.

treatment with LPS compared to those in control group (Figures 5A–D). By tea polysaccharides treatment, the mRNA levels of *tnfα*, *il-1β*, *il-6* and *il-12* were inhibited in spleen cells. Moreover, the promoted mRNA levels of *tnfα*, *il-1β*, *il-6* and *il-12* in spleen cells were alleviated in the LPS and tea polysaccharides group (Figures 5A–D). The *il-10* and *tgfβ* expression were markedly decreased in common carp spleen cells by treatment with LPS (Figures 5E, F). The *il-10* expression was significantly increased by treatment with tea polysaccharides (Figure 5E). Furthermore, the inhibited mRNA levels of *il-10* and *tgfβ* were alleviated in the LPS and tea polysaccharides treatment group (Figures 5E, F).

Discussion

As a group of heteropolysaccharides extracted from tea, tea polysaccharides reveals multiple beneficial bioactivity in previous studies (14, 15). In our study, the serum glucose levels were significantly decreased in the tea polysaccharides treatment groups. The result was similar to that in previous studies. In mice, the blood glucose level was significantly decreased after injection with tea polysaccharides (31). Furthermore, the blood glucose content of alloxan-induced diabetic mice was markedly reduced after four-week oral administration of puerh tea polysaccharides (PTPS) (32), and was suppressed increase after six days oral administration of green tea polysaccharides (GTPS) (33). In addition, the serum glucose levels were decreased by daily oral administration of tea polysaccharides in diabetic and non-diabetic mice (23, 34, 35). These results indicate tea polysaccharides can reduce blood glucose level in mammals and fish. Previous study suggested that the reduction in intestinal glucose transport by tea polysaccharides was mainly mediated by the biochemical inhibition of transport activity (36). In our study, the results also showed that the tea polysaccharides can decrease the *sglt1* and *gult2* genes expression in the foregut. We speculate that tea polysaccharides reduce the serum glucose by lowing the glucose transport in gut of common carp.

The antioxidant activity of tea polysaccharides was reported in previous studies (14, 15). In our study, the enzyme activities of

TABLE 4 Effects of tea polysaccharides on the cytokine in plasma of common carp.

	0mg/kg BW	200mg/kg BW	400mg/kg BW	800mg/kg BW
IL-1β (pg/mL)	1014.4 ± 29.0^{a}	865.3 ± 41.8^{b}	827.0 ± 60.9^{b}	783.8 ± 22.2^{b}
IL-6 (pg/mL)	41.3 ± 2.3	41.5 ± 2.4	46.3 ± 2.8	43.5 ± 4.6
IL-10 (pg/mL)	25.6 ± 1.8	26.9 ± 1.2	27.5 ± 1.1	26.9 ± 0.9
IL-12 (pg/mL)	183.7 ± 3.9^{a}	174.6 ± 7.6^{ab}	169.1 ± 6.8^{ab}	156.4 ± 5.1^{b}
TGFβ (pg/mL)	43.4 ± 1.9^{b}	47.8 ± 1.7^{b}	47.8 ± 0.9^{b}	54.4 ± 1.1^{a}
TNFα (pg/mL)	51.0 ± 2.1^{a}	44.3 ± 1.7^{b}	43.3 ± 1.8^{b}	41.9 ± 1.8^{b}

All data are shown as mean ± S.E.M. (n = 7-8). Significant differences (P<0.05) were indicated by different letters.

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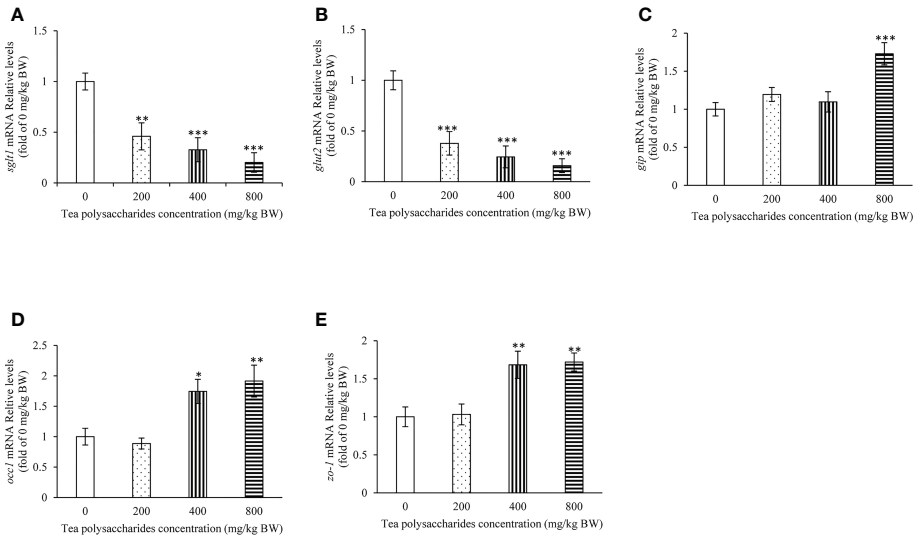

FIGURE 2
Effects of tea polysaccharides on genes expression related to glucose intake and gut barrier in foregut. **(A)** *sglt1*; **(B)** *glut2*; **(C)** *gip*; **(D)** *occ1*; **(E)** *zo-1*. At end of the experiment, fish were killed and the tissue was collected for RNA extraction and real-time PCR. All data are shown as mean ± S.E.M. (n = 10-12). Significant differences were indicated by asterisks, *,$P < 0.05$; **,$P < 0.01$; ***,$P < 0.001$.

serum SOD and GPX were promoted in the tea polysaccharides groups. However, the contents of MDA were significantly reduced in the tea polysaccharides groups. A report showed that the serum level of T-SOD was significantly promoted in rats, and the elevated serum content of MDA was attenuated in the green tea polysaccharides treated group (37). In addition, pretreatment with Keemun black tea polysaccharides (KBTP), the hepatic T-SOD and GSH levels were reduced, and the MDA content was decreased in CCl_4-intoxicated mice (38). Moreover, the study showed that the contents of MDA were reduced, and the SOD, catalase and GPX activities were increased in the plasma, liver and heart of mice after treatment with crude tea polysaccharides for 30 days (39). Furthermore, compared to MC (HFD without additional treatment) group, the content of MDA was significantly decreased and the GPX and CAT activities were increased in the Chinese Liupao tea polysaccharides (CLTPS) treatment groups (40). It is indicated that the antioxidant activity of tea polysaccharides can implement in the carbohydrate-, lipid-, toxicant-induced or normal physiological status. Furthermore, tea polysaccharides can increase the genes expression related to antioxidant capacity (*ho-1*, *gr*, *cat* and *mnsod*) in the liver of common carp in our study. Based on the above results, we speculate that tea polysaccharides increase the antioxidant capacity by lowing the levels of MDA and increasing activities and gene expression of antioxidase of common carp.

It is a crucial activity that tea polysaccharides promote immunity (14, 15, 36, 41). Tea polysaccharides activate immune cells to secrete various biological reaction mediators, such as cytokines, free radicals, and lyases (36). In the present study, the contents of IL-1β, TNFα and IL-12 were significantly decreased, and the level of TGFβ was markedly promoted in the serum of common carp after tea polysaccharides treatment. Furthermore, the contents of IL-2, IL-6, IL-10 and IFN-γ were significantly reduced in the colitis-associated cancer (CAC) mice by treatment with tea polysaccharides, and the levels of IL-10 were markedly increased in tea polysaccharides groups compared to that in Azoxymethane/Dextran sulfate sodium (AOM/DSS) group (42). Moreover, the serum IL-6 and TNFα levels were decreased, and the serum IL-2, IL-4 and IL-10 levels were increased in gastric cancer mice after tea polysaccharides treatment (43). In addition, the study of Yuan and his colleagues showed that IL-6 and TNFα levels were significantly decreased in mouse splenocytes treated with tea polysaccharides compared with that in native control (NC) group (44). Based on those results, it is indicated that improving the activity of antioxidant enzymes of tea polysaccharides was not only in mammals but also in the fish. In addition, the expression of *il-1β*, *tnfα*, *il-6* and *il-12* were significantly inhibited, and the expression of *il-10* and *tgfβ* were dramatically increased in the spleen of common carp in the tea polysaccharides groups. In the common carp spleen cells, the tea polysaccharides could relieved LPS-induced immune related genes expression. The previous study also showed that the expression levels of *il-6* and *tnfα* were reduced in white adipose tissue of rats in the tea polysaccharides treatment groups (37). Based on the above study, it is manifested that tea polysaccharides plays immunocompetence by regulating the activity of antioxidant enzymes and expression of immune factors.

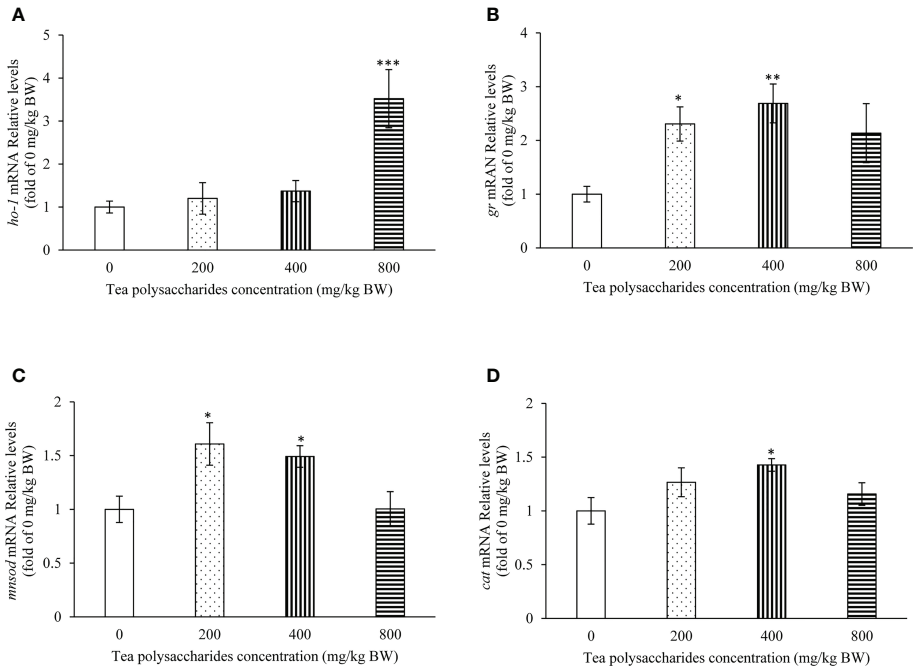

FIGURE 3
Effects of tea polysaccharides on genes expression related to antioxidant capacity in hepatopancreas. **(A)** *ho-1*; **(B)** *gr*; **(C)** *mnsod*; **(D)** *cat*. At end of the experiment, fish were killed and the hepatopancreas was collected for RNA extraction and real-time PCR. All data are shown as mean ± S.E.M. (n = 10-12). Significant differences were indicated by asterisks, *,$P < 0.05$; **,$P < 0.01$; ***,$P < 0.001$.

Tea polysaccharides also have a fat-lowing effect (36). In our study, the serum TG contents were also significantly reduced in the tea polysaccharides groups. The activity of reducing TG levels of tea polysaccharides was reported in previous studies. For instance, the black tea polysaccharides significantly reduced the TG content in serum and liver of rats compared to that in model control (Wu et al., 2016). The HMTP can decrease the CCl_4-elevated level of serum TG (22), and administration of GTPS or black tea polysaccharides (BTPS) in mice before the CCl_4 injection can resist the CCl_4-induced increases in the level

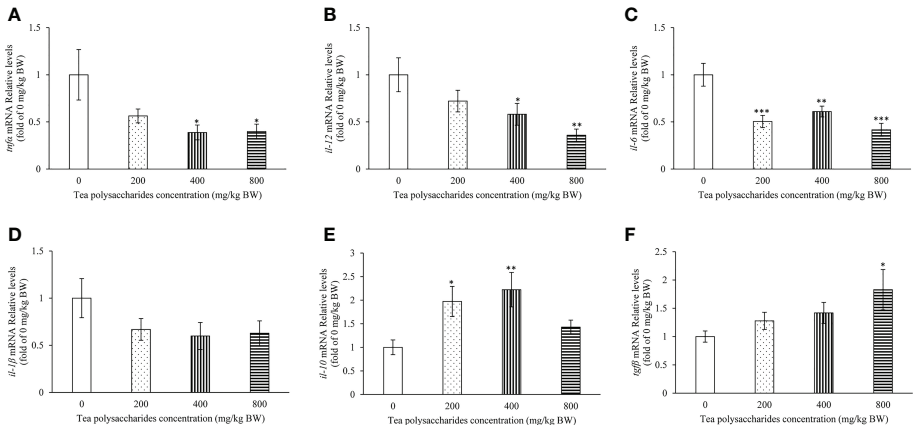

FIGURE 4
Effects of tea polysaccharides on genes expression related to immunity in spleen. **(A)** *tnfα*; **(B)** *il-12*; **(C)** *il-6*; **(D)** *il-1β*; **(E)** *il-10*; **(F)** *tgfβ*. At end of the experiment, fish were killed and the hepatopancreas was collected for RNA extraction and real-time PCR. All data are shown as mean ± S.E.M. (n = 10-12). Significant differences were indicated by asterisks, *,$P < 0.05$; **,$P < 0.01$; ***,$P < 0.001$.

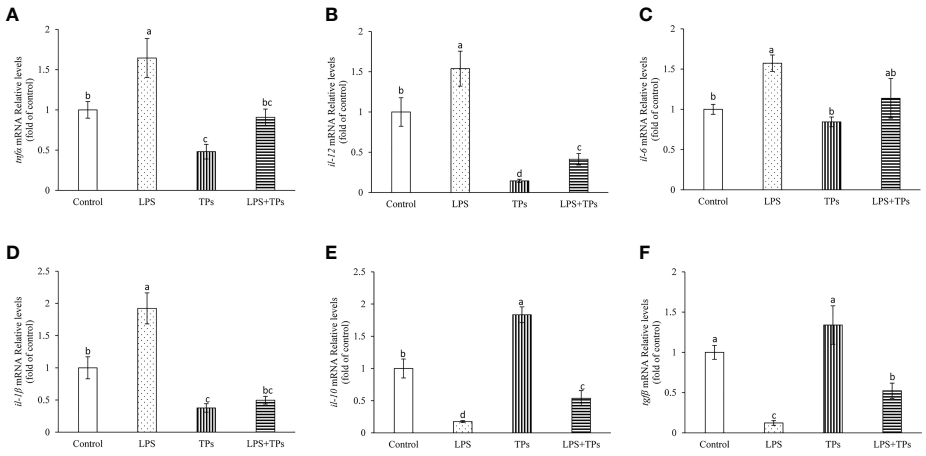

FIGURE 5
Effects of LPS and tea polysaccharides on genes expression related to immunity in common carp spleen cells. **(A)** *tnfα*; **(B)** *il-12*; **(C)** *il-6*; **(D)** *il-1β*; **(E)** *il-10*; **(F)** *tgfβ*. The cells were seeded in 24-well plates at 1×10^6 per well in 1mL DMEM/F12 with 10% FBS. The next day, cells were placed in DMEM/F12 without FBS for 1 h. Then, the cells were treated with LPS (25 μg/mL), tea polysaccharides (400 μg/mL), LPS (25 μg/mL) + tea polysaccharides (400 μg/mL) for 12 h. All data are shown as mean ± S.E.M. (n = 5-6). Significant differences ($P<0.05$) were indicated by different letters.

of TG (15). In addition, the contents of serum TG were markedly reduced in mice after Ilex Kuding tea polysaccharides (IKTP) treated with 200, 400 and 800 mg/kg BW (45). Those results indicate that the activity of reducing TG of tea polysaccharides is ubiquitous. In addition, the expression of *lpl*, *acc1*, *fas*, *srebp1c* and *pparγ*were significantly decreased in the tea polysaccharides treatment groups. Our results were similar to that in previous study, in which the tea polysaccharides effected the gene expression related to fat metabolic pathways of rats, and suppressed the accumulation and formation of fat and promoted lipolysis to prevent obesity (Wu et al., 2016). Moreover, a study reported that green tea polyphenols prevent HFD-induced obesity by increasing adiponectin levels, and alleviation of PPARγ phosphorylation (46). Another study reported that tea-supplemented reduced body fat mass of rats and down-regulated the expression of *pparγ*, *c/ebpβ* and *lpl* (47). Furthermore, the results revealed that the lipogenic-related genes expression was affected by kuding tea treatment, and that the expression of *pparγ* and lipogenic genes were inhibited in the liver of mice (48). From above results, it is indicate that the fat-lowing activity of tea polysaccharides is exerted by regulating the expression of lipid metabolism related genes in mammals and fish.

In the present study, the expression of *occ* and *zo-1* were significantly increased in the foregut of common carp in the tea polysaccharides groups. The expression of *gip* was increased, and *sglt1* and *glut2* expression were decreased in the foregut in the tea polysaccharides groups. SGLT1 and GLUT2 are the important glucose transporter in the intestines. In a previous study, the intestinal glucose transport was significantly decreased by treatment with green tea extract (GTE), water soluble polysaccharides derived from green tea (WSP), and GTE +WSP. The expression of *sglt1* was markedly decreased in the Caco-2 cells by treatment with wheat starch + GTE + WSP (49). Moreover, the protein expression of PI3Kp85, p-Akt and GLUT4 in diabetic mice were increased in the liver by orally gavage with tea polysaccharides, and the serum glucose level was accompanied decreased (34). In addition, the expression of *gys* was inhibited and the hepatopancreas glycogen content was decreased in the tea polysaccharides treatment group. The expression of *pygl* was significantly increased in the tea polysaccharides treatment group. The decreased hepatopancreas glycogen content was caused by the decreased glycogen synthesis (*gys*) and increased glycogenolysis (*pygl*) in hepatopancreas and decreased glucose absorption (*sglt1* and *glut2*) in foregut. It is indicated that the absorption of glucose and glycogenesis were affected by tea polysaccharides *via* regulating the related-genes expression. Intestinal barrier function is an important aspect for intestinal health. The study of cyclophosphamide (Cy)-induced BALB/c mice showed that the colonic TLR4/MyD88/NF-κB p65 and JAK2/STAT3 pathway was activated by pectic heteropolysaccharides and the expression of *claudin1*, *claudin5* and *occludin1* were significantly increased (50). In addition, the expression levels of *claudin1* and *claudin5* were significantly promoted in the colonic tissues of mice in the polysaccharides from the tea flower (TFPS) treatment group (51). Those results indicated that the tea polysaccharides play beneficial role in intestinal health by regulating intestinal barrier related genes expression. The regulatory mechanism underlying the impact of tea polysaccharides on intestinal barrier related genes expression of fish should be investigated in future studies.

Conclusion

In conclusion, our present results suggested that tea polysaccharides promoted immunity, antioxidant capacity and intestinal barrier function and reduced lipogenesis and glucose transporter of common carp. The results of this study will provide a theoretical foundation of tea polysaccharides application in aquaculture.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Ethics statement

The animal study was reviewed and approved by Animal Care and Use Ethics Committee of the Henan Normal University.

Author contributions

GY, XL, JH: conceived and performed the experiments, formal analysis, writing, and original draft. XM, GY: Reviewing and editing. WH, KL, XC, YZ: Supervision, reviewing, and editing. CL, YS, XZ: analyzed the data. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the National Natural Science Foundation of China (U1904118, 32273149), the Natural Science Foundation of Henan Province (212300410174).

Conflict of interest

Authors WH and KL were employed by Henan JinBaiHe Biotechnology 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.

Publisher's note

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10.3389/fimmu.2022.1074198

Glossary

TG	triglyceride
MDA	malondialdehyde
SOD	superoxide dismutase
GPX	glutathione peroxidase
ACC1	acetyl-CoA carboxylase 1
FAS	fatty acid synthase
SREBP1c	sterol regulatory element binding protein 1c
LPL	lipoprotein lipase
PPARγ	peroxisome proliferator activated receptor γ
PYGL	glycogen phosphorylase
CAT	catalase
MnSOD	manganese superoxide dismutase
HO-1	heme oxygenase-1
GR	glutathione reductase
OCC	occludin
GLUT2	glucose transporter 2
GYS	glycogen synthase
SGLT1	sodium/glucose cotransporter 1
HMTP	Huangshan Maofeng
GIP	glucose-dependent insulinotropicploypeptide
PTPS	puerh tea polysaccharides
GTPS	green tea polysaccharides
BTPS	black tea polysaccharides
KBTP	Keemun black tea polysaccharides
CLTPS	Chinese Liupao tea polysaccharides
CAC	colitis-associated cancer
AOM/DSS	Azoxymethane/Dextran sulfate sodium
NC	native control
GTE	green tea extract
WSP	water soluble polysaccharides derived from green tea
TFPS	polysaccharides from tea flower
IKTP	Ilex Kuding tea polysaccharides
TPs	tea polysaccharides.

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 14 December 2022
DOI 10.3389/fimmu.2022.1086103

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Comparative Immunology, a section of the journal Frontiers in Immunology

RECEIVED 01 November 2022
ACCEPTED 28 November 2022
PUBLISHED 14 December 2022

CITATION
Zhang Y, Guo M, Li N, Dong Z, Cai L, Wu B, Xie J, Liu L, Ren L and Shi B (2022) New insights into β-glucan-enhanced immunity in largemouth bass *Micropterus salmoides* by transcriptome and intestinal microbial composition. *Front. Immunol.* 13:1086103. doi: 10.3389/fimmu.2022.1086103

New insights into β-glucan-enhanced immunity in largemouth bass *Micropterus salmoides* by transcriptome and intestinal microbial composition

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β-glucan is widely used in aquaculture due to its immunostimulatory effects, but the specific effect and potential regulatory mechanism on largemouth bass (*Micropterus salmoides*) are still unclear. Here, we evaluated the effects of β-glucan on growth, resistance to *Aeromonas schubertii*, intestinal health, and transcriptome of largemouth bass to reveal the potential regulators, metabolic pathways, and altered differential microbiota. Four experimental diets were designed with β-glucan supplementation levels of 0 (control), 100 (LA-100), 200 (MA-200), and 300 (HA-300) mg kg^{-1}, and each diet was fed to largemouth bass (79.30 ± 0.50 g) in triplicate for 70 days, followed by a 3-day challenge experiment. Results showed that different β-glucan supplementations had no significant effects on growth performance and whole-body composition. Fish fed a diet with 300 mg kg^{-1} β-glucan significantly increased the activity of lysozyme than those fed diets with 0 and 100 mg kg^{-1} β-glucan. In addition, the survival rate of largemouth bass in β-glucan supplementation groups was significantly higher than the control group at 12- and 24-h challenge by *Aeromonas schubertii*. Transcriptome analysis showed that a total of 1,245 genes were differentially expressed [$|\log_2(\text{fold change})| \geq 1$, q-value ≤ 0.05], including 109 immune-related differentially expressed genes (DEGs). Further analysis revealed that significantly upregulated and downregulated DEGs associated with immunity were mapped into 12 and 24 pathways, respectively. Results of intestinal microflora indicated that fish fed a diet with 300 mg kg^{-1} β-glucan had higher bacterial richness and diversity as evaluated by Sobs, Chao, Ace, and Simpson indices, but no significant differences were found in the comparison groups. Furthermore, 300 mg kg^{-1} β-glucan significantly increased the relative abundance of *Mycoplasma* and decreased

Proteobacteria (mainly *Escherichia-Shigella* and *Escherichia coli*) and *Bacillus anthracis* in largemouth bass intestinal microflora. The findings of this study provided new insights that will be valuable in future studies to elucidate the mechanism of immunity enhancement by β-glucan.

KEYWORDS

largemouth bass, β-Glucan, growth, immunity, *Aeromonas schubertii*

1 Introduction

With the development and optimization of compound feed, the farming production and scale of the largemouth bass (*Micropterus salmoides*) have been expanding and now it has become one of the fastest growing cultured fish species in Chinese freshwater aquaculture (1). However, overcrowding and poor water quality due to intensive farming increased the susceptibility of fish to infection or disease (2). *Nocardia seriolae*, *Edwardsiella piscicida*, and *Aeromonas hydrophila* are the serious pathogens causing disease and death of largemouth bass (3). To alleviate disease problems, antibiotics and some drugs have been used in aquaculture, while the overuse of antibiotics will produce antibiotic-resistant bacteria and the residue and accumulation of drug will cause food safety hazards (4). Hence, eco-friendly disease prevention measures need to be found to alleviate the occurrence of disease and promote sustainable culture of fish. A promising alternative to improve the immunity of fish is supplementation with functional feed additives. Immunostimulants are effective additives that activate nonspecific immunity to improve the immune system of organisms. Numerous studies have proposed that delivery of immunostimulants as a dietary supplement in feed can improve immunity of multiple fish species (5). Thus, supplementing immunostimulants in feed is one of the effective ways to alleviate disease problems.

β-glucan has received heightened attention by feed manufacturers as a natural, safe, and economical immunostimulant that can stimulate the immune response of aquatic animals. β-glucan is a polysaccharide extracted from the cell wall of cereals, algae, yeast, or bacteria. Different sources of β-glucan have different structures and thus express different biological activities (6). Currently, most commercially available β-glucans are derived from yeast or cereal, but they are partially water-soluble or insoluble. With the development of extraction technology, microalgae have been considered as a potential source of β-glucans and can produce various β-glucans with different structures and solubilities. However, limited research has been conducted on algae-derived β-glucan in fish.

The immunostimulatory effects of β-glucan have been reported in different fish species including rohu (*Labeo rohita*), rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar* L.), red sea bream (*Pagrus major*), koi (*Cyprinus carpio koi*), mirror carp (*Cyprinus carpio* L.), and crustacean (7–13), but it has not been evaluated on largemouth bass. β-glucan can interact with the immune system to enhance the resistance of fish to pathogens. Several studies have reported that β-glucan induced increased resistance of fish to several bacterial pathogens by increasing the levels of complement and lysozyme (LZM), enhancing phagocytic and bactericidal activities of phagocytes (6, 14). In addition, studies reported that β-glucan plays an important role in improving the intestinal environment by promoting beneficial microorganisms, acidifying the intestinal tract, and reducing harmful metabolites in the intestine (6, 13). Obviously, β-glucan has multifaceted regulatory effects on the immune system. Therefore, the overall aim of this study was to investigate the effects of prolonged application of algae-derived β-glucan on growth, immunity, and resistance to *A. schubertii* in largemouth bass and to reveal the potential mechanism by which β-glucan modulates the immune system using Illumina MiSeq 16S rRNA gene and transcriptome sequencing technology.

2 Materials and methods

2.1 Experimental diets

Four isonitrogenous (~530 g kg^{-1}) and isoenergetic (~22 MJ kg^{-1}) diets were formulated to contain different levels of β-glucan (Table S1). A basal diet was supplemented with 0 (control), 100 (LA-100), 200 (MA-200), and 300 (HA-300) mg kg^{-1} β-glucan (algae-derived β-glucan). The fish meal, poultry by-product meal, soybean meal, and soy protein concentrate were used as main protein sources, and fish oil and soybean oil were used as lipid sources. Experimental diets were processed in Buhler (Changzhou) Machinery Co., Ltd., and the feed processing technology was strictly in accordance with Buhler Aquatic

processing scheme. Briefly, the cribble of all ingredients was carried out in a horizontal hammer mill (AHZC-0655), then sent to the vertical shaft micronizer (AHFL-110) for superfine grinding. The premix and superfine grinding ingredients were weighed and mixed in a single shaft paddle mixer (AHML-1000). Before extrusion, the mixed ingredients were preconditioned by conditioner (BCCC-22) to be matured in a humid and hot environment, then extruded by a twin-screw extruder (BCCG-62). The pellets were sucked into the dryer (BDBDP2G0.5C) for dying until the moisture is around 8%. The oil was vacuum-sprayed at the Feed Technology Laboratory of the Sino-European Aquatic Nutrition and Feed Resources Institute, Zhejiang Ocean University (SEANUTR-ZJOU). The oil mixture (the mix of fish oil and soybean oil was 1:1) was preheated to 50°C, then vacuum-sprayed in a vertical vacuum coating machine (ZJB-100). The pellets were quiesce for 24 h and sieved, damaged pellets were removed, and the remaining pellets were stored at -20°C until use.

2.2 Fish feeding and experimental conditions

Juvenile largemouth bass (~5 g) were obtained from a local hatchery (Hongli Aquaculture Co., Huzhou, Zhejiang) and reared in 22 m^2 fiberglass breeding pool to acclimate the laboratory conditions with commercial feed (~520 g kg^{-1} protein, ~80 g kg^{-1} lipid). The 70-day feeding trial was conducted in SEANUTR-ZJOU. A total of 600 juveniles (79.30 ± 0.50 g) were randomly assigned to 12 cylindrical fiberglass tanks (1,000 L) in recirculated aquaculture system, and each diet was assigned to three replicates with 50 fish per tank. Daily management procedure of the 70-day feeding trial followed that of a previous study (15). Briefly, largemouth bass were manually fed three times per day at 8:00 a.m., 2:00 p.m., 8:00 p.m.; all uneaten pellets were immediately siphoned out and quantified by the method of Zhang et al. (16). Tentative daily biomass of 10% was determined based on the average feed intake over the past 3 days, with more feed given at the end of each meal if fish showed signs of feeding. Each tank was supplied with seawater at a flow rate of 4–5 L min^{-1}, and water quality parameters were measured daily including ammonia nitrogen content <0.25 mg L^{-1}, nitrite nitrogen <0.5 mg L^{-1}, pH 7.0–7.5, dissolved oxygen of 5.0 ± 0.3 mg L^{-1}, and temperature 26°CC–28°CC.

2.3 *Aeromonas schubertii* challenge experiment

A. schubertii was isolated from diseased largemouth bass and cultured at 28°CC for 24 h, centrifuged at 10,000 × g for 10 min at 4°CC, and resuspended in 1 × PBS. After the feeding experiment, 25 fish per tank were fed as before and recovered from weighing and sampling stress by 2-week acclimation. Then, 300 largemouth bass (~300 g) were intraperitoneally injected with 150 μl *A. schubertii* suspension (3 × 10^9 CFU ml^{-1}) (3), while 150 μl sterile saline solution (0.85%) was also injected as the blank control group. The survival rate of largemouth bass was recorded every 12 h (0, 12, 24, 48, and 72 h) without any diet. No mortality was found in the blank control group, suggesting that no fish died because of injection stress.

2.4 Sampling

At the termination of the feeding experiment, fish were fasted for 24 h and anesthetized with MS-222. All fish were counted and weighed individually to assess the growth index [weight gain rate (WGR), specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR)]. Morphologic index including condition factor (CF), gonadosomatic index (GSI), hepatosomatic index (HSI), and viscerosomatic index (VSI) were calculated by measuring the body length and weight of the whole body, liver, gonad, and viscus from five fish per tank. Five fish from each tank were collected to analyze the whole body composition. Blood samples were collected from a further five fish per tank and centrifuged at 3,000 × g for 10 min at 4°CC, frozen in liquid N_2, then kept at -80°CC until analysis of serum biochemical parameters. Liver was collected from five fish per tank and immediately immersed in RNA keeper (Vazyme, China), prestored at 4°C for 24 h, and then transferred to -80°C until transcriptome sequencing. The hindgut of five fish from each tank was removed aseptically, collected into sterile tubes, rapidly frozen in liquid N_2, and then kept in -80°CC for intestinal microflora analyses.

2.5 Proximate compositions and hematological parameters

Pretreatment of the whole-body sample was in reference to a previous study (15). Briefly, samples were pooled per tank and homogenized by a meat grinder, dried at 120°CC for 30 min, rehomogenized in the high-speed tissue homogenizer, then dried in 75°CC oven, and finely ground into powder before analysis. Dry matter (105°C to constant weight), crude protein (Kjeldahl N, Opsis KD-310, Sweden), crude lipid (HCl hydrolysis and ether extraction, Opsis SX110A and SX-360, Sweden), ash (550°C, Muffle furnace), and gross energy (Parr, 1271, USA) in diets and whole body were analyzed by the standard methods of the Association of Official Analytical Chemists (AOAC) (17).

Hematological parameters including superoxide dismutase (SOD), catalase (CAT), and LZM were determined by the commercial kits (Nanjing Jiancheng Bio Inst, Nanjing, China) and performed according to the manufacturer's instruction.

2.6 Transcriptional analysis

2.6.1 RNA extraction and library construction

The livers obtained from the control, LA-100, MA-200, and HA-300 groups were entrusted to BGI-Wuhan Technology Service Co., Ltd., for RNA extraction, quality control, library construction, and RNA sequencing. Total RNA was extracted from the liver using TRIzol Reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. Subsequently, the concentration and quality of RNA were assessed by ND 2000 (Thermo Fisher Scientific, USA) and Agilent 2100 bioanalyzer (Thermo Fisher Scientific, MA, USA). The cDNA fragments were amplified by PCR, and products were purified by Ampure XP Beads. Library quality was validated on the Agilent 2100 bioanalyzer. High-quality RNA samples were used for library preparation and performed on an Illumina HiSeq4000 sequencer according to the manufacturer's specifications (Illumina).

2.6.2 Data analysis

All raw reads (accession number: SRR21783676, SRR21783677, SRR21783678, SRR21783679, SRR21783680, SRR21783681) were filtered with SOAPnuke software; afterward, clean reads were stored in FASTQ format. The HISAT2 and Bowtie2 software were used to align clean reads to the reference genome (GCF_014851395.1_ASM1485139v1_NCBI) and coding gene set (18, 19). Gene expression levels for each sample were calculated using RSEM software and normalized into fragment per kilobase of transcript per million base pairs sequenced (FPKM) (19, 20). The functional annotation and classification of largemouth bass transcriptome were shown in Figure S2. Differentially expressed genes (DEGs) were screened between two comparison groups (control *vs*. HA-300) using the DEGSeq2, with $|\log_2(\text{fold change})| \geq 1$ and q-value ≤0.05 (21, 22). Gene Ontology (GO) and Kyoto Encyclopedia of Gene and Genomes (KEGG) enrichment analysis of annotated DEGs were performed by Phyper based on the hypergeometric test, with q-value ≤0.05 being considered as significantly enriched.

2.7 Intestinal microbial analysis

2.7.1 Intestinal DNA extraction, PCR amplification, and illumina miSeq sequencing

DNA was extracted from the hindgut of five largemouth bass at equal concentrations in each sample. The bacterial community DNA was performed according to the instructions of EZNA® soil DNA kit (Omega Bio-tek, Norcross, GA, USA). The concentration and quality of DNA were verified using ND 2000 and 1% agarose gel electrophoresis. Amplification of the 16S rRNA gene was performed with primer pairs (338F: 5'-ACTCCTACGGGAGGCAGCAG-3' and 806R: 5'-GGACTACHVGGGTWTCTAAT-3') by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). The PCR amplification was performed with a 20 μl reaction volume containing 4 μl of 5× TransStart FastPfu buffer, 0.8 μl (each) of forward and reverse primers (5 μM), 2.0 μl dNTPs (2.5 mM), 0.4 μl TransStart FastPfu DNA polymerase, 10 ng template DNA, and ddH_2O up to 20 μl. The PCR program was 95°C for 3 min, followed by 27 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and then 72°C for 10 min. PCR products were recovered using 2% agarose gel, purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), and quantified using Quantus™ Fluorometer (Promega, USA) for the recovered products. Library construction and sequencing were performed using NEXTflexTM Rapid DNA-Seq Kit (Bioo Scientific, USA) and Illumina's MiSeq PE300/NovaSeq PE250 platform (Shanghai Meiji Biomedical Technology Co., Ltd.). All raw data were deposited into the NCBI SRA database (accession number: SRR21783450, SRR21783451, SRR21783452, SRR21783453, SRR21783454, SRR21783455).

2.7.2 Bioinformatic analysis

The raw reads were demultiplexed, quality-filtered by FASTP and merged by FLASH (23). Operational taxonomic unit (OTU) clustering of sequences (based on 97% similarity) and removal of chimeras were performed using UPARSE software (24). The taxonomy of each OTU representative sequence was analyzed by RDP Classifier against the 16S rRNA database using confidence threshold of 0.7 (25). Taxonomic richness and diversity estimators including observed richness (Sobs), Chao1 estimator (Chao), ACE estimator (Ace), Shannon diversity index (Shannon), Simpson diversity index (Simpson), and Good's coverage (Coverage) were determined using the Mothur software. The relative abundance of taxa for each sample was generated into domain, kingdom, phylum, class, order, family, genus, and species levels. The Linear discriminant analysis Effect Size (LEfSe) was determined using the LEfSe software to reflect communities or species that produced significant differential effects, with linear discriminant analysis (LDA) score >2 and Wilcoxon rank-sum test ($P < 0.05$) being used for significant difference analysis.

2.8 Statistical analysis

Statistical analysis was conducted using the SPSS 20 software (IBM SPSS Statistics 20). Results are presented as means and pooled SEM of three replicates (n = 3). All data were checked for normality and homogeneity of variances and were normalized when appropriate. Results were analyzed by one-way ANOVA to investigate differences among treatments followed by Duncan's multiple range test, with $P < 0.05$ being considered as a significantly different level. The calculations were listed in the Supplementary Materials.

3 Results

3.1 Growth performance and body composition

As shown in Tables 1, 2, β-glucan supplementation did not result in a significant difference in growth performance (FI, WG, FCR, and SGR), morphologic index (HSI, VSI, GSI, and CF), body composition (moisture, protein, fat, ash, gross energy), protein retention efficiency, and energy retention efficiency in largemouth bass ($P > 0.05$).

3.2 Serum biochemical parameters

Effects of different levels of β-glucan supplementation on activities of enzymes related to immunity and oxidation resistance in largemouth bass serum are presented in Table 3.

TABLE 1 Growth performance and morphologic index of largemouth bass fed diets with different levels of β-glucan.

Items	Diet				*P*-value	PooledSEM[1]
	Control	LA-100	MA-200	HA-300		
FBW, g fish^{-1}	295	295	295	292	0.98	10.2
FI, g DM fish^{-1}	190	193	191	188	0.83	7.05
WGR, %	272	271	273	268	0.98	14.3
FCR, g FI (g WG)$^{-1}$	0.89	0.90	0.89	0.88	0.49	0.01
SGR, % d^{-1}	1.90	1.90	1.90	1.89	0.98	0.06
HSI, %	1.71	1.76	1.61	1.51	0.13	0.14
VSI, %	8.74	8.74	8.35	8.75	0.59	0.48
GSI, %	1.54	1.83	0.93	1.34	0.38	0.69
CF, g cm^{-3}	2.98	3.12	3.06	2.98	0.14	0.08

[1] Pooled standard error of means.
Values are means and pooled SEM (n = 3); different superscript letters indicate significant differences among treatments ($P < 0.05$).
CF, condition factor; FBW, final body weight; FCR, feed conversion ratio; FI, feed intake; GSI, gonadosomatic index; HSI, hepatosomatic index; SGR, specific growth rate; VSI, viscerosomatic index; WGR, weight gain rate.

TABLE 2 Whole body composition and nutrient retention efficiency of largemouth bass fed diets with different levels of β-glucan.

Items	Diet				*P*-value	PooledSEM
	Control	LA-100	MA-200	HA-300		
Moisture, g kg^{-1}	661	657	659	660	0.32	3.65
Crude protein, g kg^{-1}	176	177	177	178	0.18	1.26
Crude fat, g kg^{-1}	123	126	125	121	0.39	4.02
Ash, g kg^{-1}	36.2	36.1	36.5	35.9	0.89	1.12
Gross energy, MJ kg^{-1}	8.79	8.97	8.87	8.80	0.41	0.16
Protein retention efficiency, %	38.0	38.4	38.4	38.4	0.77	0.69
Energy retention efficiency, %	47.6	47.9	47.6	47.9	0.98	1.29

Values are means and pooled SEM (n = 3); different superscript letters indicate significant differences among treatments ($P < 0.05$).

TABLE 3 Serum biochemical parameters of largemouth bass fed diets with different levels of β-glucan.

Items	Diet				*P*-value	PooledSEM
	Control	LA-100	MA-200	HA-300		
SOD, U ml^{-1}	21.5	20.1	22.4	20.3	0.34	1.86
CAT, U ml^{-1}	3.22	2.72	2.61	2.93	0.88	1.08
LZM, μg ml^{-1}	83.3^{b}	74.3^{b}	92.2^{ab}	105^{a}	0.03	13.3

Values are means and pooled SEM (n = 3); different superscript letters indicate significant differences among treatments ($P < 0.05$).
CAT, catalase; LZM, lysozyme; SOD, superoxide dismutase.

10.3389/fimmu.2022.1086103

Fish fed a diet with 300 mg kg^{-1} β-glucan significantly had increased activity of LZM compared to those fed diets with 0 and 100 mg kg^{-1} β-glucan ($P < 0.05$), while no differences were found in activities of SOD and CAT in largemouth bass serum ($P > 0.05$).

3.3 Survival rate of largemouth bass after *Aeromonas schubertii* challenge

As shown in Figure 1, the survival rate of largemouth bass in β-glucan supplementation groups (LA-100, MA-200, and HA-300) was significantly higher than that of the control group at 12 and 24 h ($P < 0.05$). The highest survival rate of largemouth bass after *A. schubertii* challenge was found in fish fed a diet containing 300 mg kg^{-1} β-glucan than those fed diets with 0 and 100 mg kg^{-1} β-glucan at 36 h ($P < 0.05$). Notably, all fish fed a diet without β-glucan supplementation died after the 12-h challenge, but fish in the β-glucan supplementation groups survived after the 72-h challenge, suggesting that β-glucan could improve the resistance of largemouth basses to *A. schubertii*.

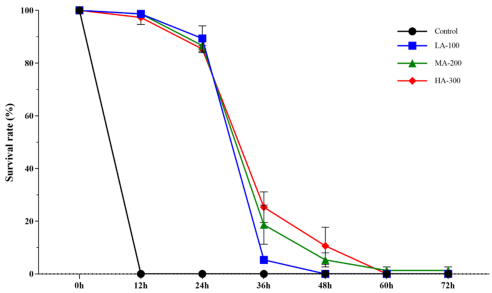
3.4 Transcriptional analysis of largemouth bass liver

3.4.1 Sequencing and mapping

As shown in Table 4, a total of six cDNA libraries including three control libraries (control-1, control-2, control-3) and three HA-300 libraries (HA-300-1, HA-300-2, HA-300-3) with 43.8 million raw reads were constructed. After filtration, the clean reads range from 42.8 to 43.0 million (clean read ratio is about 98%). The percentages of Q20 and Q30 were above 98.0% and 94.3%, indicating that the quality of all samples was qualified and could be used for subsequent data analysis. The sequence length of all unigenes is shown in Figure S1, and the length of most transcripts is longer than 3,000 nt.

3.4.2 Identification of differentially expressed genes (DEGs)

The transcriptome analysis was performed between comparison groups (control *vs.* HA-300) to identify DEGs [$|\log_2$(fold change)$| \geq 1$ and q-value ≤ 0.05] in response to different levels of β-glucan supplementation. Specifically, a total of 1,245 DEGs were obtained; fish fed a diet with 300 mg kg^{-1} β-glucan showed



time	Diets				*P*-value	Pooled S.E.M.
	Control	LA-100	MA-200	HA-300		
0 h, %	100	100	100	100	0	0
12 h, %	0^{b}	98.67^{a}	98.67^{a}	97.33^{a}	0.00	3.27
24 h, %	0^{b}	89.33^{a}	86.67^{a}	85.33^{a}	0.00	5.66
36 h, %	0^{c}	5.33^{bc}	18.67^{ab}	25.33^{a}	0.02	9.52
48 h, %	0	0	5.33	10.67	0.22	7.54
60 h, %	0	0	1.33	0	0.44	1.33
72 h, %	0	0	1.33	0	0.44	1.33

FIGURE 1
Survival rate of largemouth bass within 72 h after the challenge with *Aeromonas schubertii*. Values are means and pooled SEM (n = 3); different superscript letters indicate significant differences among treatments ($P < 0.05$).

TABLE 4 Summary of transcriptome sequencing and mapping for largemouth bass.

Group	Sample	Raw reads (10^6)	Clean reads (10^6)	Clean bases (Gb)	Q20 (%)	Q30 (%)	Clean reads ratio (%)	Total mapping (%)
Control	Control-1	43.8	43.0	6.45	98.2	94.8	98.1	95.9
	Control-2	43.8	42.9	6.43	98.1	94.4	97.8	95.3
	Control-3	43.8	43.0	6.45	98.1	94.4	98.1	95.7
HA-300	HA-300-1	43.8	42.8	6.43	98.1	94.6	97.8	95.1
	HA-300-2	43.8	43.0	6.45	98.0	94.3	98.1	95.4
	HA-300-3	43.8	43.0	6.46	98.1	94.5	98.2	95.4

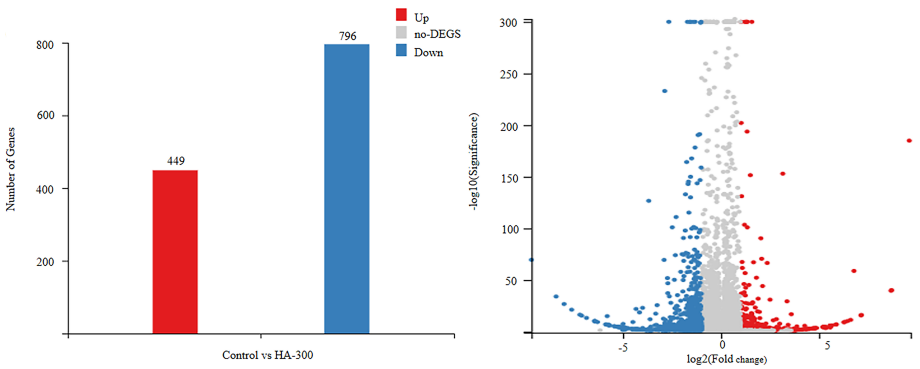


FIGURE 2
Differentially expressed genes (DEGs) [$|\log_2$(fold change)$| \geq 1$, q-value ≤ 0.05] in the liver transcriptome of largemouth bass fed diets with 0 and 300 mg kg^{-1} β-glucan. The blue dots and column represent significantly downregulated DEGs, and the red blue dots and column represent significantly upregulated DEGs, the gray signifies no DEGs.

449 significantly upregulated DEGs and 796 significantly downregulated DEGs compared with the control group (Figure 2).

3.4.3 GO annotations and KEGG classification of DEGs

As shown in Figure 3, DEGs were divided into three categories, including biological process (38.96%), cellular component (28.02%), and molecular function (33.02%). According to KEGG terms, all DEGs were classified into five categories, including organismal systems (27.77%), metabolism (23.95%), environmental information processing (19.68%), cellular processes (16.70%), and genetic information processing (11.90%). GO and KEGG classifications of all unigenes in the live transcriptome of largemouth bass are shown in Figure S2.

3.4.4 KEGG enrichment analysis of immune-related DEGs

To further investigate the effect of β-glucan on the immunity of largemouth bass, KEGG enrichment analysis was performed (q-value <0.05, Table 5). The significantly upregulated DEGs associated with immunity were mapped to 12 pathways, including chemokine signaling pathway, NOD-like receptor signaling pathway, complement and coagulation cascades, interleukin (IL)-17 signaling pathway, and NF-kappa B signaling pathway (top 5 pathways). Accordingly,

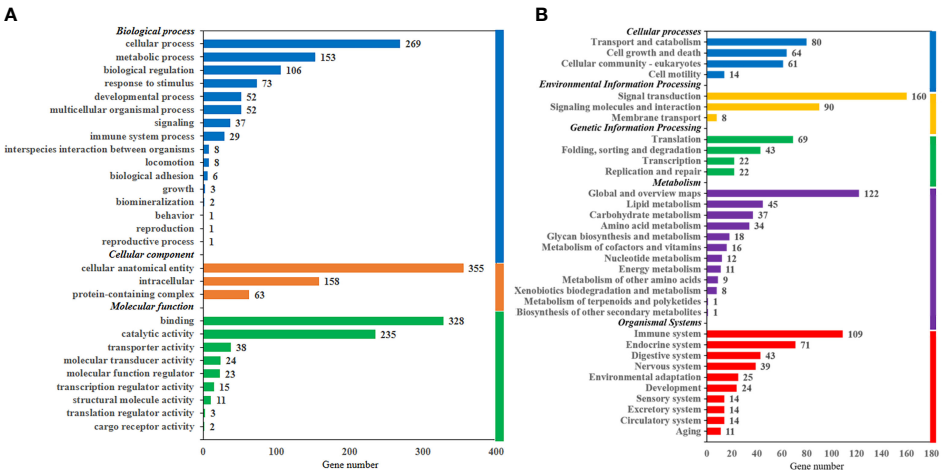


FIGURE 3
Gene Ontology annotations **(A)** and Kyoto Encyclopedia of Gene and Genomes classification **(B)** of differentially expressed genes in the liver transcriptome of largemouth bass.

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TABLE 5 The significantly enriched immune-related pathways and corresponding DEGs in the liver transcriptome of largemouth bass (*q*-value <0.05).

Pathway ID	Pathway name	*q*-value	Rich ratio	DEGs in the corresponding pathway[1]
Upregulated				
Ko04062	Chemokine signaling pathway	2.80e-5	0.03	*xcr1*, *pak1*, *cxcr1*, *ptk2b*-like, *il8*-like, *ccl7*, *ccl5*, permeability factor 2-like
Ko04621	NOD-like receptor signaling pathway	1.87e-4	0.02	*nlrc3, trmp2-like, il8, nlrp12, ccl7*, *vdac2*-like, *lrrc39*, permeability factor 2-like
Ko04610	Complement and coagulation cascades	4.01e-4	0.03	*cfh*-like, urokinase plasminogen activator surface receptor-like, *f10*-like, B2 bradykinin receptor-like
Ko04657	IL-17 signaling pathway	4.01e-4	0.03	*mmp18*-like, *il8*-like, *ccl7*, protein S100-B-like, *kiaa1522*
Ko04064	NF-kappa B signaling pathway	8.11e-4	0.03	*il8*-like, *trim110*, *ccl5*, permeability factor 2-like
Ko04670	Leukocyte transendothelial migration	9.79e-4	0.02	*ptk2b*-like, *cldn11a*
Ko04622	RIG-I-like receptor signaling pathway	1.22e-3	0.04	*cyld*-like, *il8*-like, *trim110*, permeability factor 2-like
Ko04060	Cytokine-cytokine receptor interaction	2.03e-3	0.02	chemokine XC receptor 1-like, *cxcr1*-like, *il8*-like, *ccl5*, permeability factor 2-like
Ko04620	Toll-like receptor signaling pathway	3.37e-3	0.03	*il8*-like, *map2k7*, *ccl5*, permeability factor 2-like
Ko04072	Phospholipase D signaling pathway	0.01	0.02	*cxcr1*-like, *ptk2b*-like, *kitb*, permeability factor 2-like
Ko04672	Intestinal immune network for IgA production	0.04	0.02	*mpz*-like, *icosl*-like, *clm1*
Ko04668	TNF signaling pathway	0.04	0.02	*ccl7*, protein jagged-1a-like, *map2k7*
Downregulated				
Ko04672	Intestinal immune network for IgA production	1.31e-8	0.06	*plgr*-like, nectin-4-like, *tnfrsf13b*-like, *vtcn1*-like, *il10*
Ko04623	Cytosolic DNA-sensing pathway	5.71e-8	0.09	*tnip1*-like, *il1β*, *rpac1*-like, *polr3d*, *polr3c*, *polr2h*
Ko04625	C-type lectin receptor signaling pathway	1.74e-5	0.04	*tnip1*-like, *septin2*-like, *il1β*, *egr2b*, proto-oncogene tyrosine-protein kinase Src-like, *lyg*-like, *rhes*-like, *il10*
Ko04621	NOD-like receptor signaling pathway	3.45e-4	0.02	*tnip1*-like, *septin2*-like, *syngr3a*, *il1β*, *lyg*-like, *syngr1*-like
Ko04064	NF-kappa B signaling pathway	7.35e-4	0.04	*tnip1*-like, *syngr3a*, *il1β*, *syngr1*-like
Ko04650	Natural killer cell mediated cytotoxicity	8.39e-4	0.03	*syngr1*-like, *rhes*-like, *tnfrsf10a*-like
Ko04662	B cell receptor signaling pathway	1.29e-3	0.03	*tnip1*-like, protein FAM110A-like, low-affinity immunoglobulin gamma Fc region receptor II-c-like, *rhes*-like
Ko04666	Fc gamma R-mediated phagocytosis	1.40e-3	0.03	low-affinity immunoglobulin gamma Fc region receptor II-c-like, *marcksl1b*, phospholipid phosphatase 1-like
Ko04640	Hematopoietic cell lineage	1.42e-3	0.03	*fam110a*-like, *il1β*, *tfr1b*
Ko04664	Fc epsilon RI signaling pathway	2.02e-3	0.04	*rhes*-like, *ncoa7*-like
Ko04620	Toll-like receptor signaling pathway	2.07e-3	0.03	*tnip1*-like, *syngr3a*, *il1β*, *lyg*-like, *syngr1*-like, *tlr9*
Ko03020	RNA polymerase	2.39e-3	0.04	*rpac1*-like, *polr3d*, *polr3c*, *polr2h*
Ko04062	Chemokine signaling pathway	2.39e-3	0.02	*tnip1*-like, *arrb1*, *arr3b*, *il8*, proto-oncogene tyrosine-protein kinase Src-like, *rhes*-like, *ttc27*-like
Ko04072	Phospholipase D signaling pathway	0.01	0.02	*rhes*-like, phospholipid phosphatase 1-like, *thada*
Ko04145	Phagosome	0.01	0.02	low-affinity immunoglobulin gamma Fc region receptor II-c-like, *tfr1b*, *ctsl.1*
Ko04010	MAPK signaling pathway	0.01	0.01	*arrb1*, *arr3b*, *syngr3a*, *il1β*, *syngr1*-like, *rhes*-like, *hspa1l*, *ttc27*-like
Ko04670	Leukocyte transendothelial migration	0.02	0.02	*myl7*, *cldn5*-like
Ko04668	TNF signaling pathway	0.02	0.02	*tnip1*-like, *syngr3a*, *il1β*, *lyg*-like, *syngr1*-like
Ko04217	Necroptosis	0.03	0.02	*septin2*-like, *il1β*, *lyg*-like, *tnfrsf10a*-like,
Ko04612	Antigen processing and presentation	0.03	0.03	protein disulfide-isomerase A3-like, *hspa4a*, *hspa1l*, *ctsl.1*
Ko04060	Cytokine-cytokine receptor interaction	0.03	0.02	*il1β*, *il8*, *tnfrsf13b*-like, *il12rb2l*, *il10*, *tnfrsf10a*-like
Ko04340	Hedgehog signaling pathway	0.03	0.04	*arr3b, arrb1*, *ttc27*-like
Ko04380	Osteoclast differentiation	0.03	0.02	*tnip1*-like, *syngr3a*, *il1β*, low-affinity immunoglobulin gamma Fc region receptor II-c-like, *syngr1*-like
Ko04610	Complement and coagulation cascades	0.03	0.02	*f13a*-like, *thada*, *at Ⅲ*-like

[1] Abbreviations for Table 5 were listed in Table S2 of the Supplementary Material.

significantly downregulated DEGs associated with immunity were mapped to 24 pathways, including intestinal immune network for IgA production, cytosolic DNA-sensing pathway, C-type lectin receptor signaling pathway, NOD-like receptor signaling pathway, and NF-kappa B signaling pathway (top 5 pathway). The 109 immune-related DEGs were summarized in Table S2.

3.5 Intestinal microbial analysis

3.5.1 Intestinal microflora structure at the phylum and class levels

As shown in Figure 4A, the dominant bacteria at the phylum level were *Fusobacteria*, *Firmicutes*, and *Proteobacteria*. Specifically, *Fusobacteria*, *Firmicutes*, and *Proteobacteria* were 59.29%, 22.30%, 13.03% in the control group and 48.92%, 41.00%, and 3.64% in the HA-300 group, respectively. The dominant bacteria of largemouth bass at the class level were *Fusobacteria*, *Bacilli*, and *Gammaproteobacteria* (Figure 4B). Specifically, *Fusobacteria*, *Bacilli*, and *Gammaproteobacteria* were 9.30%, 22.44%, and 12.18% in the control group and 48.92%, 40.92%, 2.64% in the HA-300 group, respectively. Heatmaps were used to further compare the relative abundance of intestinal microflora between the control and HA-300 groups at the phylum (Figure 4C) and class (Figure 4D) levels. *Fusobacteriota*, *Proteobacteria*, *Actinobacteriota*, *Bacteroidota*, and *Verrucomicrobiota* were more abundant in the control group, and *Firmicutes* and *Cyanobacteria* were more abundant in the HA-300 group at the phylum level. *Fusobacteriia*, *Gammaproteobacteria*, *Clostridia*, *Bacteroidia*, and *Verrucomicrobiae* were more abundant in the control group, and *Bacilli* was more abundant in the HA-300 group at the class level.

3.5.2 Alpha diversity analysis

Sobs, Ace, and Chao reflect community richness, and Shannon and Simpson represent community diversity, with Coverage being used to evaluate community coverage. No significant difference was found in the alpha diversity analysis

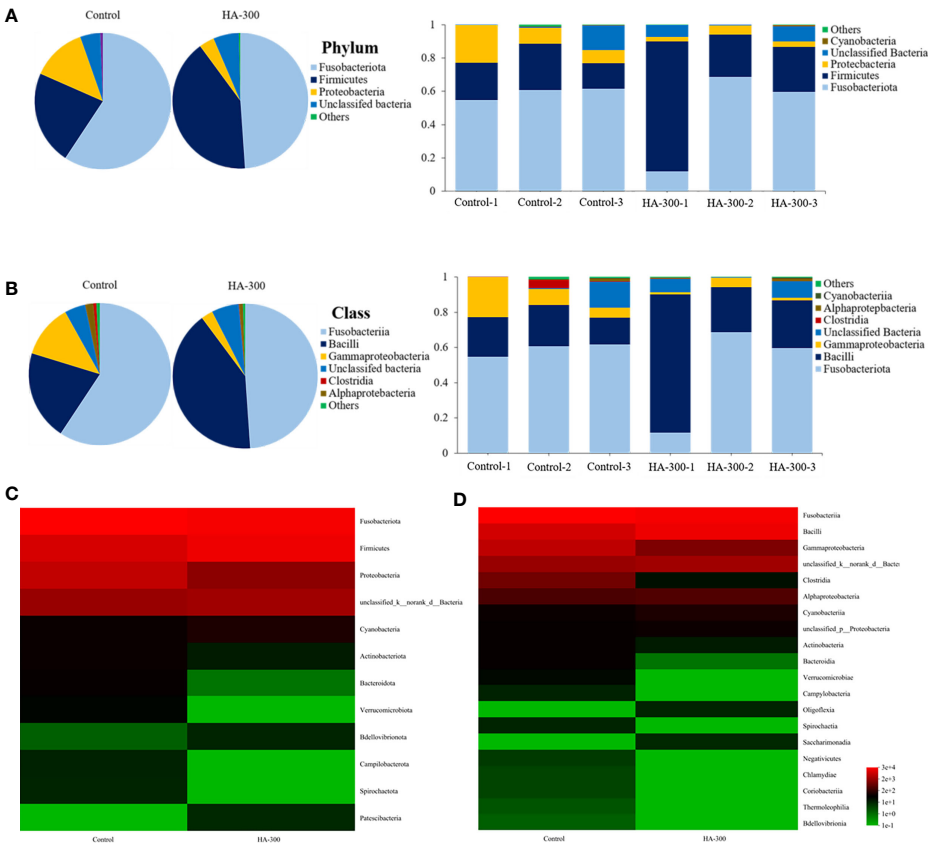

FIGURE 4
Comparisons of the intestinal microflora structure at the phylum and class levels of largemouth bass (n = 3). **(A)** Percentage distribution and relative abundance of intestinal microflora at the phylum level. **(B)** Percentage distribution and relative abundance of intestinal microflora at the class level. **(C, D)** Heatmap of intestinal microflora abundance at the phylum and class levels.

including Sobs, Chao, Ace, Shannon, Simpson, and Coverage indices between the control and HA-300 groups (Figure 5). However, fish fed a diet with 300 mg kg^{-1} β-glucan showed higher values in the Sobs, Chao, Ace, and Simpson indices.

3.5.3 LEfSe analysis

LEfSe analysis showed that a total of 10 taxa with significant differences between the control and HA-300 groups were found (Figure 6). Fish fed diet containing 300 mg kg^{-1} β-glucan significantly increased the relative abundance of *Bacilli* (mainly *Mycoplasmatales*, *Mycoplasmataceae*, *Mycoplasma*) and significantly decreased *Proteobacteria* (mainly *Gammaproteobacteria*, *Escherichia-Shigella*, and *Bacillus anthracis*) (LDA score >2 and $P < 0.05$).

4 Discussion

β-glucan have been proven to be a highly efficient stimulator of cellular and humoral branches in mammals and also is a potential stimulant with pronounced immune effects in fish. The effects of dietary β-glucan on growth have been evaluated in different species of aquatic animals, but inconsistent results have been obtained. A study in *L. rohita* showed that 250 and 500 mg kg^{-1} β-glucan significantly enhanced SGR (7). Similarly, Dawood et al. (11) found that 250–1,000 mg kg^{-1} β-glucan supplementation in the feed significantly increased WGR and SGR of red seabream (*Pagrus major*). Conversely, other studies have reported that dietary β-glucan had no significant effect on growth performance (26–29). A study showed that diet supplemented with different levels of β-glucan had no adverse effects on Nile tilapia (*Oreochromis niloticus*) during 10 weeks of feeding (30), which was in accordance with the results of this study. Contradictory results on the influence of β-glucan on growth performance may be due to species, feed composition, breeding environment, or other experimental conditions. Actually, it is generally believed that β-glucan has no direct growth-promoting effect on animals, but affects growth performance by improving immunity. Thus, we further investigated the effect of β-glucan on the immunity of largemouth bass.

The fish immune system is composed of two components, innate and adaptive immunity, in which innate immunity plays a major immune conditioning role. The components of innate immunity are divided into humoral molecules, in which LZM can destroy the cell wall of Gram-positive bacteria and prevent bacterial invasion. In addition, LZM has been recognized as a biomarker of immune defense mechanisms in fish (31, 32). Results of this study showed that LZM activity significantly increased with increasing dietary β-glucan supplementation, suggesting that β-glucan could improve the immunity of largemouth bass. Similar results were also found in other fish species, including the Persian sturgeon (*Acipenser persicus*), Nile tilapia, and hybrid striped bass (*Morone chrysops* × *M. saxatilis*) (33–35). Both *in vivo* and *in vitro* studies revealed that β-glucan significantly improved serum LZM activity in hybrid striped bass (33). Misra et al. (7) reported that serum LZM activity significantly increased after feeding rohu with β-glucan for 28–42 days. The Persian sturgeon fed diets with 200 and 300 mg kg^{-1} β-glucan showed higher LZM activity than those fed 0 and 100 mg kg^{-1} β-glucan (34), which was highly similar to the results of this study. To further investigate the effects of β-glucan on the immunity of largemouth bass, we conducted a challenge experiment with *A. schubertii*. *A. schubertii* is widely distributed in aquatic environments and also a common pathogen in aquaculture. *A. schubertii* belongs to *Aeromonas mesophilic*, which is one of the most serious bacteria in fish farming (36). Infected fish with *A. schubertii* shows fin rot and hemorrhage on the body surface, mainly causing bacterial septicemia and bacterial enteritis, leading to mass mortality and serious economic losses (37). A study in largemouth bass has confirmed that fish (~15 g) infected with *A. schubertii* (5×10^6 CFU ml^{-1}) showed slight hyperemia and hemorrhage of the anus and caudal fin and severe hyperemia of the liver with white nodules (3). The injection concentration of this experiment is higher than the above study mainly due to the fish weight specification. In the present study, all fish fed diets without β-glucan supplementation died after the 12-h challenge, but fish in the β-glucan supplementation groups survived after the 72-h challenge, suggesting that β-glucan could improve the resistance of largemouth basses against *A. schubertii*. Similar results were also found in other studies. A study on Nile tilapia reported that β-glucan can enhance the antioxidant and immune responses to avoid *A. hydrophila* (a branch of *A. schubertii*) infection (38). Meshram et al. (39) reported that a diet supplemented with 1 g kg^{-1} β-glucan enhanced immunity and resistance against *A. hydrophila* in freshwater prawn (*Macrobrachium rosenbergii*).

The transcriptome results showed that a total of 1,245 DEGs were obtained, fish fed a diet with 300 mg kg^{-1} β-glucan showed 449 significantly upregulated DEGs and 796 significantly downregulated DEGs compared with the control group. Meanwhile, 109 immune-related DEGs was screened, with 47 significantly upregulated immune-related DEGs enriched into 12 immune pathways, among which the chemokine signaling pathway and NOD-like receptor signaling pathway have important physiological functions in fish. The chemokine signaling pathway regulates leukocyte migration and plays an important role in nonspecific and specific immune responses in fish (40). Fish chemokines are involved in almost all functions of lymphocytes and can recruit and activate leukocytes to act at the infected site and participate in the immune response (41). In this study, diet supplementation of β-glucan significantly upregulated the expression levels of chemokine family genes, including chemokine XC receptor 1 (*xcr1*), C-X-C chemokine receptor type 1 (*cxcr1*), C-C motif chemokine 7 (*ccl7*), and C-C motif chemokine 5 (*ccl5*), suggesting that the chemokine signaling

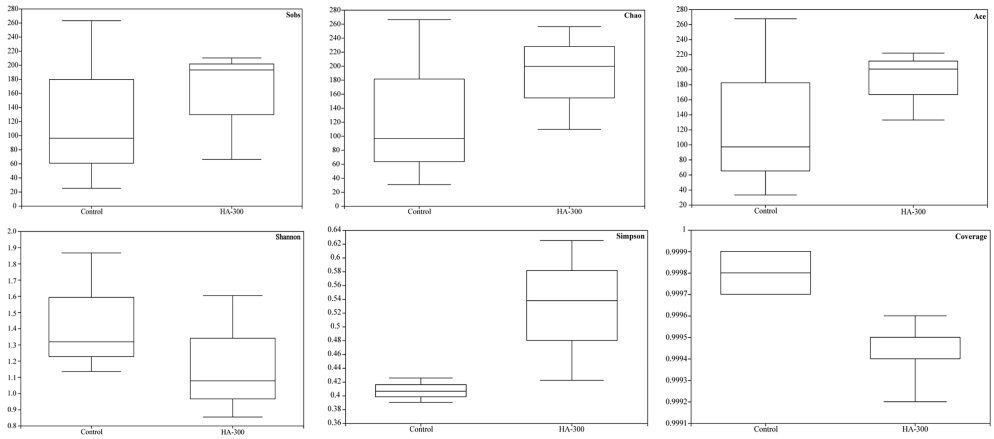

FIGURE 5
Boxplot for evaluating diversity and richness of intestinal microflora of largemouth bass based on the Sobs, Chao, Ace, Shannon, Simpson, and Coverage indices (n = 3).

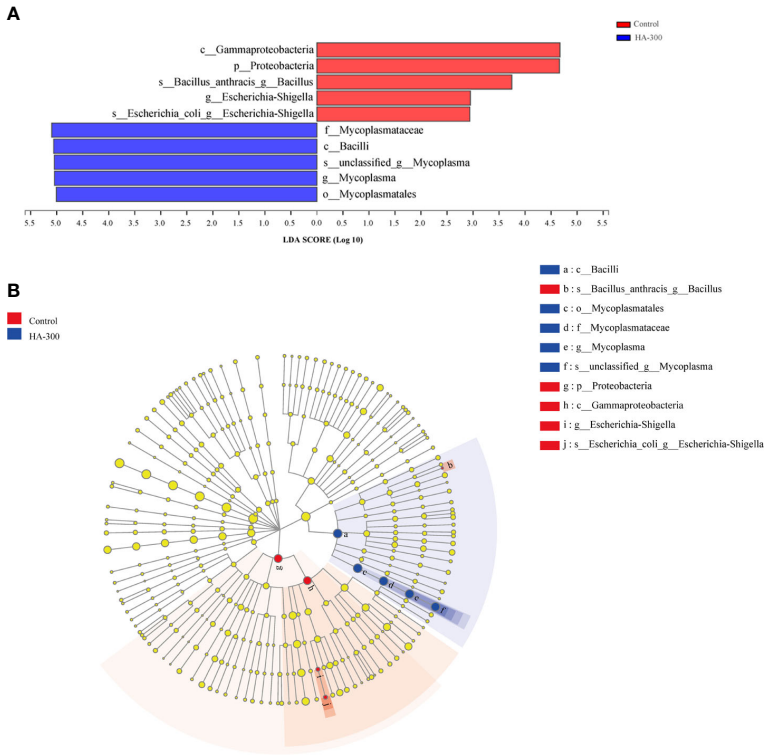

FIGURE 6
Linear discriminant analysis effect size (LEfSe) analysis of intestinal microflora of largemouth bass. **(A)** Histogram of linear discriminant analysis (LDA) value, with the length representing the LDA score (LDA >2). **(B)** Evolutionary branch diagram, with yellow nodes indicating no significant difference in intestinal microflora, and the red and blue nodes representing the differential microbiota classes that play a significant role in the control and HA-300 groups, respectively.

pathway plays an important role in β-glucan-mediated immune enhancement. In addition, β-glucan significantly upregulated the expression level of protein (Cdc42/Rac)-activated kinase 1 (*pak1*), which is a serine-threonine kinase and plays an important role in regulating key nodes of cellular function and angiogenesis (42). Recently, Ren et al. (43) reported that the activity of LZM significantly decreased in coelomic fluid of sea cucumber (*Apostichopus japonicus*) after inhibition expression of *pak1*. Therefore, the enhanced LZM activity of largemouth bass fed a diet with β-glucan supplementation may be related to the upregulated expression level of *pak1*. Another β-glucan-related immune pathway identified in this study is NOD-like receptor signaling pathway, which plays a key role in pathogen recognition and nonspecific immune responses and lead to the initiation of antimicrobial and antiviral immune responses. Nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) are important pathogen recognition receptors in this pathway and play an important role in the nonspecific immune response of teleost fish (44). Studies have confirmed that β-glucan activates NOD-like receptor signaling pathway through NLRs (45). The Nile tilapia *nlrc3* gene was expressed in tissues as NOD1 and NOD2, and the expression was significantly upregulated after *Streptococcus agalactiae* infection (46). Significantly upregulated NLR family genes were also observed in this study, such as NLR family CARD domain-containing protein 3 (*nlrc3*), indicating that the NOD-like receptor signaling pathway is another important pathway in which β-glucan modulates immunity. Furthermore, we also found 62 significantly downregulated immune-related DEGs enriched into 24 immune pathways, among which intestinal immune network for IgA production, cytosolic DNA-sensing pathway, and C-type lectin receptor signaling pathway have important physiological functions in fish. The intestinal immune network for IgA production protects the host from pathogen invasion (47). DNA-directed RNA polymerase (*polr*) complexes play an important role in the immune-related cytosolic DNA-sensing pathway (48). In addition, studies had shown that the C-type lectin receptor signaling pathway stimulated by β-glucan can activate the NF-kappa B signaling pathway to produce an inflammatory response (49), while genes regulated in these pathways including nectin-4-like, *il-10*, *il-1β*, TNFAIP3-interacting protein 1-like (*tnip1*-like), polymerase (RNA) III polypeptide D (*polr3d*), polymerase (RNA) III polypeptide C (*polr3c*), and RNA polymerase II, I and III subunit H (*polr2h*) were significantly downregulated. *Nectin-4* belongs to the family of immunoglobulin-like cell adhesion molecules and causes viral nervous necrosis in grouper (*Epinephelus fuscoguttatus*♀× *Epinephelus lanceolatus*♂) (50). *Il-10* is an anti-inflammatory factor that inhibits the expression of pro-inflammatory factor *il-1β* and plays a central role in regulating inflammatory responses (51). Studies had shown that β-glucan could downregulate the expression of *il-1β* in common carp (*Cyprinus carpio*), which was consistent with the results of this study (52). *Tnip1* is an inflammation-related gene with multiple roles and expression in multiple cell types (53), while genes of *polr3d*, *polr3c*, and *polr2h* associated with pathogens (54). Overall, transcriptome results of this study demonstrated that diet supplementation of β-glucan upregulated the chemokine signaling pathway and NOD-like receptor signaling pathway (including genes of *xcr1*, *cxcr1*, *ccl7*, *ccl5*, *pak1*, and *nlrc3*) and downregulated the intestinal immune network for IgA production, cytosolic DNA-sensing pathway, and C-type lectin receptor signaling pathway (including genes of *nectin-4*-like, *il-10*, *il-1β*, *tnip1*-like, *polr3d*, *polr3c*, and *polr2h*) to reduce inflammation and enhance immunity in largemouth bass.

Intestinal microorganisms form a complex microbial community in the gastrointestinal tract of animals, which plays an important role in immune function and prevention of pathogen invasion, and are an important indicator for evaluating fish health status. In this study, *Fusobacteria*, *Firmicutes*, and *Proteobacteria* were the dominant bacteria in largemouth bass at the phylum level, which was consistent with results of other studies in largemouth bass (55–58). Some pathogenic bacteria in the intestine can cause reduced immunity in fish, such as *Escherichia-Shigella* and *Escherichia coli* in *Proteobacteria* and *B. anthracis*. Studies have shown that increased relative abundance of *Proteobacteria* is a marker of community instability and intestinal inflammatory response (57–59). *E. coli*, including the closely related genus *Shigella*, is a representative bacterium of highly pathogenic bacterium *Escherichia* (60). *Escherichia-Shigella* is a pathogen that causes intestinal disease, which is positively correlated with intestinal inflammation and negatively correlated with growth performance in fish (61, 62). *B. anthracis* belongs to the genus *Bacillus*, which is a well-known pathogen that can infect skin, lungs, and intestines and cause severe damage to tissues and organs (63). Brown et al. (64) reported that the abundance of potential pathogenic *Vibrio* appeared to be inversely correlated with *Mycoplasma*, suggesting that *Mycoplasma* may be potentially beneficial to rainbow trout. In this study, LEfSe analysis showed that fish fed a diet containing 300 mg kg^{-1} β-glucan significantly decreased the abundance of *Proteobacteria* (mainly *Escherichia-Shigella* and *E. coli*) and *B. anthracis*, suggesting that β-glucan can maintain intestinal health by reducing harmful bacteria.

5 Conclusion

In conclusion, long-term oral administration of β-glucan (<300 mg kg^{-1}) had no adverse effects on largemouth bass. In addition, 300 mg kg^{-1} β-glucan supplementation stimulated the nonspecific immune system of largemouth bass and improved resistance against *A. schubertii*. Transcriptome analysis revealed that fish fed a diet supplemented with β-glucan significantly

upregulated the chemokine signaling pathway and NOD-like receptor signaling pathway and downregulated the intestinal immune network for IgA production, cytosolic DNA-sensing pathway, and C-type lectin receptor signaling pathway. In addition, β-glucan can maintain intestinal health by decreasing harmful bacteria *Proteobacteria* (mainly *Escherichia-Shigella* and *E. coli*) and *B. anthracis* in largemouth bass intestine.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/ , SRR21783450, SRR21783451, SRR21783452, SRR21783453, SRR21783454, SRR21783455 https://www.ncbi.nlm.nih.gov/ , SRR21783676, SRR21783677, SRR21783678, SRR21783679, SRR21783680, SRR21783681.

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

MG performed formal analysis, investigation and writing original draft. YZ and BS performed conceptualization, designed experiment, funding acquisition, supervision and writing review and editing. NL performed data curation and project administration. ZD and JX performed methodology and validation. LC and BW performed data curation and validation. LL and LR performed project administration. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by National Key R&D Program of China (Grant No: 2019YFD0900203), National Engineering Research Laboratory of marine biotechnology and Engineering, Key Laboratory of Aquacultural Biotechnology, Collaborative Innovation Center for Zhejiang Marine High-efficiency and Healthy Aquaculture and Key Laboratory of Marine Biotechnology of Zhejiang Province.

Acknowledgments

The authors are grateful to Kemin AquaScience for the support and valuable help.

Conflict of interest

Authors NL, LL and LR are employed by Kemin Industries, Inc, United States

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1086103/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 15 December 2022
DOI 10.3389/fimmu.2022.1075128

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Comparative Immunology, a section of the journal Frontiers in Immunology

RECEIVED 20 October 2022
ACCEPTED 28 November 2022
PUBLISHED 15 December 2022

CITATION
Zhou J, Feng M, Zhang W, Kuang R, Zou Q, Su J and Yuan G (2022) Oral administration of hepcidin and chitosan benefits growth, immunity, and gut microbiota in grass carp (*Ctenopharyngodon idella*). *Front. Immunol.* 13:1075128. doi: 10.3389/fimmu.2022.1075128

Oral administration of hepcidin and chitosan benefits growth, immunity, and gut microbiota in grass carp (*Ctenopharyngodon idella*)

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Intensive high-density culture patterns are causing an increasing number of bacterial diseases in fish. Hepcidin links iron metabolism with innate immunity in the process of resisting bacterial infection. In this study, the antibacterial effect of the combination of hepcidin (Cihep) and chitosan (CS) against *Flavobacterium columnare* was investigated. The dosing regimen was also optimized by adopting a feeding schedule of every three days and every seven days. After 56 days of feeding experiment, grass carp growth, immunity, and gut microbiota were tested. *In vitro* experiments, Cihep and CS can regulate iron metabolism and antibacterial activity, and that the combination of Cihep and CS had the best protective effect. *In vivo* experiments, Cihep and CS can improve the growth index of grass carp. After challenge with *Flavobacterium columnare*, the highest survival rate was observed in the Cihep+CS-3d group. By serum biochemical indicators assay and Prussian blue staining, Cihep and CS can increase iron accumulation and decrease serum iron levels. The contents of lysozyme and superoxide dismutase in Cihep+CS-3d group increased significantly. Meanwhile, Cihep and CS can significantly reduce the pathological damage of gill tissue. The 16S rRNA sequencing results showed that Cihep and CS can significantly increase the abundance and diversity of grass carp gut microbiota. These results indicated that the protective effect of consecutive 3-day feeding followed by a 3-day interval was better than that of consecutive 7-day feeding followed by a 7-day interval, and that the protective effect of Cihep in combination with chitosan was better than that of Cihep alone. Our findings optimize the feeding pattern for better oral administration of Cihep in aquaculture.

KEYWORDS

hepcidin, chitosan, *Ctenopharyngodon idella*, growth, immunity, gut microbiota

Introduction

In China, freshwater fish farming is an important industry, accounting for 70% of the world's aquatic products (1). Grass carp (*Ctenopharyngodon idella*), a herbivorous freshwater fish, is one of the four domestic fishes in China, and it ranks the first in the production yield among freshwater farmed fishes (2). Data from the 2020 Fisheries Yearbook show that grass carp production yield reached 5.533 million tons in 2019, accounting for 18.36% of freshwater aquaculture production. However, the frequent occurrence of diseases has become an important limiting factor for the sustainable and healthy development of grass carp farming industry (3). Intensive high-density culture patterns are causing an increasing number of bacterial diseases in fish. *Flavobacterium columnare* (*F. columnare*) is a common pathogen of freshwater cultured fish. *F. columnare* can cause bacterial gill rot disease. In recent years, there have been outbreaks of the disease due to intensive farming (4, 5).

Antibiotics are widely used for controlling diseases. However, the overuse of antibiotics can lead to excessive accumulation of antibiotics in the water environment as well as in aquatic organisms, which in turn can be harmful to human health (6). In addition, overuse of antibiotics may also lead to bacterial resistance, thereby reducing the immunity of aquatic animals (7, 8). Therefore, avoiding the excessive use of antibiotics in the aquaculture industry has become one of the major challenges facing human health and the aquaculture sector (9).

Antimicrobial peptides (AMPs) are seen as the most promising alternative to antibiotics (10, 11). Hepcidin is a cysteine-rich peptide hormone synthesized mainly by hepatocytes (12–14). Krause and Park et al. successively extracted hepcidin from human plasma and urine in 2001 (15, 16). Hepcidin can combine with ferroportin and cause the ubiquitination of ferroportin, thus reducing the serum iron content of the body (17). Hepcidin can promote the expression of certain immune-related genes, thereby enhancing the defense function of fish against pathogens (18–20). Hepcidin can also limit the uptake and utilization of iron by pathogens by decreasing the iron level in organism (21, 22). Our previous studies have demonstrated that hepcidin can reduce mortality caused by bacterial infections in fish by regulating iron metabolism and immune-related gene expression (23–26). Therefore, hepcidin-regulated iron removal is an important method to control bacterial growth and improve survival.

Chitosan is an important immune enhancer and is the only alkaline polysaccharide present in nature, mainly found in the shells of aquatic animals such as shrimps, crabs, and shellfish (27). In recent years, chitosan has been widely used in aquaculture industry for its advantages of promoting aquatic animal growth and improving immunity and disease resistance of aquatic animals (28–30). Furthermore, dietary chitosan has no toxic effect on its biodegradability and biocompatibility (31). Studies have shown that chitosan can increase the microbial diversity of the fish gut and participate in the immune regulatory system of the body by regulating the ratio of beneficial and pathogenic microorganisms (32). More specifically, beneficial bacteria secrete digestive enzymes and nutrients to promote intestinal absorption, which in turn improves fish growth rate and feed efficiency (32). In addition, adding chitosan to feed can improve the antioxidant properties of aquatic organisms (33).

Previous studies in our laboratory have shown that hepcidin have dual functions as iron-regulating and antimicrobial activity. Chitosan, as a widely used immune polysaccharide in aquaculture, also plays an important role in improving the immunity of fish. In view of this, we explored the effectiveness of different hepcidin feeding methods or its combination with chitosan on growth, immunity, and gut microbiota of grass carp. Meanwhile, we analyzed the iron-regulation function of hepcidin and the specific role in the immune system of grass carp infected with *F. columnare*. This study aims to develop new green and safe antibiotic alternatives so as to provide theoretical basis for controlling diseases of cultured fish.

Materials and methods

The grass carp management

Grass carp (150.53 ± 1.80 g) obtained from the Da Bei Nong Recirculating Water Farming Base (Wuhan, China) were maintained and acclimated in a recirculating freshwater system with daily feeding. Before experiment, the grouped grass carp were temporarily raised for 7 days for acclimatization. During the experiments, feeding was scheduled at 8: 00 am and 16:00 pm twice a day, and the feeding rate was 2%. During the experiment, the water temperature was maintained at 25-28 °C, the pH was 7.0-7.5, the dissolved oxygen was greater than 5 mg/L, the concentration of ammonia was less than 0.2 mg/L, and nitrite concentration was less than 0.01 mg/L. Treatment methods of experimental animals involved in this experiment comply with the principles of experimental animal welfare and have been approved by the Animal Committee of Huazhong Agricultural University. The ethics number is HZAUFI-2021-0012.

Experimental design

Fish cultivation experiment lasted 56 days. At the start of the experiment, 450 grass carp were randomly distributed

into 15 fiberglass tanks (300 L of water) with 30 grass carp per tank. The grouping was as follows: the control group (Control) was fed with basal diet on daily basis; the Cihep group (Cihep-3d) was fed with Cihep for three days on end followed by three-day interval; the Cihep group (Cihep-7d) was fed with Cihep for seven days on end followed by seven-day interval; the Cihep+chitosan group (Cihep+CS-3d) was fed with Cihep and chitosan for three days on end followed by three-day interval; and the Cihep+ chitosan group (Cihep +CS-7d) was fed with Cihep and chitosan for seven days on end followed by seven-day interval. The feed formula of each group is shown in Table 1.

The prokaryotic expression of grass carp hepcidin

DNAs encoding the open reading frame (ORF) of the *C. idella* hepcidin gene (Cihep; GenBank: JQ246442.1) were inserted into the *E. coli* expression vector pGEX-KG (Novagen). The confirmed recombinant expression construct pGEXKG-Cihep and the expression construct pGEX-KG were transformed into competent *E. coli* BL21 (DE3) cells (Novagen). Then, the transformed cells were inoculated into 600 mL LB medium (LB, L3027, Sigma, Shanghai, China) in the fermenter, incubated at 37°C at 150 rpm for 3 h, and cultured to an OD600 of 2.0–5.0. It was then cooled to 25°C, before a final concentration of 1 mM IPTG was added to induce expression for 5 h. After induction, the bacterial solution was centrifuged at 20,000 r/min, collected, weighed, and stored at −80°C. The cell pellet was then resuspended in PBS buffer at 25°C. The cells were subsequently broken by passing them through a French pressure cell press at 1.0×10^3 bar for 5 min. After centrifugation at 12,000g for 1 h at 4°C, the recombinant Cihep was obtained. Next, recombinant protein was prepared as freeze-drying powder and detected using SDS-PAGE electrophoresis. Finally, stored at -20 °C for the subsequent use.

Western blot analysis

Western blot analysis was used to confirm the existence and molecular weights of the obtained Cihep protein. The recombinant Cihep protein was separated on a 12% SDS-PAGE gel, transferred to nitrocellulose filter membrane at 9 V for 30 min. The membrane was blocked with 5% skimmed milk at room temperature for 1 h, incubated with a 1:4000 dilution of a anti-GST tag mouse monoclonal antibody for 1 h, washed with 1 × TBS + 0.1% Tween and incubated with a 1:5000 dilution of HRP Goat Anti-Mouse secondary antibody (1:5000; 1 mg/mL) (ab6789, Abcam) for 1 h.

Cell treatment and sample preparation

Grass carp liver cells (L8824) were cultured with M199 medium (Gibco, Beijing, China) in an incubator at 28°C. L8824 cells were directly stimulated by the addition of GST-Cihep fusion protein or CS. The stimulated cells were continued at 28°C. After 24 hours, the medium was decanted and the cells were collected. Cellular was extracted using the Trizol (Aidlab, Beijing, China) method for detection of hepcidin expression. Labile iron Pool (LIP) levels were assessed with reference to the methods covered in previous articles from our laboratory (25).

We assessed the preventive effect of Cihep and CS by culturing L8824 cells with *F. columnare*. Firstly, L8824 cells were cultured in 24-well plates (BeyoGold, Shanghai, China). After 24 hours, cells were continued to be cultured with M199 containing PBS (10,100,147, Gibco, Beijing, China), Cihep, CS, and Cihep + CS. After 3 hours, bacterial suspension (multiplicity of infection MOI=50) was added to each well and the culture was continued for 24 hours. Finally, the cells were fixed with 4% paraformaldehyde solution, and then stained with crystal violet solution for 25 minutes. Finally, the cells were washed and drained. The preventive effect of Cihep and CS was evaluated using crystal violet staining.

TABLE 1 Experimental feed formula.

IngredientsContent (g/1000 g feed)	Control	Cihep-7d	Cihep-3d	Cihep+CS-3d	Cihep+CS-7d
Soybean meal	360.01	360.01	360.01	359.98	359.98
Rapeseed meal	260.00	260.00	260.00	260.00	260.00
Vegetable cake	115.00	115.00	115.00	115.00	115.00
Wheat	170.00	170.00	170.00	170.00	170.00
Soybean oil	50.00	50.00	50.00	50.00	50.00
Vitamin premix	12.00	12.00	12.00	12.00	12.00
Mineral premix	8.00	8.00	8.00	8.00	8.00
$Ca(H_2PO_4)$	25.00	25.00	25.00	25.00	25.00
Hepcidin	0	0.09	0.09	0.09	0.09
Chitosan	0	0	0	0.03	0.03

Challenge experiments

F. columnare for the challenge experiment was provided by Professor Xie Haixia. The 80 μL of cryopreserved *F. columnare* was inoculated onto a Shieh Agar plate at 26 °C for 48 h to form single colony. The selected single colony was inoculated into the selective medium and incubated at 26 °C for 48 hours. According to the pre-experiment results, the median lethal concentration was 3 $\times 10^9$ CFU/mL. During the formal challenge, 25 fish were taken from each group for the challenge experiment. Each fish was injected with 3 $\times$ 10^9 CFU/mL of *F. columnare* (500 μL), and the cumulative death curve was obtained in the following 7 days.

Sample collection and biological analyses

At the end of the 56-day feeding experiment (Pre-challenge), and at 7 d post-challenge, 5 fish were randomly selected from each cylinder after 24 h fasting and anesthetized. Body weight and length were measured to calculate growth index, and blood was collected from the tail vein for analysis of serum biochemical indexes. The fish body was dissected with aseptic dissecting scissors, and the contents of foregut were collected for the analysis of gut microbiota. Hepatopancreas, spleen, and intestines were used for the expression analysis of immune-related genes and iron-related genes by qPCR. The visceral mass and hepatopancreas of grass carp were weighed, and the visceral body ratio and hepatosomatic index were calculated. The hepatopancreas and gill were fixed in 4% paraformaldehyde for histopathological analysis, and the remaining fish were used for challenge experiment.

Tissue staining

Parts of gill and hepatopancreas tissue were dissected using sterile dissection and immediately fixed with 10% paraformaldehyde. HE staining of gill tissues and Prussian blue staining of hepatopancreas were performed by Borf Biotechnology.

Serum biochemical parameters analysis

Blood samples were placed for 12 h at 4°C. After centrifugation (3000 g, 4°C, 15 min), the serum was collected for enzyme activity detection, and the remaining serum was stored at −80°C. Serum iron was assayed by colorimetric method. Lysozyme was measured by turbidimetric method. Superoxide gasification enzyme was measured by the hydroxylamine method. Test kits were purchased in Nanjing Jiancheng Bioengineering Institute. The determination was carried out according to the manufacturer's instructions. The experiment was performed in triplicates.

RNA extraction and qRT-PCR analysis

The expression levels of iron-related genes *hepcidin* and *ferroportin*, and the expression levels of immune-related genes *IL-1β, IL-10, TNF-α* and *MHC-II* were detected in hepatopancreas, spleen and intestine. Total RNA was extracted from with RNAiso Plus kit (Takara, Dalian, China) according to the manufacturer's instructions, followed by DNase I treatment. Total RNA was quantified by absorbance ratio of 260 nm and 280 nm with NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, USA), and the quality was evaluated using 1% agarose gel electrophoresis. The cDNA was synthesized using the HiScript® II Q Select RT SuperMix (Vazyme, Nanjing, China) according to the manufacturer's instruction for subsequent qPCR. All the cDNA products were diluted to 250 ng/μL, and the qPCR was performed using the AceQ® qPCR SYBR® Green Master Mix (Vazyme, Nanjing, China) on a Roche LightCycle® 480 System (Roche, Basel, Switzerland) according to the manufacturer's instructions. The primers mentioned in previous literatures are shown in Table 2. A melting curve was constructed for every qPCR product to confirm the specificity of the assays. A dilution series were prepared to check the efficiency of the reaction. 18S rRNA and β-actin were used as the housekeeping genes, and the relative mRNA expression level was calculated with the $2^{-\Delta\Delta CT}$ method (34).

DNA extraction and 16s rRNA gene sequencing

Microbial genomic DNA was extracted from intestinal contents by using PowerFecal® DNA Isolation Kit (Mo Bio, CA, USA) following manufacturer's instruction. DNA concentrations and purity were assessed with a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA). DNA quality was checked by 1% agarose gel electrophoresis. The V3-V4 hypervariable regions of the bacteria 16S rRNA gene were amplified with primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') by thermocycler PCR system (GeneAmp 9700, ABI, USA). The PCR conditions were as follows: 3 min of denaturation at 95 oC, 27 cycles of 30 s at 95° C, 30 s for annealing at 55°C, and 45s for elongation at 72 oC, and a final extension at 72°C for 10 min. PCR was performed in triplicate in a total volume of 20 μL containing: 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 L of each primer (5 μM), 0.4 μL of FastPfu Polymerase and 10 ng of template DNA.

10.3389/fimmu.2022.1075128

TABLE 2 Primers used in this study.

Gene	Primer	Primer Sequence (5′-3′)
Hepcidin	Forward	CAGCCGTTCCGTTCGTACA
	Reverse	AGCCTTTGTTACGACAGCAG
Ferritin	Forward	TCCTGTGCTTCGTGCGTGT
	Reverse	ACCTTCAGTCCGTCCTCGTG
Ferroportin	Forward	CCTCGGACATGCTCTGTCAA
	Reverse	CAGTCCATACACGGCTGTCA
IL-1β	Forward	AAGTTCCCGCTTTGGAGAGTA
	Reverse	GCCACATACCAGTCGTTCAGT
IL-10	Forward	CTCAAGCGGGATATGGTCAAA
	Reverse	TGGCAGAATGGTCTCCAAGTAG
MHC-II	Forward	ACAAGCCTCAGTGTGACGAG
	Reverse	TGTGTCCGGAATCTCATGGC
TNF-α	Forward	ATTTATCTCGGTGCGGCCTT
	Reverse	GCTTACAGAGCAAACACCCC
18S rRNA	Forward	ATTTCCGACACGGAGAGG
	Reverse	CATGGGTTTAGGATACGCTC
β-actin	Forward	GATGATGAAATTGCCGCACTG
	Reverse	ACCGACCATGACGCCCTGATGT

Finally, add ddH2O to 20 μL. PCR products were gel purified using a 2% agarose gel and the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor ™ -ST (Promega, USA) according to the manufacturer's protocol. Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq PE300 platform (Illumina, San Diego, USA), according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

Raw FASTQ files were de-multiplexed using in-house perl script, and then quality-filtered by fastp version 0.19.6 and merged by FLASH version 1.2.7 with the following criteria: (i) the 300 bp reads were truncated at any site receiving an average quality score of<20 over a 50 bp sliding window, and the truncated reads shorter than 50 bp were discarded, reads containing ambiguous characters were also discarded; (ii) only overlapping sequences longer than 10 bp were assembled according to their overlapped sequence. The maximum mismatch ratio of overlap region is 0.2. Reads that could not be assembled were discarded; (iii) reads containing ambiguous bases were removed; and (iv) sequences with >= 10 bp overlap longer were merged. Operational taxonomic units (OTUs) were clustered with 97% similarity cut-off using UPARSE 7.1 and chimeric sequences were identified and removed using UCHIME. Taxonomy was assigned by RDP classifier algorithm against the Silva 16S rRNA database using a confidence threshold of 70%. The average read length was approximately 400 bp after quality trimming. A phylogenetic tree was constructed by the neighbor joining method with CLUSTAL X and MEGA 6.0.

Bioinformatic analyses of gut microbiota was performed on the Majorbio Cloud Platform (https://cloud.majorbio.com). Based on the OTUs information, rarefaction curves and alpha diversity indices including observed OTUs, Chao1 richness, and Shannon index were calculated with Mothur v1.30.1. The similarity among the microbial communities in different samples was determined by principal coordinate analysis (PCoA) based on Bray-curtis dissimilarity using Vegan v2.5-3 package. All 16S rRNA sequence data used in this study were deposited in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under the BioProject accession number PRJNA900049.

Statistical analyses

The results were expressed as the means ± standard deviation. We analyzed the experimental data through Multi-factorial ANOVA using the SPSS software (p<0.05). Different superscript letters in each group (a, b, and c) denote significant variations. The mortality rate was analyzed by Mantel-Cox test. Use the following formula to calculate: Weight gain (WG), specific growth rate (SGR), condition factor (CF), hepatosomatic index (HSI), visceral body ratio (VIS) and the mortality rate were calculated by the following formula:

$$WG(\%) = 100 \times (W_2 - W_1)/W_1$$

$$SGR(\%) = 100 \times (\ln W_2 - \ln W_1)/TW_1$$

$$CF(\%) = 100 \times \text{body weight(g)}/\text{body length(cm)}^3$$

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

$$VIS(\%) = 100 \times \text{Visceral weight(g)}/\text{body weight(g)}$$

$$\text{Cumulative mortality}(\%) = 100 \times (N_0 - N_F)/N_0$$

where W_1 is the initial weight (g), W_2 is the final weight (g), T is time (d), N_0 is the initial number of fish and N_F is the final number of fish.

Results

Stability of Cihep proteins

We obtain the Cihep fusion protein in the prokaryotic expression system. A target band of 28 kDa was confirmed by SDS-PAGE (Figure 1A) and Western blot (Figure 1B). To determine the stability of Cihep protein, the freeze-dried powder was stored at -20°C refrigerator and sampled at day 1,

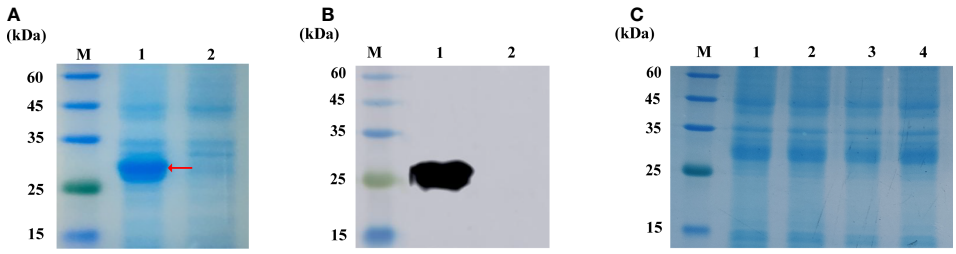

FIGURE 1
Expression and activity verification of Cihep. **(A)** Analysis by SDS-PAGE. M=protein marker; 1= pGEX-KG-hepcidin after overnight induction; 2= pGEX-KG-hepcidin before induction. **(B)** Western blotting assay of anti-GST antibody. M=protein marker; 1= pGEX-KG-hepcidin after overnight induction; 2= pGEX-KG-hepcidin samples before induction. **(C)** Protein lyophilized powder stability at -20°C. M=protein marker; 1, 2, 3, 4= represent D1, D7, D14 and D28 respectively.

7, 14 and 28, respectively. Then, the stability of Cihep protein was determined by SDS-PAGE (Figure 1C). The results showed that the properties of the protein remained unchanged at -20°C for 28 days.

Synergistic effect of Cihep and CS

The results showed that hepcidin mRNA was significantly increased after incubation of chitosan with hepatocytes (Figure 2A). Subsequently, we examined the change in the labile iron pool (LIP) after 12 h Cihep and CS incubated hepatocytes (Figure 2B). The results showed that Cihep and CS stimulated the cells to increase the intracellular LIP content. To further investigate whether hepcidin and chitosan have synergistic effects, we investigated the preventive effect of hepcidin and chitosan on L8824 cells infected with *F. columnare*. Crystalline violet staining showed that large amount of cells died in the control group, while the Cihep+CS group had less cells death (Figure 2C). After 3 h of infection with *F. columnare*, all the hepcidin treatment group, chitosan treatment group, and hepcidin combined with chitosan treatment group had obvious protective effect, among which, the hepcidin combined with chitosan treatment group had the most significant protective effect, indicating that hepcidin and chitosan had a good synergistic effect.

Growth performance

The growth performance parameters of grass carp fed with supplemented with hepcidin were summarized in Table 3. Compared with those in the control group, the WG, SGR, CF, and HSI in four experience groups were increased. Among all five groups, Cihep-3d group exhibited the highest WG, SGR, and VIS, and the optimal weight gain effect.

Reduction in mortality and establishment of a protective immune state by Cihep and CS in grass carp

After bacterial challenge, the mortality rate of the four experimental groups was lower than that of the control group (65.00%). Among them, Cihep+CS-3d group displayed the lowest mortality rate (45.00%) (Figure 3A). Results showed the mortality rate in the Cihep+CS-3d group was significantly lower than that of the control group ($p<0.05$). Our results shows that hepcidin and CS have a significant preventive effect.

Before and after challenge, the serum iron in all four experimental groups was lower than that in the control group. The serum iron level was extremely significantly lower in the Cihep + CS-3d group than in the control group (Figure 3B). The trend of changes in LZM (Figure 3C) was significantly higher. The serum enzyme activity of Cihep+CS-3d group was significantly higher than those of the other four groups before and after challenge (Figure 3C). The T-SOD activity of Cihep-3d group and Cihep + CS-3d group was significantly higher than that of the control group (Figure 3D). Overall, the Cihep-3d and Cihep+CS-3d groups, especially the Cihep+CS-3d group, can effectively exhibit higher serum innate immunity levels than other groups.

Protective effect of Cihep and CS against pathological damage of gill tissue

The healthy gill filaments were neatly distributed and the gill lamellae were well arranged. At 7 d post infection, the gill tissue epithelial cells of the control group were severely detached, and necrotic. The gill filaments showed symptoms such as bleeding and curling. Compared with the control group, the lesions in the four experimental groups were mild and the gill lamellae were more neatly arranged (Figure 4).

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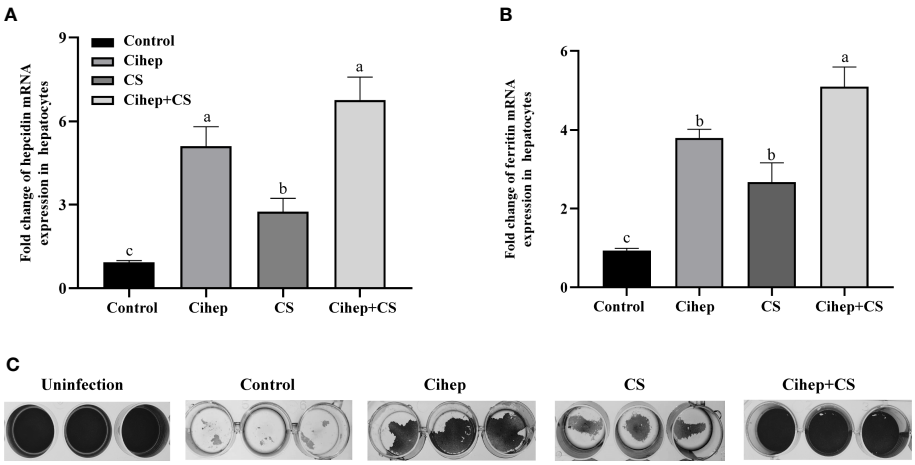

FIGURE 2
Preventive effects of Cihep and CS in L8824-infected bacteria by iron metabolism. **(A)** qRT-PCR assay of mRNA level of *hepcidin* in hepatocytes treated with Cihep and CS. **(B)** Fluorescence assay of LIP level in hepatocytes treated with Cihep and CS. **(C)** Bacterial suspension in M199 was incubated with L8824 cells for 3 h and then treated with PBS, Cihep, CS and Cihep+CS at 28°C for 24 h. Cell density was determined using crystal violet staining. Uninfected cells were used as blank control. Different small letters in each group (a, b, and c) denote significant variations suggested by the multifactorial ANOVA statistics ($p < 0.05$).

Alleviation of *F. columnare*-induced inflammation by Cihep and CS

The expressions of *IL-1β*, *TNF-α* and *MHC-II* in the hepatopancreas were significantly higher in the four experimental groups than in the control group after injection with *F. columnare* (Figure S1A). In the spleen, the expression level of *IL-1β* was also significantly higher in four experimental groups than that the control group. In addition, the expression level of *IL-10* in the spleen was significantly lower than that in the control group (Figure S1B). In the intestine, the expression levels of *IL-1β*, *TNF-α* and *MHC-II* were also significantly higher in four experimental groups than in the control group.

Regulation of individual iron levels by Cihep and CS

The obvious blue was observed in the four experimental groups, but much less obvious in the control group, indicating that adding hepcidin to the diet increased the accumulation of iron ions in the hepatopancreas of grass carp (Figure 5A). The results showed that iron ion aggregation was significantly higher in the Cihep+CS-3d group than in the other three groups ($p<0.01$).

Before challenge, in the hepatopancreas, the expression level of *hepcidin* was significantly higher in both Cihep -7d and Cihep + CS-3d groups than in the other groups (Figure 5C),

TABLE 3 Effects of dietary supplementation of Cihep and CS on growth performance of grass carp.

Items	Control	Cihep-3d	Cihep-7d	Cihep+CS-3d	Cihep+CS-7d
Initial weight (g)	150.50 ± 1.80	149.53 ± 0.60	149.71 ± 1.30	149.15 ± 0.70	149.33 ± 0.60
Final Weight (g)	401.82 ± 7.35^{b}	408.56 ± 3.90^{a}	403.75 ± 8.52^{b}	402.15 ± 11.91^{b}	400.83 ± 5.20^{b}
Weight gain (WG, g)	166.97 ± 4.60^{b}	173.27 ± 2.70^{a}	169.62 ± 3.90^{b}	169.67 ± 8.90^{b}	168.51 ± 2.50^{b}
Specific growth rate (SGR, %/d)	1.71 ± 0.03^{b}	1.79 ± 0.02^{a}	1.77 ± 0.03^{b}	1.77 ± 0.06^{b}	1.76 ± 0.02^{b}
Condition factor (CF, g/cm^{3})	1.13 ± 0.10^{b}	1.23 ± 0.11^{a}	1.15 ± 0.20^{b}	1.16 ± 0.10^{b}	1.15 ± 0.12^{b}
Hepatosomatic index (HSI, %)	1.65 ± 0.24^{c}	1.70 ± 0.42^{b}	1.78 ± 0.32^{a}	1.71 ± 0.30^{b}	1.70 ± 0.30^{b}
Visceral body ratio (VIS, %)	8.85 ± 0.60^{b}	9.09 ± 0.82^{a}	8.86 ± 1.02^{b}	8.85 ± 0.70^{b}	8.57 ± 0.80^{b}

Value are presented as mean ± SD. Means in the same row with different superscripts are significantly different (P< 0.05).

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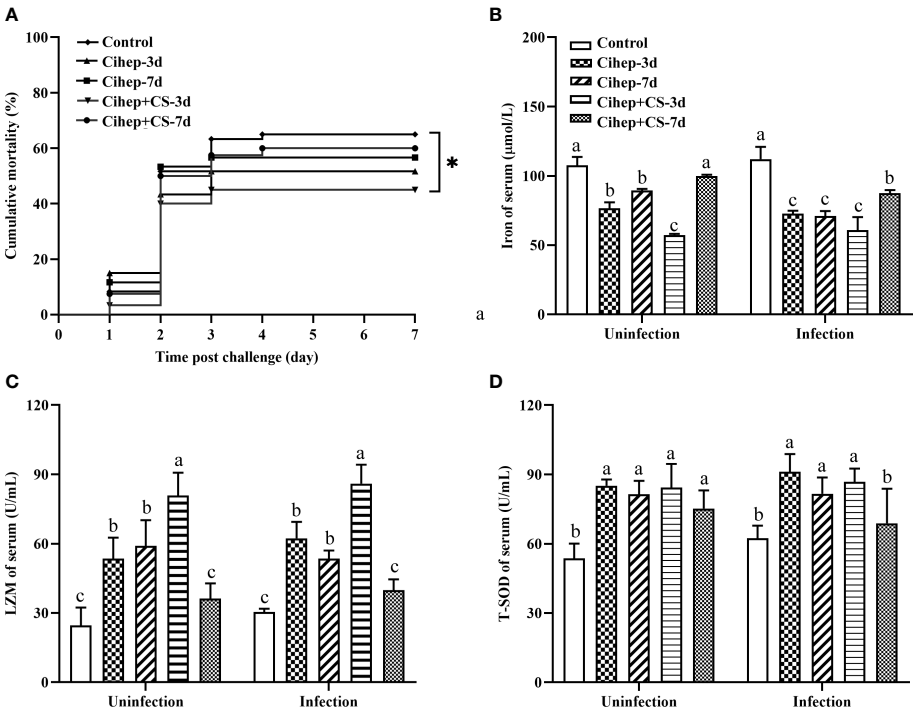

FIGURE 3
Mortality and Serum innate immune indexes statistics. **(A)** Fish in each group (n = 20) were challenged with 500 μL F. columnare (1×10^9 CFU/mL), and death events in each group were monitored on the next 7 days. p values were calculated by Log-rank (Mantel–Cox) Test (* $p < 0.05$). **(B-D)** Serum innate immune indicators. Iron of serum **(B)**, LZM **(C)** and T-SOD **(D)** were measured by commercial kits (Nanjing Jiancheng Institute of Biological Engineering, Nanjing, China). Different small letters in each group (a, b, and c) denote significant variations suggested by the multifactorial ANOVA statistics ($p < 0.05$).

but the expression level of *ferroportin* was lower than that in the control group (Figure 5D). After challenge, the expression levels of *hepcidin* were significantly higher in the four experimental groups than in the control group (Figure 5C).

Improving the diversity gut microbial by Cihep and CS in grass carp

As shown in Figure 6A, the stable curves indicated that the sequencing results were reliable. The alpha diversity of samples was analyzed based on Chao 1 index and Shannon index. As shown in Figures 6B, C, the Shannon index and Chao 1 index in Cihep-3d group and Cihep+cs-3d groups were significantly higher than those in the control group, but there was no difference between Cihep-3d group and Cihep+CS-3d group. As shown in Figure 6D, all samples of Cihep-3d group and Cihep-7d group were clustered together, and the samples of Cihep + CS-3d group and Cihep + CS-7d group were divided into two different clusters. Moreover, the samples of the four experimental groups and the control group are divided into different clusters, indicating that there are significant differences in gut microbiota between the experimental groups and the control group. In addition, we also found that the gut microbiota communities of grass carp fed with hepcidin alone and hepcidin + chitosan were significantly separated.

The distribution of gut microbiota of grass carp in each group was shown in Figure 7. At the phylum level (Figure 7A), the gut microbiota of grass carp mainly included *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Chloroflexi*. Among them, the abundance of *Proteobacteria* in the four experimental groups was significantly higher than that in the control group, but the abundance of *Fusobacteria* and *Chloroflexi* was significantly lower (Figure 7B). At the genus level (Figure 7C), Bosea and Caulobacteraceae-unclassified exhibited the highest abundance in the five groups. The abundance of Bradyrhizobium in the four experimental groups was significantly higher than that in the control group. It should be noted that Fusobacterium only existed in the control group (Figure 7D). Hierarchical cluster heatmap analysis showed that the gut microbiota in the control group and the experimental groups were not clustered in the same branch (Figure 8). In the Cihep + CS-3d group, the content of beneficial bacteria such as Lactobacillus and Bacillus increased significantly, while the content of harmful bacteria such as Enterobacter decreased significantly.

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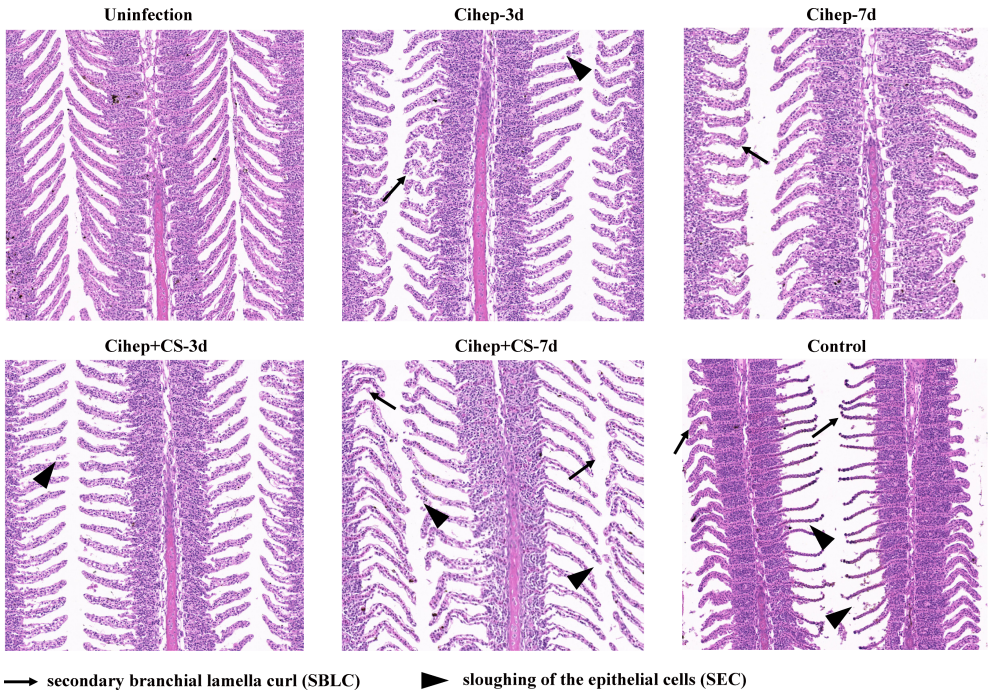

FIGURE 4
Gill tissue lesion post bacterial infection in grass carp. HE staining of gill, bar = 120 μm. Secondary branchial lamella curl (⟶), Sloughing of the epithelial cells (►), Epithelial hyperplasia (✚).

Discussion

Hepcidin is an antimicrobial peptide expressed in the liver. It has an ability to inhibit a variety of pathogens, regulate gut microbiota, and enhance immune functions (35–37). Many studies have revealed that antimicrobial peptides as feed additives can have a positive effect on the growth performance of numerous farmed animals (38–40). In this experiment, we found that the addition of hepcidin to the feed improved the growth performance and immunity function of grass carp. The highest WG, and SGR were observed in Cihep-3d group and Cihep+CS-3d group, and the second highest WG in the Cihep-7d group and Cihep+CS-7d group. This is in line with the previous findings that antimicrobial peptides can significantly improve growth performance in cultured fish (41–43).

Bony fish are at the lower stage of phylogeny and the acquired immune system is not developed enough. The innate immune system plays a crucial role in the resistance of fish to invasion by pathogenic organisms. The mortality is an important indicator reflecting organism's resistance to disease. The mortality of grass carp in the control group was higher than that in the four experimental groups after infection with *F. columnare*, indicating that the addition of hepcidin into feed can enhance the resistance of grass carp to *F. columnare* infection. Our results are in line with the previous report that the addition of Tilapia hepcidin 2-3 to grouper feed activated the innate immune system and significantly increased the survival rate of grouper infected with *V. alginolyticus* (44). The health status of the gills is highly dependent on the antioxidant capacity and immunity of the fish body, which can be well reflected by the disease resistance (45, 46). In this experiment, the disease resistance could be widely determined by intraperitoneal injection of *Flavobacterium columnare*. The mortality of grass carp after challenge was significantly reduced and the pathological symptoms of gill tissue were significantly improved by feeding hepcidin and chitosan. In addition, the activity of superoxide dismutase and the expression of immune genes were also significantly increased. These results suggested that Cihep and CS could significantly enhance serum enzyme activity and immune gene regulation compared with the control group.

Hepcidin, a major regulator of systemic iron homeostasis, regulates the expression of ferroportin on cell surface, thus reducing dietary iron uptake, eventually lowering plasma iron concentrations (17). The hepatopancreas is the main tissue synthesizing ferritin and storing iron (47). All biological processes require the involvement of iron, and iron ions play a key role in the interaction between the host and the pathogen (48, 49). Based on this, we hypothesized that iron regulators could prevent fish diseases by regulating iron metabolism.

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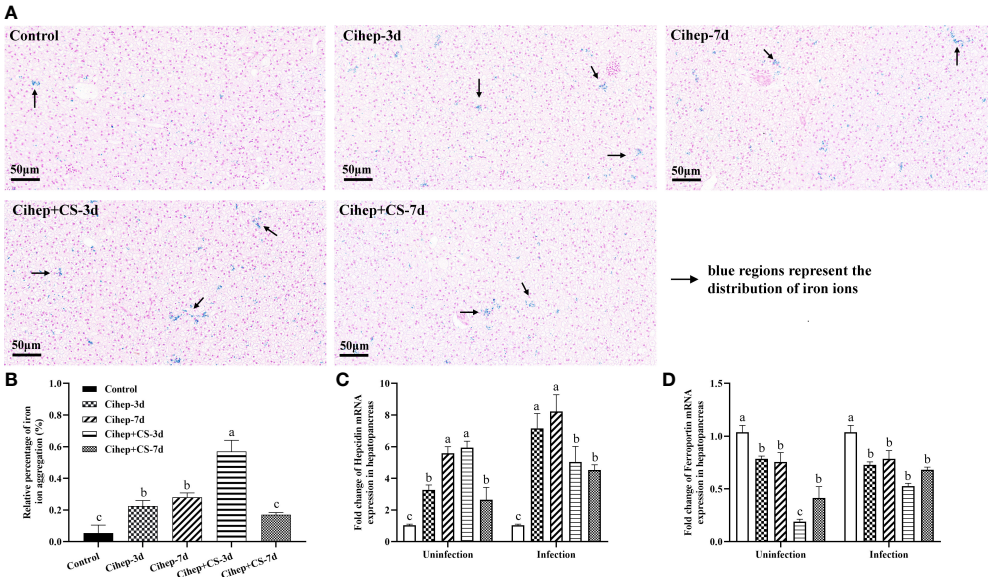

FIGURE 5
Regulation of hepatopancreas iron and iron metabolism-related genes by Cihep and CS. **(A)** Iron distribution in hepatopancreas. Using the PBS group as the control, the iron content in hepatopancreas was stained by Prussian blue, bar = 50 μm. Arrowheads in the image show the iron content in hepatopancreas. **(B)** The Prussian blue-stained sections from **(A)** were analyzed with ImageJ image-analysis software to calculate the relative percentage of iron ion aggregation. **(C)** mRNA expression expression of hepcidin gene in hepatopancreas. **(D)** mRNA expression expression of ferroportin gene in hepatopancreas. Different small letters in each group (a, b, and c) denote significant variations suggested by the multifactorial ANOVA statistics ($p < 0.05$).

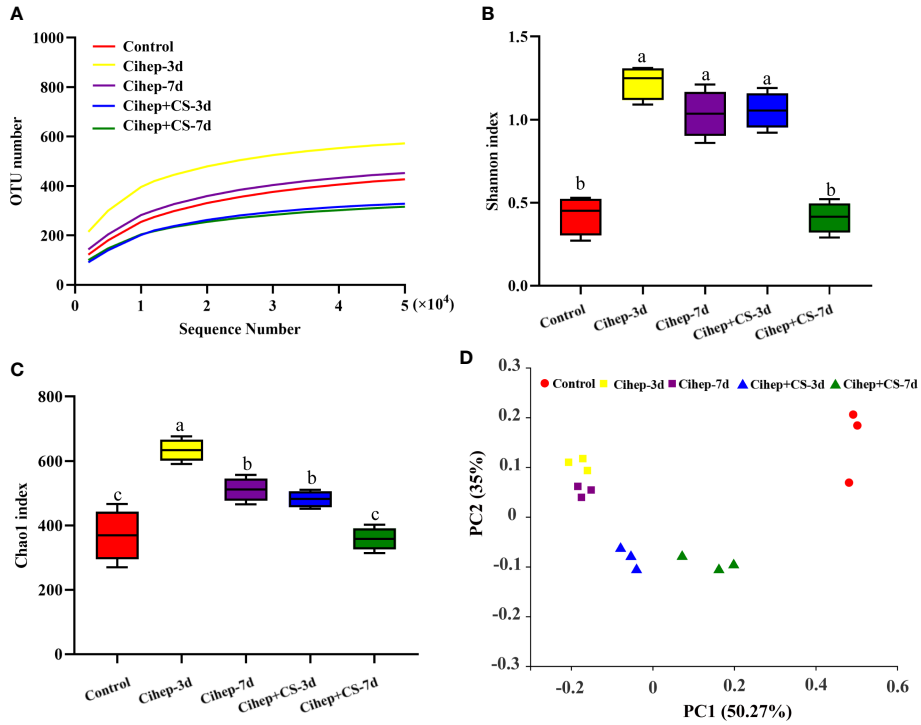

FIGURE 6
Intestinal 16S sequencing analysis of grass carp. **(A)** Rarefaction Observed species curves of OTUs clustered at 97% sequence (Y-axis indicates number of observed species). **(B, C)** Alpha-diversity index difference based on Chao1 metric and. Shannon index. **(D)** Beta-diversity index difference based on unweighted UniFrac. Different small letters in each group (a, b, and c) denote significant variations suggested by the multifactorial ANOVA statistics ($p < 0.05$).

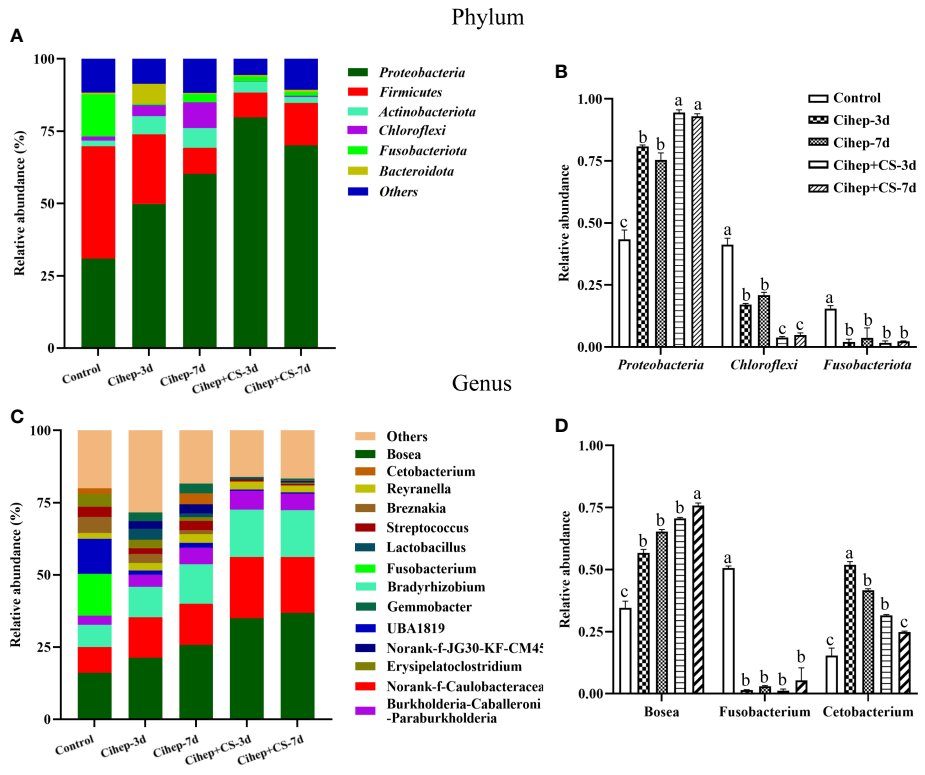

FIGURE 7
Sequencing analysis of gut microbiota species relative abundance in grass carp. **(A, B)** Relative abundance in phylum level. "Others" includes the sum of different phyla which are less than 1% in the sample. The representative differential phyla were statistically analyzed. **(C, D)** Relative abundance in genus level. "Others" includes the sum of different genera which are less than 1% in the sample. The representative differential genera were statistically analyzed. Different small letters in each group (a, b, and c) denote significant variations suggested by the multifactorial ANOVA statistics ($p < 0.05$).

Gut microbiota is a key factor in body development, immunity, and nutrient transformation (50). Research has proven that microbial-directed therapy is an excellent approach to improve the health of the host (51). Diet is the main factor affecting the proportion and content of microbiota in the gut (52). The gut microbiota of fish are remarkably plastic and can enhance host health by applying exogenous substances to improve the existing microbiota of fish (53). Therefore, this study focused on the correlation between gut microbiota and fish health. We found that hepcidin increased the gut microbiota α-diversity and β-diversity, thus significantly altering the community composition of grass carp gut microbiota. The microbial community with the highest abundance in the gut of grass carp mainly included *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Chloroflexi* in our study. Further, we found that the abundance of *Firmicutes* and *Bacteroidota* was significantly higher in the Cihep + CS-3d group than in the other groups. Short-chain fatty acids (SCFAs) are reported to be produced mainly by *Firmicutes* and *Bacteroidetes*, and SCFAs have anti-inflammatory effects to prevent metabolic diseases, especially acetate, propionate and butyrate which promote the absorption of inorganic ions in the intestine and have a role in the gut microbiota (54). Dietary supplementation of sodium butyrate has been reported to improve growth performance, antioxidative capacity, and intestinal absorption in grass carp (55). Butyrate supplementation to snapper feed also has a promotion effect on growth performance and intestinal metabolism (56). The genus Cetobacterium is one of the main bacterial species in the gut of herbivorous fish, and it is related to the digestive function of fish (57), which might explain why our Cihep-3d group achieved the most weight gain (WG) in grass carp.

The 3-day consecutive feeding followed by 3-day interval exhibited better protection effect than the 7-day consecutive feeding followed by 7-day interval. Immunological indicators of serum reflect the level of immunity of the body (58). In this experiment, compared with the control group, the activity of lysozyme in the serum of grass carp was higher at before and after infection. In particular, the up-regulation of lysozyme activity in Cihep -3d group and Cihep+CS-3d group was the most obvious after infection. Lysozyme, as a strong antibacterial enzyme, can inhibit the proliferation and colonization of bacteria by disrupting cell wall polysaccharides, thus leading to bacterial damage and death, and Lysozyme participates in body

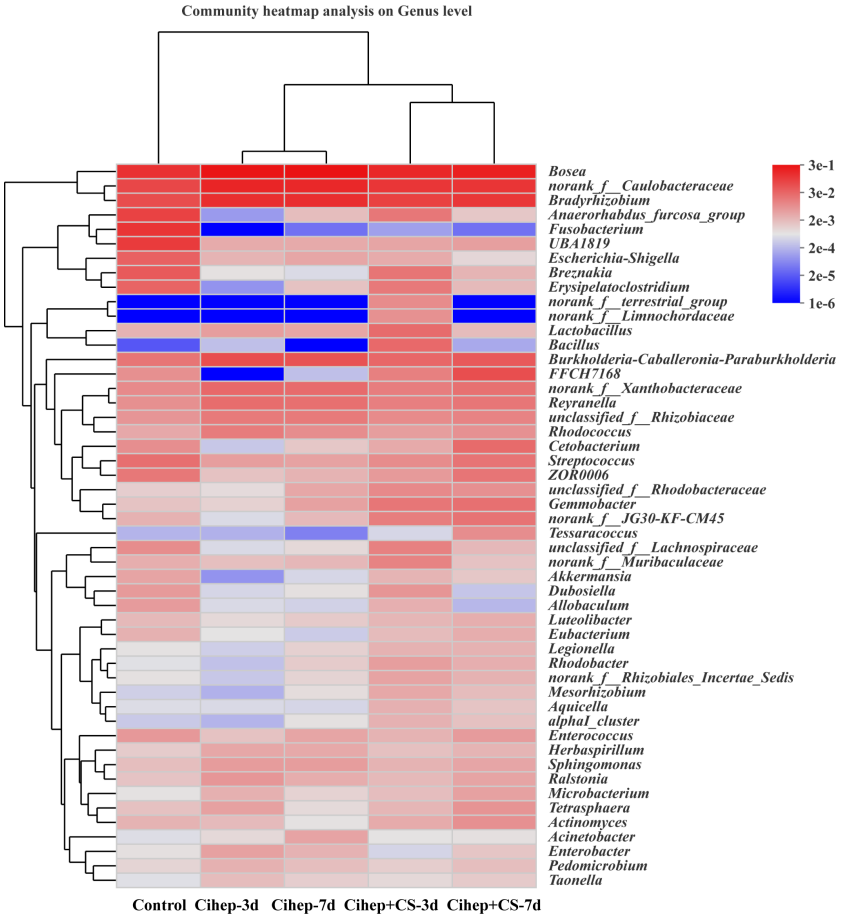

FIGURE 8
Heatmap of hierarchy cluster results for the relative abundance of all samples at genus level. The sample information is shown along the horizontal axis. Annotations are shown along the vertical axis on the right. The genera clustering tree is shown along the vertical axis on the left. The sample clustering tree is shown above. The color represents the Z-Score, which is the standardized relative abundance.

defense and immune regulation, thus it is an important indicator of fish innate immunity (59–61). In this study, the T-SOD content in Cihep-3d group and Cihep+CS-3d group was higher than that in the other three groups before and after the challenge. When bacterial infection in fish body causes inflammation, the body produces the large number of free radicals to resist the invasion of pathogenic bacteria (62). The accumulation of free radicals will cause the lipid peroxidation of the cell membrane, thus leading in membrane fission, eventually causing cell damage and even death (63). T-SOD is the most important and the best free radical scavenger in the organism, and it can maintain the metabolic balance of the body (7, 64). The above findings jointly explain why 3-day consecutive feeding followed by 3-day interval significantly improved the viability of grass carp. Therefore, our feeding mode is a very meaningful feeding strategy, which can provide an important reference for adding immune enhancers in aquaculture.

Our data indicated that the combined feeding of hepcidin and chitosan exhibited a significant better protective effect than feeding of hepcidin alone. Chitosan is a common feed additive due to low toxicity, high biocompatibility, low cost, and easy-to-handle properties (65, 66). The addition of chitosan to the diet of juvenile crucian carp (*Carassius auratus*) has significantly reduced the abundance and diversity of intestinal bacteria (67). Similarly, the addition of chitosan to the diet has a regulatory effect on certain non-specific immune functions (68, 69). These previous findings were supported by our results that the Cihep+CS-3d group had the lowest cumulative mortality. Chitosan can exert the immunostimulatory effect possibly by promoting the function of inflammatory cells and inducing an inflammatory response (70). We also found that the expression of *IL-1β* and *TNF-α* in the Cihep+CS-3d group was significantly higher than that in the control group. *IL-1β* and *TNF-α* are the most effective molecules in the innate immune system, and they

jointly mediate the immune response (71, 72), which might explain why the combined application of hepcidin and chitosan can significantly improve the immunity of fish in our study. In conclusion, the protective effect of hepcidin in combination with chitosan is superior to that of hepcidin alone. Our findings provide a new perspective for the combined application of immune enhancers.

Conclusion

In summary, this study showed that oral administration of recombinant hepcidin improved the growth performance and regulated the iron metabolism. The immunity and survival rate of grass carp were improved. The 3-day consecutive feeding followed by 3-day interval exhibited better protection effect than the 7-day consecutive feeding followed by 7-day interval, and hepcidin in combination with chitosan was better than that of hepcidin alone.

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 *via* accession ID: PRJNA900049.

Ethics statement

The animal study was reviewed and approved by the Ethical Committee on Animal Research at Huazhong Agricultural University (ID Number: HZAUFI-2021-0012).

Author contributions

JZ and MF conceived and designed the experiments, and wrote the manuscript. MF and WZ performed the experiments and analyzed the data. QZ and RK provided guidance on the experimental design. JS and GY revised the manuscript critically. All authors reviewed the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was jointly supported by the National Natural Science Foundation of China (32273179) and the Fundamental Research Funds for the Central Universities (2662019PY068).

Acknowledgments

Thank you very much to Dr. Xiaoling Liu, Miss Manquan Fu, Mr. Jiahui Jiang, and Mr. Weicheng Wang for their technical advice and assistance in experiments.

Conflict of interest

Author JZ and QZ were employed by Wuhan DaBeiNong Aquaculture Technology Co. LTD, China.

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1075128/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 21 December 2022
DOI 10.3389/fimmu.2022.1079677

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Comparative Immunology, a section of the journal Frontiers in Immunology

RECEIVED 25 October 2022
ACCEPTED 08 December 2022
PUBLISHED 21 December 2022

CITATION
Liu Y, Ma S, Lv W, Shi H, Qiu G, Chang H, Lu S, Wang D, Wang C, Han S and Liu H (2022) Effects of replacing fishmeal with cottonseed protein concentrate on growth performance, blood metabolites, and the intestinal health of juvenile rainbow trout (*Oncorhynchus mykiss*). *Front. Immunol.* 13:1079677. doi: 10.3389/fimmu.2022.1079677

Effects of replacing fishmeal with cottonseed protein concentrate on growth performance, blood metabolites, and the intestinal health of juvenile rainbow trout (*Oncorhynchus mykiss*)

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Cottonseed protein concentrate (CPC) is a potential non-food protein source for fishmeal replacement in fish feed. However, a high inclusion level of CPC in diets may have adverse effects on the metabolism and health of carnivorous fish. This study aimed to investigate CPC as a fishmeal alternative in the diet of rainbow trout *Oncorhynchus mykiss* based on growth performance, blood metabolites, and intestinal health. Five isonitrogenous (46% crude protein) and isolipidic (16% crude lipid) diets were formulated: a control diet (30% fishmeal) and four experimental diets with substitution of fishmeal by CPC at 25%, 50%, 75%, and 100%. A total of 600 fish (mean body weight 11.24g) were hand-fed the five formulated diets to apparent satiation for eight weeks. The results showed no adverse effects on growth performance when 75% dietary fishmeal was replaced by CPC. However, reduced growth and feed intake were observed in rainbow trout fed a fishmeal-free diet based on CPC (CPC100%). Changes in serum metabolites were also observed in CPC100% compared with the control group, including an increase in alanine aminotransferase (ALT), a decrease in alkaline phosphatase (ALP), alterations in free amino acids, and reductions in cholesterol metabolism. In addition, the CPC-based diet resulted in reduced intestinal trypsin, decreased villus height and width in the distal intestine, upregulated mRNA expression levels of inflammatory cytokines in the

intestine, and impaired gut microbiota with reduced bacterial diversity and decreased abundance of *Bacillaceae* compared with the control group. The findings suggest that the optimum substitution rate of dietary fishmeal by CPC for rainbow trout should be less than 75%.

KEYWORDS

cottonseed protein concentrate, rainbow trout, growth, blood metabolites, intestinal health

1 Introduction

Fishmeal replacement is one of the dominant research fields in aquaculture due to the urgent need for reducing fishmeal to the maximum extent in aquafeed for sustainable aquaculture (1, 2). A variety of protein sources have been investigated as fishmeal alternatives, including terrestrial animal proteins, plant products, and single-cell protein sources (3–5). Among these alternative protein sources, plant proteins possess certain positive characteristics, including wide availability, high production, competitive price, relatively high protein content, and reasonably balanced amino acid profiles (6). Some plant feedstuffs have been widely used in aquafeed, including soy products, corn, wheat, and rapeseed (7, 8). However, most of the plant feedstuffs have nutritional limitations (e.g., adverse antinutrient and toxic effects), resulting in low substitution rates of fishmeal in the diets of carnivorous fish species (6, 9). More importantly, nearly all current plant production can be consumed by humans, resulting in food-feed competition (10). Competition for the use of plant-based ingredients in aquafeed also comes from livestock, agriculture sectors, and biofuel production (3, 10).

Cottonseed protein concentrate (CPC) is a novel non-food protein derived from cottonseed. It contains a high protein content (crude protein: 60-70%) with a relatively balanced amino acid profile and low levels of anti-nutrients (e. g., gossypol) (11). Previous studies have shown that CPC can partially replace fishmeal in the diets of carnivorous fish, but the optimum substitution rate of fishmeal varied among species. It was reported that substituting 12-36% fishmeal protein exhibited no negative effects on growth performance of hybrid grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatu*) (12). A recent study on largemouth bass (*Micropterus salmoides*) revealed that the optimum substitution rate of dietary fishmeal should be less than 75% (13). However, excessive inclusion of CPC in diets may result in growth suppression, liver inflammation, and impaired intestinal histology (12–14).

Rainbow trout (*Oncorhynchus mykiss*) is a typical widely cultured carnivorous fish species, with an annual worldwide production of over 0.8 million tons (15). The dietary protein requirement for rainbow trout is generally over 40%, and high-quality fishmeal is still one of the primary protein sources in commercial trout feed (9). Soy product is currently the most dominant plant protein used in the diets of rainbow trout (10, 16). However, the nutritional limitations and competition from human food and livestock diets limit its further application in trout feed. CPC is a potential alternative protein source for rainbow trout. Previous studies have shown that there were no adverse effects on growth or intestinal histomorphology in rainbow trout when 10-50% dietary fishmeal was replaced by CPC (17). However, replacing dietary fishmeal in diets at higher inclusion levels by CPC for rainbow trout has not been reported. Therefore, the purpose of the present study was to investigate CPC as a fishmeal alternative at high replacement levels in the diets of rainbow trout based on growth performance, blood metabolites, and intestinal health.

2 Materials and methods

2.1 Diet

Five isonitrogenous (46% crude protein) and isolipidic (16% crude lipid) diets were formulated, including a control diet (C1, 30% fishmeal) and four experimental diets with graded levels of CPC, corresponding to the substitution rates of 25% (C2), 50% (C3), 75% (C4), and 100% (C5) for dietary fishmeal protein (Tables 1, 2). The CPC was obtained from Xinjiang Jinlan Vegetable Protein Co. Ltd. (China). It contained 65% crude protein and 230mg/kg free gossypol. The feed was processed using a laboratory granulator (HKJ-218, Tongli Grain Machinery, China) and made into 1.5-mm (diameter) pellets. All diets were kept at 4°C for use after being dried in a ventilated oven (60°C, 1.5 h).

2.2 Fish and feeding trial

Rainbow trout used in the experiment were obtained from Agrimarine Industries Inc. (Benxi, China). After a three-week acclimation, a total of 600 healthy fish with similar size were selected and randomly distributed among 20 tanks (30 individuals/tank) with the five diets in quadruplicate. The feeding trial was conducted in a recirculating aquaculture system with a total water

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

Diet	FM	CPC25%	CPC50%	CPC75%	CPC100%
Ingredients					
Fishmeal[1]	30.00	22.50	15.00	7.50	0.00
Black soldier fly meal	5.00	5.00	5.00	5.00	5.00
Cottonseed protein concentrate[2]	0.00	7.50	15.00	22.50	30.00
Corn protein concentrate	12.00	12.00	12.00	12.00	12.00
Soy protein concentrate	15.00	15.00	15.00	15.00	15.00
Wheat gluten meal	2.00	2.00	2.00	2.00	2.00
Wheat flour	19.58	19.38	19.23	19.03	18.83
Fish oil	12.00	12.00	12.00	12.00	12.00
Premix[3]	1.00	1.00	1.00	1.00	1.00
L-lysine[4]	1.00	1.20	1.35	1.55	1.75
DL-methionine[5]	0.30	0.30	0.30	0.30	0.30
Taurine[6]	0.50	0.50	0.50	0.50	0.50
Sodium alginate	1.00	1.00	1.00	1.00	1.00
Chloride choline[7]	0.60	0.60	0.60	0.60	0.60
BHT[8]	0.02	0.02	0.02	0.02	0.02
Approximate chemical composition					
Crude protein	46.08	46.00	45.89	45.82	45.74
Crude lipid	16.09	16.07	16.05	16.05	16.02
Gross energyMJ/kg	21.79	21.96	22.12	22.28	22.45

[1]Fishmeal: from TASA Fish Product Co., Ltd., Peru.
[2]Cottonseed protein concentrate: Xinjiang Jinlan Co. Ltd, China.
[3]Premix (mg/kg or IU/kg diet): VA 750000 IU, VD_3 200000 IU, VE 6000 mg, VK_3 2000 mg, VB_1 1200 mg, VB_2 1200 mg, VB_6 1200 mg, VB_{12} 8 mg, VC 21000 mg, D-calcium pantothenate 2000 mg, niacinamide 9000 mg, folic acid 370 mg, D-biotin 15 mg, nositol 10000 mg, $MgSO_4$ 6000 mg, $ZnSO_4$ 4000 mg, $MnSO_4$ 2500 mg, $CuSO_4$ 2500 mg, $FeSO_4$ 2500 mg, $CoSO_4$ 160 mg, $Ca(IO_3)_2$ 200 mg, Na_2SeO_3 40 mg.
[4]L-lysine, 98%, Macklin Inc., Shanghai, China.
[5]DL-methionine, 99%, Macklin Inc., Shanghai, China.
[6]Taurine, 99%, Macklin Inc., Shanghai, China.
[7]Chloride choline, 50%, Jujia Biotech Co., Ltd, Shandong, China.
[8]BHT, butylated hydroxytoluene, >99.0%, Aladdin Biochemical Technology, Co., Ltd, Shanghai, China.

volume of 8 m^3. During the feeding trial, juvenile rainbow trout were hand-fed to apparent satiation twice daily (8:30 and 16:00). Water temperature was controlled at 12.5°C~13.5°C, and the inflow rate was maintained at 0.4 m/s. Dissolved oxygen was kept above 8 mg/L with a water change of 15–20% daily and a photoperiod of 12L: 12D. The feeding trial lasted eight weeks.

2.3 Sample collection

At the end of the feeding trial, all fish were weighed in batches with measurement for body lengths after anaesthetization with MS-222 (200 mg/L). Five fish from each tank were randomly selected for blood sampling and further serum samples. The viscera and liver of each fish were also weighed for the calculation of viscerosomatic index and hepatosomatic index. Afterwards, gut contents, intestine, and liver samples were collected. All gut contents and liver samples were stored at liquid nitrogen until further analysis. Three intestine samples were also kept in liquid nitrogen, and another two intestine samples, including proximal intestine and distal intestine, were stored in 10% neutral formalin (RightTech, Changchun, China) for histological analysis.

2.4 Biochemical analysis and histological analysis

Proximate composition and content of amino acids in ingredients, diets, and fish samples were determined by

10.3389/fimmu.2022.1079677

TABLE 2 Amino acid composition of the experimental diets (% dry matter).

Amino Acids	FM	CPC25%	CPC50%	CPC75%	CPC100%
Met	1.15	1.10	1.06	1.01	0.97
Cys	0.48	0.57	0.66	0.71	0.78
Lys	2.88	2.85	2.78	2.73	2.69
Thr	1.60	1.55	1.51	1.46	1.41
Ile	1.75	1.71	1.62	1.58	1.50
His	1.00	1.02	1.03	1.05	1.07
Val	2.01	1.98	1.96	1.93	1.90
Leu	3.52	3.48	3.45	3.41	3.37
Arg	2.45	2.74	3.08	3.34	3.70
Phe	1.91	1.97	2.08	2.14	2.22
Tyr	1.74	1.67	1.61	1.54	1.48
Glu	6.42	6.75	7.13	7.47	7.84
Asp	3.36	3.37	3.37	3.38	3.39
Gly	2.20	2.02	1.87	1.70	1.55
Ser	1.92	1.91	1.90	1.89	1.89
Ala	2.38	2.25	2.12	2.04	1.90
Pro	2.60	2.47	2.30	2.15	2.03

standard methods of AOAC (2012) (18). The proximate composition included moisture, crude protein, crude lipid, and ash. Serum biochemical indicators were measured using a BECKMAN CX4 automatic analyzer with commercial kits (Beckman, CA, USA). Targeted metabolomic analyses were conducted to determine free amino acids and free sterols using UPLC-MS/MS according to previously reported methods (19–21). Digestive enzyme activity in the intestine was measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, China), including those for trypsin, lipase, and amylase. Proximal and distal intestine for histological analysis was examined by light microscopy after dehydration with ethanol, embedding in paraplast, and staining with hematoxylin and eosin.

2.5 Analysis of gut microbiota

Total DNA of bacteria was extracted from gut contents using a MP FastDNA Spin Kit (MP Biomedicals, Irvine, CA, USA) (n = 7). The quality of the extracted DNA was evaluated by 1% agarose gel electrophoresis. The V3-V4 hypervariable regions of the bacterial 16S rRNA were amplified by universal primers 338F and 806R using previously reported conditions (22). After purification, all amplicons were sequenced using an Illumina MiSeq platform (Illumina, San Diego, CA, USA).

For bioinformatic analysis of gut bacteria, assignment of qualified reads was performed based on operational taxonomic units (OTUs) at a similarity of 97% using the QIIME pipeline. (version 1.8.0) (23). The α-diversity was evaluated based on Sobs, Shannon, ACE, Chao, and Coverage indices. The β-diversity analysis was conducted by PCA using the Vegan package (Community Ecology Package) in the R language. Unique and shared OTUs between groups were analyzed by Venn diagrams. Differences of intestinal bacteria between groups were identified at the phylum and genus levels.

2.6 Gene expression

Total RNA was isolated from intestine (n = 6) using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and then was reverse transcribed to cDNA (Takara, Dalian, China). Quantitative real-time PCR was conducted in a Real-Time PCR System (Applied Biosystems 7500) in triplicate using Takara TB Green Premix Ex Taq II (Tli RNaseH Plus) following the manufacturer's instructions. Efficiencies of the specific primers used in this

study were evaluated according to previously reported methods (24) (Table S1). The primers of the target and reference genes are listed in (43–46) (Table 3). Gene expression levels were calculated according to the threshold cycle ($2^{-\Delta\Delta Ct}$) method (24).

2.7 Statistical analysis

Differences among the five groups were analyzed based on one-way ANOVA with Duncan's multiple range test using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Comparisons between two groups were analyzed by independent samples t-test using the same software. Significant differences were determined by $P< 0.05$.

3 Results

3.1 Growth and feed utilization

At the end of the feeding trial, no significant differences in growth performance were observed among the control group (FM), CPC25%, CPC50%, and CPC75% ($P > 0.05$). However, complete fishmeal replacement by CPC (CPC100%) resulted in reduced feed intake and growth ($P < 0.05$). There was a significant decrease of condition factor (CF) in CPC75% and CPC100% compared with other groups ($P < 0.05$). Reductions of viscerosomatic index (VSI) and hepatosomatic index (HSI) were also found in experimental fish fed diets containing graded CPC

TABLE 3 Specific primers for Real-time PCR.

Target gene	Primer	Sequence (5′→3′)	Accession number
β-actin[43]	F	ACAGACTGTACCCATCCCAAAC	AJ438158
	R	AAAAAGCGCCAAAATAACAGAA	
C3[44]	F	GGCCAGTCCCTGGTGGTTA	L24433
	R	GGTGGACTGTGTGGATCCGTA	
C4[44]	F	TCTACAACCCTACACAGCAAGTGAG	AJ544262
	R	TGCCCGCAGCATTAAAAATAG	
IL1β[45]	F	ACCGAGTTCAAGGACAAGGA	AJ223954
	R	CATTCATCAGGACCCAGCAC	
IL8[45]	F	CACAGACAGAGAAGGAAGGAAAG	AJ279069
	R	TGCTCATCTTGGGGTTACAGA	
TNF-α[43]	F	GGGGACAAACTGTGGACTGA	AJ277604
	R	GAAGTTCTTGCCCTGCTCTG	
IL10[45]	F	CGACTTTAAATCTCCCATCGAC	AB118099
	R	GCATTGGACGATCTCTTTCTTC	
TGF-β[45]	F	AGATAAATCGGAGAGTTGCTGTG	AJ007836
	R	CCTGCTCCACCTTGTGTTGT	
OCLN[45]	F	CAGCCCAGTTCCTCCAGTAG	GQ476574
	R	GCTCATCCAGCTCTCTGTCC	
TRIC[45]	F	GTCACATCCCCAAACCAGTC	KC603902
	R	GTCCAGCTCGTCAAACTTCC	
ZO1[45]	F	AAGGAAGGTCTGGAGGAAGG	HQ656020
	R	CAGCTTGCCGTTGTAGAGG	
CLD1[46]	F	GAGGACCAGGAGAAGAAGG	BK008768
	R	AGCCCCAACCTACGAAC	

C3, complement component 3; C4, complement component 4; IL1β, interleukin 1 beta; IL8, interleukin 8; TNFα, tumor necrosis factor alpha; IL10, interleukin 10; TGFβ, transforming growth factor beta; OCLN, occludin; TRIC, tricellulin; ZO-1, zonula occludens-1; CLD1, claudin-1.

10.3389/fimmu.2022.1079677

TABLE 4 Growth, feed utilization and survival of rainbow trout fed diets containing graded CPC (mean ± S.E.).

Item	FM	CPC25%	CPC50%	CPC75%	CPC100%
Initial body weight (g)	11.26 ± 0.12	11.24 ± 0.10	11.27 ± 0.03	11.17 ± 0.14	11.22 ± 0.04
Final body weight (g)	46.15 ± 0.81^{b}	44.50 ± 1.03^{b}	44.76 ± 0.17^{b}	44.34 ± 0.83^{b}	40.36 ± 0.43^{a}
Weight gain (%)	309.88 ± 4.39^{b}	295.85 ± 8.02^{b}	297.11 ± 2.78^{b}	297.03 ± 3.42^{b}	260.62 ± 3.12^{a}
SGR (%/day)	2.52 ± 0.02^{b}	2.46 ± 0.04^{b}	2.46 ± 0.01^{b}	2.46 ± 0.02^{b}	2.29 ± 0.02^{a}
FR (%/day)	2.27 ± 0.04^{b}	2.22 ± 0.03^{b}	2.22 ± 0.03^{b}	2.20 ± 0.02^{b}	2.06 ± 0.02^{a}
FCR	1.04 ± 0.01	1.04 ± 0.02	1.04 ± 0.01	1.03 ± 0.01	1.03 ± 0.02
CF (g cm^{-3})	1.51 ± 0.01^{b}	1.54 ± 0.02^{b}	1.56 ± 0.02^{b}	1.43 ± 0.02^{a}	1.39 ± 0.01^{a}
VSI (%)	17.34 ± 0.28^{c}	16.40 ± 0.36^{b}	15.81 ± 0.40^{ab}	15.52 ± 0.24^{ab}	14.95 ± 0.26^{a}
HSI (%)	1.50 ± 0.04^{c}	1.48 ± 0.04^{c}	1.33 ± 0.04^{b}	1.30 ± 0.03^{ab}	1.22 ± 0.04^{a}
Survival rate (%)	99.17 ± 0.83	99.17 ± 0.83	99.17 ± 0.83	99.17 ± 0.83	97.50 ± 0.83

IBW, Initial body weight; FBW, Final body weight;
Weight gain (WG, %)=100×[(FBW − IBW) × IBW^{-1}].
Specific growth rate (SGR, %/day)=100×(ln FBW − ln IBW)/days;
Feeding rate (FR, %/day)=100×FI/[days×(FBW − IBW)/2];
Feed conversion ratio (FCR)=FI/WG;
Condition factor (CF, g cm^{-3}) = 100×body weight/(body length)^{3}, n=16;
Viscerosomatic index (VSI, %) = 100×viscera wet weight/body weight, n=16;
Hepatosomatic index (HSI, %) = 100 ×liver wet weight/body weight, n=16;
Survival rate = 100×(final number of fish)/(initial number of fish).
values with different superscripts in the same column are significantly different (P<0.05).

TABLE 5 Chemical composition of whole-body fish fed diets containing graded CPC (mean ± S.E, *n*=8).

Item	Moisture	Crude protein	Crude lipid	Ash
FM	68.06 ± 0.44	17.14 ± 0.20	10.55 ± 0.09	1.54 ± 0.03
CPC25%	67.71 ± 0.57	17.69 ± 0.28	10.58 ± 0.11	1.60 ± 0.03
CPC50%	68.15 ± 0.46	17.35 ± 0.33	10.28 ± 0.08	1.53 ± 0.04
CPC75%	67.91 ± 0.67	17.15 ± 0.13	10.41 ± 0.10	1.57 ± 0.03
CPC100%	67.31 ± 0.34	17.89 ± 0.20	10.61 ± 0.14	1.60 ± 0.04

Values with different superscripts in the same column are significantly different (P<0.05).

($P < 0.05$). Survival rates in all groups were higher than 97%, with no significant difference ($P > 0.05$) (Table 4).

3.2 Chemical composition of whole fish

No significant difference in whole-body composition was found among groups, including moisture, crude protein, crude lipid, and ash ($P > 0.05$) (Table 5).

3.3 Blood metabolites

An increase in serum ALT and a reduction of serum ALP were observed in groups with graded CPC ($P < 0.05$). There was also a significant decrease in serum TC of CPC100% compared with that in FM ($P < 0.05$) (Table 6).

Analysis of free amino acids in the serum showed that lysine, serine, proline, glutamic acid, methionine, tyrosine, and cysteine in CPC100% were significantly lower than those in FM ($P < 0.05$). However, valine, leucine, histidine, arginine, and tryptophan in CPC100% were significantly higher than those in FM ($P < 0.05$) (Table 7).

Targeted metabolomics for free cholesterol in serum showed no significant difference in free cholesterol between FM and CPC100%. However, decreases in ergosterol, lathosterol, and cholestanol and increases in desmosterol and 7-dehydrocholesterol in CPC100% were observed compared with FM (Table 8).

TABLE 6 Effects of fishmeal replacement by cottonseed protein concentrate on serum chemistry of rainbow trout (Mean ± S.E., *n*=6).

Item	FM	CPC25%	CPC50%	CPC75%	CPC100%
ALT(U/L)	201.48 ± 5.17^{a}	262.18 ± 10.35^{b}	255.50 ± 3.16^{b}	275.88 ± 14.97^{bc}	306.93 ± 19.74^{c}
GLU(g/L)	22.45 ± 0.46	22.98 ± 0.33	23.35 ± 0.64	23.40 ± 0.12	22.90 ± 0.36
ALP(U/L)	428.83 ± 22.25^{d}	315.03 ± 22.96^{c}	323.73 ± 36.78^{c}	240.25 ± 20.18^{b}	142.18 ± 7.27^{a}
TP(g/L)	40.05 ± 0.44	40.83 ± 0.39	41.63 ± 1.01	41.58 ± 0.17	40.68 ± 0.64
ALB(g/L)	17.72 ± 0.33	17.98 ± 0.28	18.28 ± 0.38	18.38 ± 0.08	17.78 ± 0.32
TC(mmol/L)	10.14 ± 0.26^{b}	10.31 ± 0.29^{b}	10.20 ± 0.70^{b}	9.16 ± 0.30^{ab}	8.45 ± 0.33^{a}
TG(mmol/L)	7.18 ± 0.57	6.96 ± 0.58	6.23 ± 0.34	7.24 ± 0.20	6.38 ± 0.33
HDL-C(mmol/L)	3.72 ± 0.12	3.73 ± 0.22	3.87 ± 0.14	3.44 ± 0.11	3.44 ± 0.13
LDL-C(mmol/L)	7.22 ± 0.15	7.32 ± 0.40	7.52 ± 0.24	6.71 ± 0.23	6.67 ± 0.25
TBA(μmmol/L)	4.78 ± 0.53	5.58 ± 0.92	5.66 ± 0.33	5.54 ± 0.63	5.12 ± 0.43

TP, total protein; ALB, albumin; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglyceride; TBA, total bile acid; GLU, glucose; ALT, alanine aminotransferase; ALP, alkaline phosphatase;
Values with different superscripts in the same column are significantly different (P<0.05).

TABLE 7 Free amino acid in serum of rainbow trout fed different diets (Mean ± S.E., *n*=6).

Amino acid (μmol/L)	FM	CPC100%
Lysine	238.85 ± 4.03^{b}	205.83 ± 9.93^{a}
Glycine	1237.42 ± 77.53	1062.86 ± 51.43
Alanine	509.76 ± 21.49	501.62 ± 11.20
Serine	171.38 ± 11.55 ^{b}	130.65 ± 3.83 ^{a}
Proline	271.79 ± 32.64^{b}	117.80 ± 10.66^{a}
Valine	404.93 ± 8.64^{a}	454.49 ± 15.78^{b}
Threonine	195.80 ± 13.69	165.10 ± 17.15
Leucine	453.09 ± 16.09^{a}	535.21 ± 25.02^{b}
Isoleucine	95.74 ± 11.40	109.90 ± 10.99
Asparagine	51.87 ± 4.35	66.71 ± 8.44
Aspartic acid	48.79 ± 2.80	39.73 ± 2.91
Glutamine	322.60 ± 35.60	323.62 ± 19.57
Glutamic acid	148.36 ± 6.87^{b}	112.81 ± 7.54^{a}
Methionine	242.56 ± 23.44^{b}	142.75 ± 3.63^{a}
Histidine	182.13 ± 8.57^{a}	312.95 ± 16.25^{b}
Phenylalanine	164.39 ± 6.18	184.35 ± 6.60
Arginine	98.49 ± 3.18^{a}	128.98 ± 6.96^{b}
Tyrosine	107.48 ± 6.28^{b}	84.31 ± 4.91^{a}
Tryptophan	29.17 ± 1.77 ^{a}	36.05 ± 1.84 ^{b}
Cysteine	3.60 ± 0.12^{b}	2.20 ± 0.08^{a}

Values with different superscripts in the same column are significantly different (P<0.05).

10.3389/fimmu.2022.1079677

TABLE 8 Free sterols in serum of rainbow trout fed different diets (Mean ± S.E., *n*=6).

Amino acid (nmol/L)	FM	CPC100%
Pregnenolone	6.76 ± 0.65	6.07 ± 0.29
Cholesterol 5α,6α-epoxide	270.47 ± 13.96	264.05 ± 3.33
Desmosterol	2995.46 ± 217.69[a]	3758.26 ± 61.17[b]
7-Dehydrocholesterol	6658.35 ± 551.79[a]	8578.67 ± 139.63[b]
Ergosterol	96.93 ± 8.92[b]	28.47 ± 1.61[a]
Lathosterol	1592.53 ± 235.85[b]	868.10 ± 74.58[a]
Brassicasterol	22.50 ± 5.87	19.83 ± 1.76
Cholesterol	2240990.41 ± 76364.64	2153811.15 ± 52256.36
Lanosterol	3531.64 ± 516.72	3248.41 ± 208.40
Campesterol	8147.26 ± 478.64	9506.76 ± 478.28
Cholestanol	7540.16 ± 556.70[b]	4615.27 ± 62.12[a]
β-Sitosterol	19876.29 ± 1672.66	24641.22 ± 2290.78

Values with different superscripts in the same column are significantly different (P<0.05).

3.4 Digestive enzyme activity in the intestine

The activity of intestinal trypsin in CPC75% and CPC100% was significantly lower than that in FM ($p < 0.05$), and there were no significant differences among FM, CPC25%, and CPC50% ($P > 0.05$) (Figure 1A). An increase in activity of intestinal lipase was observed in groups with graded CPC, and the activity of intestinal lipase in CPC50%, CPC75%, and CPC100% was significantly higher than that in FM and CPC25% ($P < 0.05$) (Figure 1B). No significant difference in activity of intestinal amylase was observed among groups ($P > 0.05$) (Figure 1C).

3.5 Intestinal histology

Villus height and width in the distal intestine decreased in groups with graded CPC, and a significant reduction was observed in CPC25%, CPC50%, CPC75% and CPC100% compared with FM ($P < 0.05$) (Table 9, Figure 2). However, no significant difference in muscular thickness of the distal intestine was observed between groups ($P > 0.05$) (Table 9, Figure 2). In addition, no significant differences in muscular thickness, villus height, or width in the proximal intestine were found among groups with graded CPC ($P < 0.05$) (Table 10, Figure 3).

3.6 Expression of immune genes and tight junction protein genes in intestine

Upregulations in mRNA expression levels of inflammatory cytokines were observed in groups with graded CPC, including increases of IL-1β, IL-8, and TNF-α in CPC75% and CPC100% compared with FM, and an increase of IL-10 in CPC100% compared with other groups ($P < 0.05$) (Figures 4A, B). However, no significant difference in the mRNA expression level of TGF-β was found between groups ($P > 0.05$) (Figure 4B). Also, there was an increase in the mRNA expression level of complement system genes *c3* and *c4* in the intestine with graded CPC in diets ($P < 0.05$) (Figure 5). In addition, no significant differences in the mRNA expression levels of tight junction protein genes (ZO1, TRIC, OCLN, CLD1) in the intestine were observed between groups ($P > 0.05$) (Figure 6).

3.7 Gut microbiota

A total of 1961271 clean sequences were obtained from 35 samples (seven per group) with an average length of 416 bp. The rarefaction curves tended to saturation, indicating that the raw data can be used for further analysis (Figure S2). The analysis of alpha diversity indices showed that there were decreases in the Sobs, Shannon, Ace, and Chao indices with graded CPC in diets, including reductions of Sobs and Shannon indices in CPC50%, CPC75% and CPC100%, and decreases in the Ace and Chao indices in CPC25%, CPC50%, CPC75% and CPC100% compared with those in FM ($P < 0.05$) (Table S2). Sequencing coverage in each group was above 99%, and no significant difference was observed between groups ($P > 0.05$) (Table S2). In particular, there was a clear separation between FM and CPC100% based on the PCA plot (Figure S2).

The Venn diagram analysis showed a total of 179 shared OTUs between groups, and the numbers of unique OTUs in FM, CPC25%, CPC50%, CPC75% and CPC100% were 61, 24, 44, 11,

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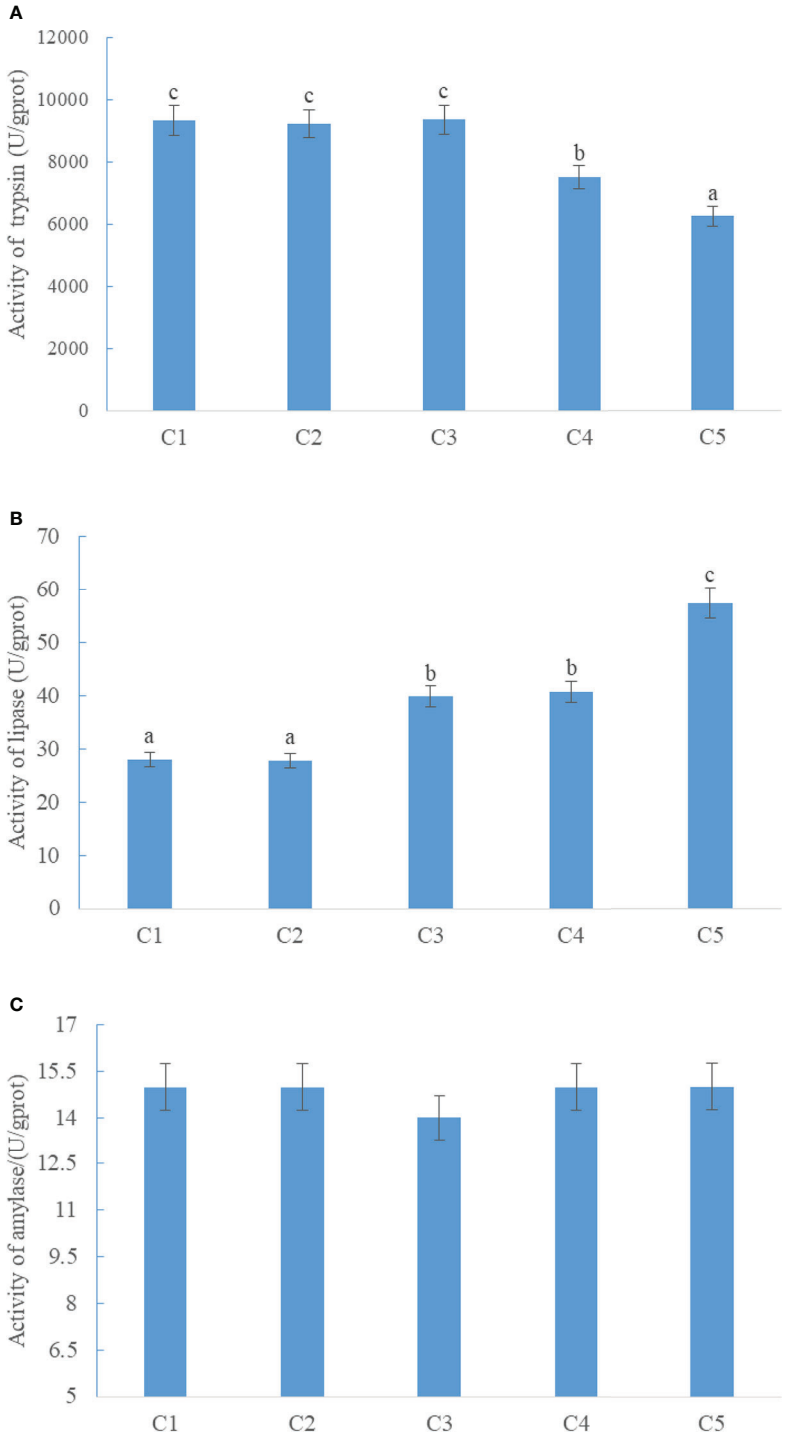

FIGURE 1
Effects of fishmeal replacement by cottonseed protein concentrate on the activity of intestinal trypsin **(A)**, lipase **(B)**, and amylase **(C)** in rainbow trout (Mean ± S.E.). Different letters on the bar chart indicate significant difference (P <0.05).

TABLE 9 Effects of fishmeal replacement by cottonseed protein concentrate on histology of distal intestine in rainbow trout (Mean ± S.E.).

Item	FM	CPC25%	CPC50%	CPC75%	CPC100%
Villus height (μm)	924.90 ± 58.04^{c}	834.43 ± 38.06^{bc}	817.82 ± 16.07^{bc}	743.02 ± 47.96^{b}	558.86 ± 93.99^{a}
Villus width (μm)	487.87 ± 39.40^{b}	347.58 ± 16.86^{a}	331.18 ± 13.79^{a}	297.40 ± 24.01^{a}	285.66 ± 11.14^{a}
Muscular thickness (μm)	140.05 ± 6.33	170.54 ± 18.60	143.49 ± 13.16	144.65 ± 13.19	167.42 ± 11.12
Values with different superscripts in the same column are significantly different (P<0.05).					

and 36, respectively (Figure S3). At the phylum level, fishmeal replacement by CPC in diets resulted in a decrease in Actinobacteriota and an increase in Firmicutes (Figure 7). At the genus level, there were decreases in *Corynebacterium*, *Staphylococcus*, *norank_f_Bacillaceae*, *virgibacillus*, *Brevibacterium*, and an increase in *Clostridium_sensu_stricto_1* in groups with graded CPC in diets (Figure 8).

LEfSe analysis revealed that the dominant bacterial genera in FM were *norank_f_Bacillaceae*, *virgibacillus*, *Staphylococcus*, *unclassified_f_Bacillaceae*, *Corynebacterium*, *Georgenia*, and *Tissierella*, and the main bacterial genera in CPC100% were *Clostridium_sensu_stricto_1* (Figure 9).

4 Discussion

4.1 Growth performance

Previous studies have demonstrated that dietary fishmeal can be partially replaced by CPC in carnivorous fish species without negative effects on growth performance (11, 25–27). It has been reported that CPC can replace up to 50% of dietary fishmeal (40% fishmeal in the control group) for largemouth bass (*Micropterus salmoides*) with no detrimental effects on growth performance, but a substitution rate of 75% for dietary fishmeal (fishmeal level: 10%) resulted in reductions in weight

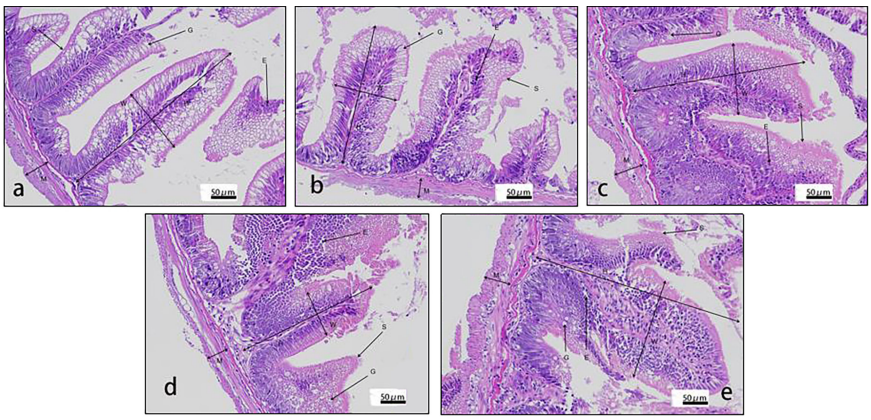

FIGURE 2
Histology of distal intestine in rainbow trout fed graded cottonseed protein concentrate. **(A)** FM; **(B)** CPC25%; **(C)** CPC50%; **(D)** CPC75%; **(E)** CPC100%. W-villus width, H-villus height, M-muscular layer thickness, S-striatum; G-goblet cells and E-epithelial cells.

TABLE 10 Effects of fishmeal replacement by cottonseed protein concentrate on histology of proximal intestine in rainbow trout (Mean ± S.E.).

Item	FM	CPC25%	CPC50%	CPC75%	CPC100%
Villus height (μm)	980.71 ± 30.75	1084.60 ± 36.64	1002.18 ± 54.63	982.93 ± 31.51	1006.23 ± 56.60
Villus width (μm)	301.43 ± 9.30	290.80 ± 28.51	277.86 ± 17.57	316.33 ± 16.62	265.26 ± 7.18
Muscular thickness (μm)	168.20 ± 8.31	208.25 ± 18.54	172.88 ± 18.53	165.92 ± 4.74	183.71 ± 18.94

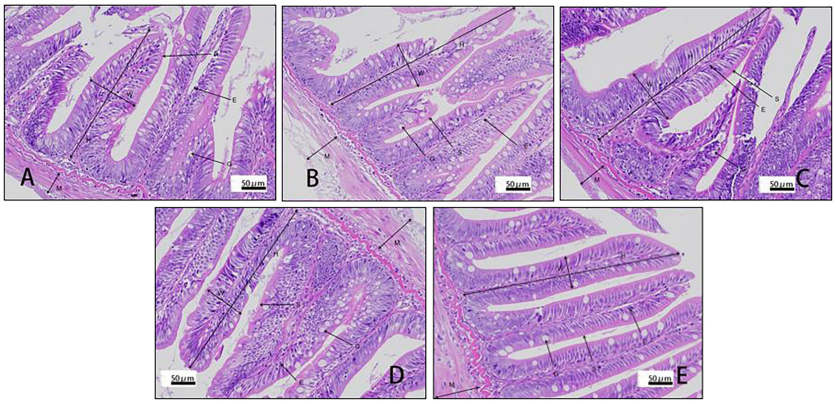

FIGURE 3
Histology of proximal intestine in rainbow trout fed graded cottonseed protein concentrate. **(A)** FM; **(B)** CPC25%; **(C)** CPC50%; **(D)** CPC75%; **(E)** CPC100%. W-villus width, H-villus height, M-muscular layer thickness, S-striatum; G-goblet cells and E-epithelial cells.

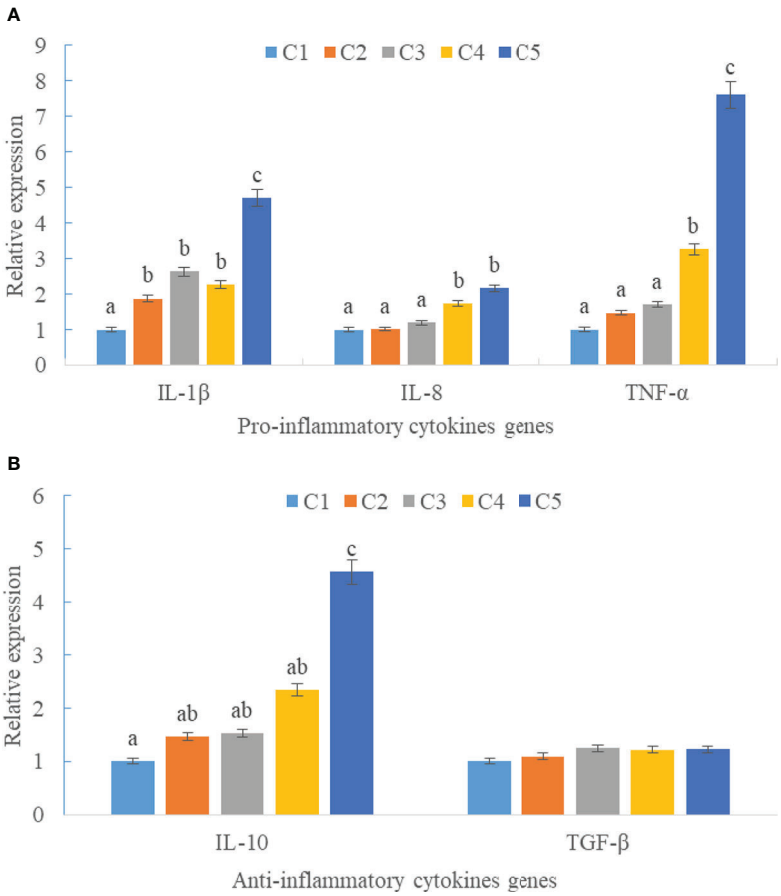

FIGURE 4
Effects of fishmeal replacement by cottonseed protein concentrate on the mRNA expression levels of inflammatory cytokines in intestine of rainbow trout (Mean ± S.E.). **(A)** Pro-inflammatory cytokines genes; **(B)** Anti-inflammatory cytokines genes. Different letters on the bar chart indicate significant difference ($P < 0.05$).

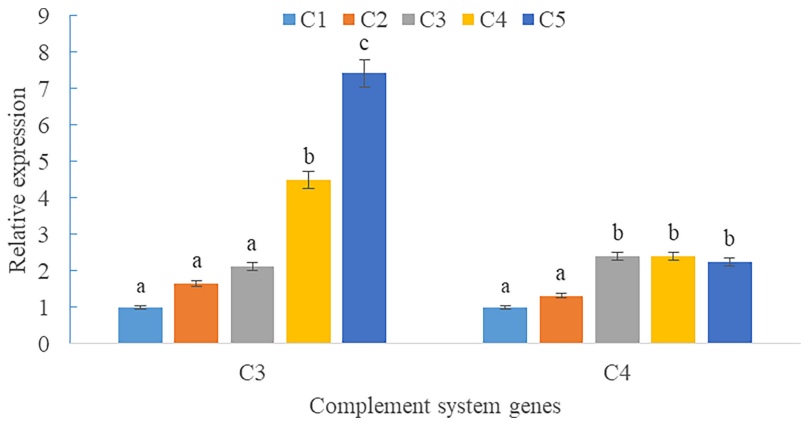

FIGURE 5
Effects of fishmeal replacement by cottonseed protein concentrate on the mRNA expression levels of complement system genes *c3* and *c4* in intestine of rainbow trout (Mean ± S.E.). Different letters on the bar chart indicate significant difference (P <0.05).

gain, feed efficiency, and condition factor (13). No adverse effects on growth were observed in juvenile golden pompano (*Trachinotus ovatus*) when 60% dietary fishmeal was replaced by CPC with 13.6% fishmeal and 20.4% CPC in the diet (28). For rainbow trout, a recent study showed that replacing 50% dietary fishmeal (40% fishmeal in the control group) by concentrated dephenolized cottonseed protein (CDCP) had no significant adverse effects on growth performance, survival rate, or feed utilization (17). However, there was no higher substitution level of fishmeal by CDCP in that study (17). In the present study, no significant detrimental effects on growth performance or feed intake were observed in rainbow trout when 75% dietary fishmeal was replaced by 22.5% CPC and only 7.5% fishmeal in the diet, indicating that CPC has the potential to replace fishmeal at a higher inclusion level. However, the significantly reduced feed intake and weight gain in the fishmeal-free diet based on CPC (CPC100%) suggests that it is still challenging to completely replace dietary fishmeal by CPC.

4.2 Metabolism

In the present study, no significant difference was observed in the proximate composition of whole-body fish when dietary fishmeal was replaced by graded CPC. However, the significant changes in contents of blood metabolites indicated alterations in metabolism in rainbow trout fed graded CPC. In this study, an increased substitute rate of fishmeal by CPC resulted in elevation of serum alanine aminotransferase (ALT) activity. ALT is a widely used indicator for hepatic function and metabolic

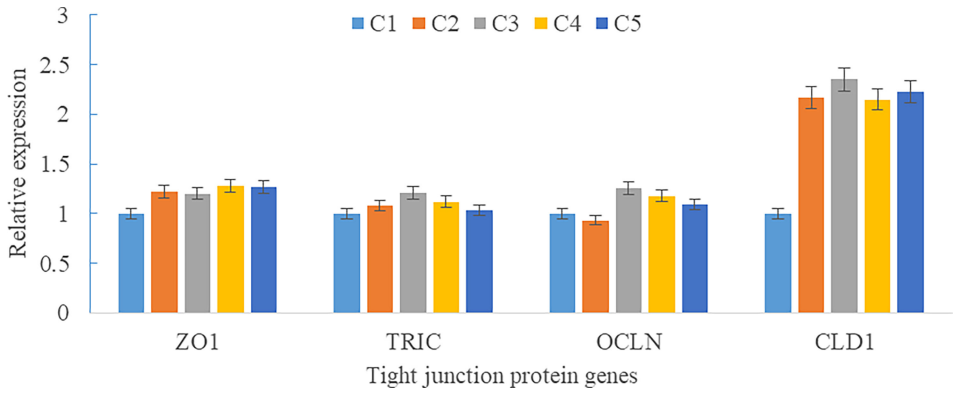

FIGURE 6
Effects of fishmeal replacement by cottonseed protein concentrate on the mRNA expression levels of tight junction protein genes in intestine of rainbow trout (Mean ± S.E.). Different letters on the bar chart indicate significant difference (P <0.05).

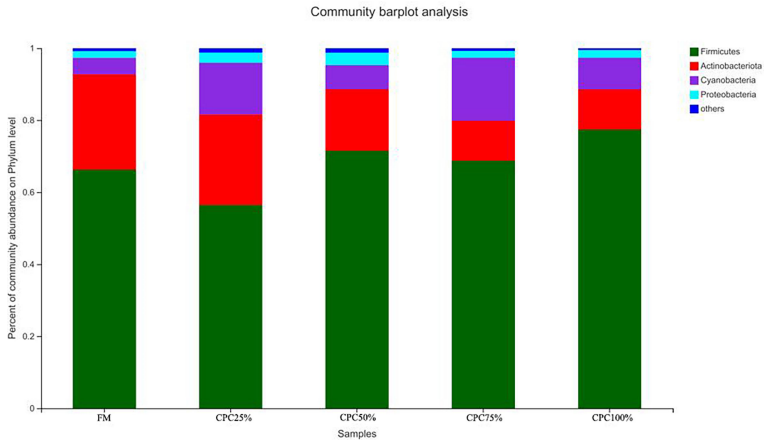

FIGURE 7
Effects of fishmeal replacement by cottonseed protein concentrate on relative abundance of intestinal microbes in juvenile rainbow trout at the phylum level.

syndrome (29–31). The increased serum ALT in rainbow trout fed graded CPC suggests that the inclusion of CPC in the diet could result in potential adverse effects on liver health. Previous studies also showed that detrimental effects of plant protein sources for fishmeal replacement could be due to the lack of cholesterol in plant proteins (9, 32). It has been reported that high inclusion levels of plant proteins in diets could reduce the level of blood cholesterol and result in hypocholesterolemia in carnivorous fish species (33, 34). In the present study, complete replacement of fishmeal by CPC resulted in reduced total cholesterol in serum. Further targeted metabolomic analysis for free sterols in serum of rainbow trout revealed no significant difference in free cholesterol of serum between the control group and fishmeal-free diet group based on CPC. However, the decreases of ergosterol, lathosterol, and cholestanol and increases of desmosterol and 7-dehydrocholesterol in serum of the fishmeal-free diet group based on CPC demonstrated an alteration in sterol metabolism, indicating that the free cholesterol level in serum could be balanced through metabolic regulation. Some studies have shown that supplementation of cholesterol in high plant protein diets could improve feed intake and growth performance in carnivorous fish species, but the effects of cholesterol supplementation varied with plant proteins (32, 34–36).

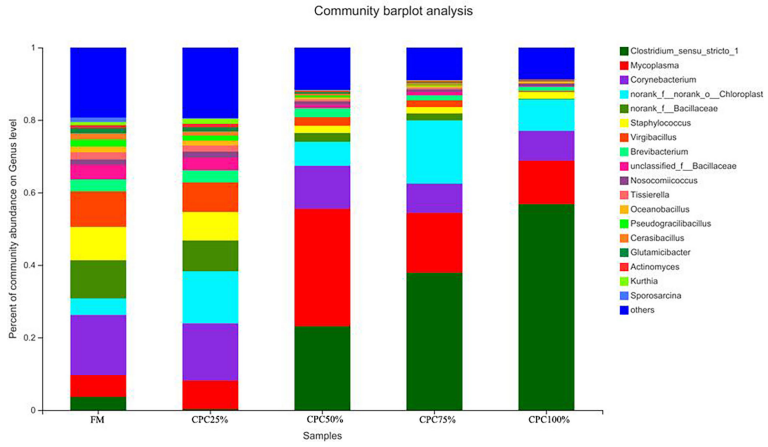

FIGURE 8
Effects of fishmeal replacement by cottonseed protein concentrate on relative abundance of intestinal microbes in juvenile rainbow trout at the genus level.

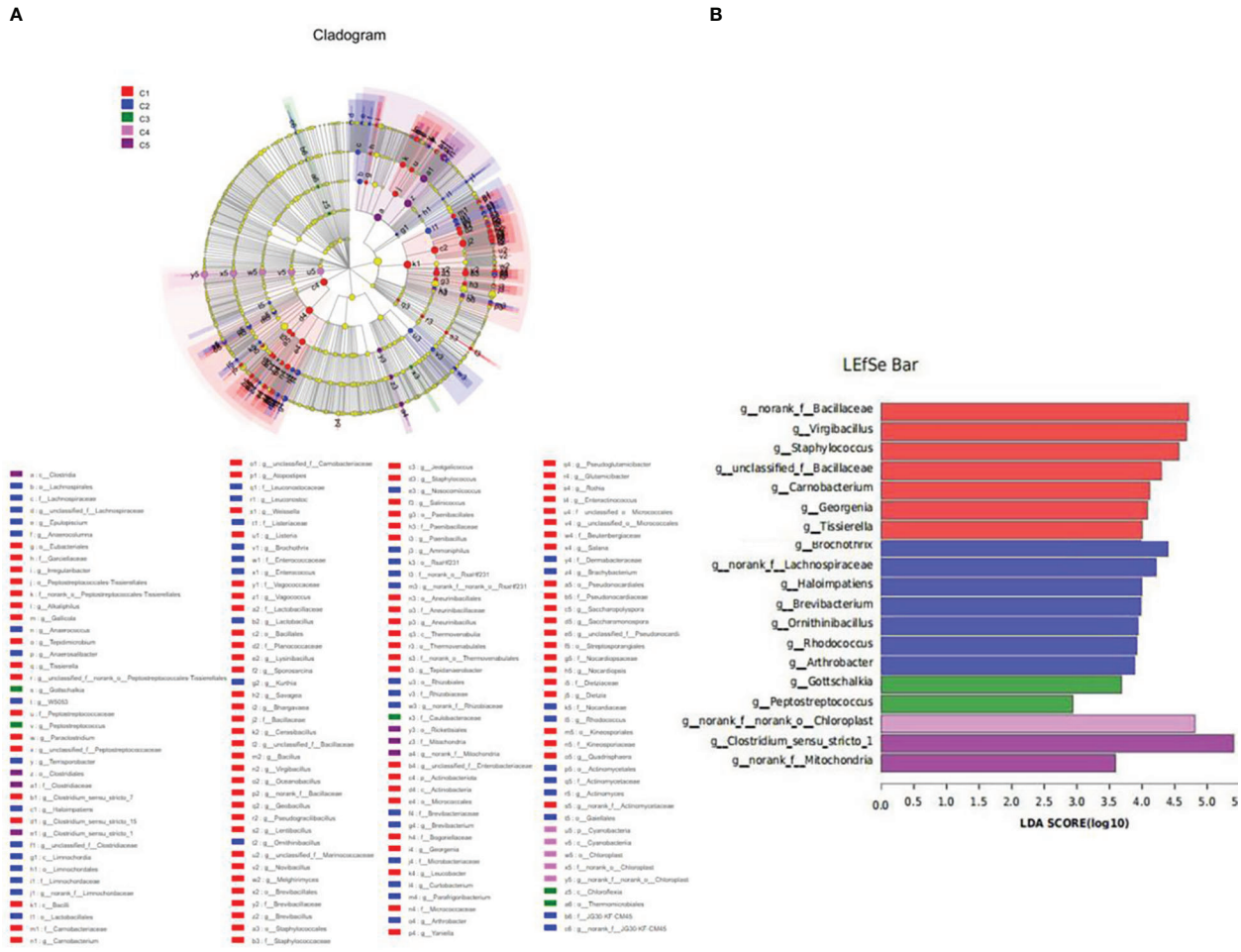

FIGURE 9
Inter-group variation in the relative abundance of intestinal microbial communities at the genus level. **(A)** Cladogram from Lefse. **(B)** LDA score from Lefse-PICRUSt.

Therefore, it is essential to investigate the effects of cholesterol supplementation in CPC-based diets on growth and feed intake in rainbow trout in the future.

In this study, complete replacement of fishmeal by CPC resulted in reductions of some free amino acids (e.g., lysine, methionine) in the serum of rainbow trout, although amino acids in diets were balanced by supplementation of crystalline amino acids. The reason for this could be the decreases in feed intake and digestibility. Previous studies in other carnivorous fish species also observed reduced feed intake and digestibility in fish fed high CPC. For example, dietary fishmeal replacement by CPC caused decreased feed intake and apparent digestibility in pearl gentian groupers (*Epinephelus fuscoguttatus*♀ × *E. lanceolatus*♂) (25). Further study is needed for efficient use of CPC in aquafeed through improving feed intake and digestibility.

4.3 Intestinal health

High inclusion of CPC in diets can result in impaired intestinal health of carnivorous fish species. Previous research on largemouth bass (*Micropterus salmoides*) revealed that 75% dietary fishmeal replacement induced impaired intestinal morphology, including reduced villus height and width (13). In rainbow trout, it has been reported that 50% fishmeal protein substitution by CPC showed no detrimental effects on intestinal morphology (17). However, the effects of dietary fishmeal replacement by CPC at higher inclusion levels on intestinal morphology were not reported in that study. In the present study, decreased villus height and width of the distal intestine was observed in rainbow trout fed diets with 75% fishmeal substitution, indicating that high inclusion levels of CPC could result in impaired intestinal morphology.

It is widely accepted that cytokines play important roles in the regulation of the immune system in vertebrates, and pro- and anti-inflammatory cytokines have been used as markers in assessment of intestinal health of fish (11, 37). In this study, 75% dietary fishmeal replacement induced upregulation of pro-inflammatory cytokines including IL1β, IL8, and TNFα, indicating an elevation of the inflammatory response. The results were in line with previous studies on hybrid grouper in which promotion of intestinal inflammation was found in the fish fed diets containing graded CPC (14, 25). The reason could involve the possible persistence of certain anti-nutritional factors.

In the present study, dietary fishmeal replacement by CPC reduced bacterial diversity in the intestine and altered the composition of the gut microbiota. Similarly, previous studies on carnivorous fish species showed reduction of bacterial diversity by CPC substitution (11, 12, 28). Meanwhile, fishmeal replacement by CPC decreased Actinobacteriota abundance and increased Firmicutes abundance in the present study. The results were in agreement with previous research where increased Firmicutes was observed in largemouth bass fed diets containing graded CPC (11). However, the decreased Actinobacteriota abundance was not found in other studies on CPC substitution, where alterations in Proteobacteria were often observed in largemouth bass (38). The discrepancy could be due to variation in fish species and feed formulations. At the genus level, reduced *Bacillaceae* was observed with graded CPC substitution in the present study. Previous studies demonstrated that *Bacillus* supplementation exhibited a variety of beneficial properties for carnivorous fish fed diets containing high levels of plant protein (e.g., soybean meal), including growth promotion, improvement in immune response, and general health (39–42). Therefore, the reduced Baci, llaceae could be responsible for the impaired intestinal health in experimental fish, and *Bacillus* may improve utilization of CPC-based diets for rainbow trout and other carnivorous fish species.

In conclusion, this study systematically evaluated CPC as a fishmeal alternative in the diets of rainbow trout, and the suggested substitution rate of fishmeal by CPC should be less than 75%. Excessive substitution of fishmeal by CPC and less than 75 g fishmeal/kg in the diet caused suppression of growth, alterations in blood metabolites, and impairment of intestinal health.

Data availability statement

The raw sequences based on 16S rRNA gene can be found in the NCBI Sequence Read Archive (SRA) (Accession Number: PRJNA841419).

Ethics statement

The animal study was reviewed and approved by Committee for the Welfare and Ethics of Laboratory Animals of Heilongjiang River Fisheries Research Institute of Chinese Academy of Fishery Sciences (CAFS).

Author contributions

YL designed the study and wrote the manuscript. SM, HC, and GQ conducted the feeding trial and analyzed experimental data. WL, SL, DW, CW, and SH provided technical assistance. HL reviewed the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by China Agriculture Research System of MOF and MARA (Grant No. CARS-46), Central–Level Non–profit Scientific Research Institutes Special Funds in China (Grant No. HSY202104M), Postdoctoral Scientific Research Developmental Fund in Heilongjiang Province, China (Grant No. LBH-Q20194), and Scientific Research Fund for Returned Overseas Scholars in Heilongjiang Province, China (Hljlxzz2022.43).

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1079677/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 23 December 2022
DOI 10.3389/fimmu.2022.1093567

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Comparative Immunology, a section of the journal Frontiers in Immunology

RECEIVED 09 November 2022
ACCEPTED 06 December 2022
PUBLISHED 23 December 2022

CITATION
Wang Y, Jia X, Guo Z, Li L, Liu T, Zhang P and Liu H (2022) Effect of dietary soybean saponin Bb on the growth performance, intestinal nutrient absorption, morphology, microbiota, and immune response in juvenile Chinese soft-shelled turtle (*Pelodiscus sinensis*). *Front. Immunol.* 13:1093567. doi: 10.3389/fimmu.2022.1093567

Effect of dietary soybean saponin Bb on the growth performance, intestinal nutrient absorption, morphology, microbiota, and immune response in juvenile Chinese soft-shelled turtle (*Pelodiscus sinensis*)

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Soybean meal is widely applied in the aquafeeds due to the limitation of fish meal resources. Numerous studies have manifested that dietary soybean saponin, an anti-nutrient factor in soybean meal, may slow growth and induce intestinal inflammation in aquatic animals, but the possible causes are unclear. The juvenile *Pelodiscus sinensis* (mean initial body weight: 6.92 ± 0.03 g) were fed basal diet (CON group) and 2.46% soybean saponin Bb-supplemented diet (SAP group) for 35 days to further explore the effects of dietary soybean saponin Bb on the growth performance, apparent digestibility coefficients, intestinal morphology, the gut microbiota, intestinal transporters/channels, and immune-related gene expression. The results indicated that dietary soybean saponin Bb significantly decreased final body weight, specific growth rate, protein deposition ratio, and apparent digestibility coefficients (dry matter, crude protein, and crude lipid) of nutrients in *Pelodiscus sinensis*, which may be closely correlated with markedly atrophic villus height and increased lamina propria width in the small intestine. In addition, plasma contents of cholesterol, calcium, phosphorus, potassium, lysozyme, and C3 were significantly decreased in the SAP group compared with the control group. Soybean saponin Bb significantly downregulated the mRNA levels of glucose transporter 2, fatty acid binding protein 1 and fatty acid binding protein 2, amino acid transporter 2, $b^{0,+}$-type amino acid transporter 1, and sodium-dependent phosphate transport protein 2b in the small intestine. At the same time, the expressions of key transcription factors (*STAT1*, *TBX21*, *FOS*), chemokines (*CCL3*), cytokines (*TNF-α*, *IL-8*), and aquaporins (*AQP3*, *AQP6*) in the inflammatory response were increased by soybean saponin Bb in the large

intestine of a turtle. Additionally, dietary supplementation of SAP significantly reduced the generic abundance of beneficial bacteria (*Lactobacillus*, *Bifidobacterium*, and *Bacillus*) and harmful bacteria (*Helicobacter* and *Bacteroides*). In a nutshell, dietary supplementation of 2.46% soybean saponin not only hindered the growth performance by negatively affecting the macronutrients absorption in the small intestine but also induced an inflammatory response in the large intestine possibly by damaging the intestinal morphology, disturbing the intestinal microbiota and decreasing intestinal epithelial cell membrane permeability.

KEYWORDS

Soybean saponin Bb, growth performance, macronutrients and minerals absorption, intestine morphology, gut microbiota, intestine health, *Pelodiscus sinensis*

1 Introduction

The Chinese soft-shelled turtle (*Pelodiscus sinensis*) has a high edible value ascribing to its relatively high protein content, unsaturated fatty acids, and essential trace elements (1) and has become a significant freshwater-cultured species in China with an annual production of more than 330,000 tons (2). As a carnivorous omnivore, the turtle has a comparatively high protein requirement, which is mainly supplied by white fishmeal in the diet. In view of the high price and low availability of white fishmeal, searching for suitable fishmeal alternative protein sources or manufacturing low-fishmeal diets has become a major concern for researchers and formulators in the feed industry. Soybean meal (SBM) is a common fishmeal substitute in aquafeed due to its steady source and relatively balanced amino acid profile (3, 4). However, high doses of dietary soybean meal supplementation led to an obvious decrease in growth rate and feed utilization efficiency in Atlantic salmon (*Salmo salar*) (5), rainbow trout (*Oncorhynchus mykiss*) (6), turbot (*Scophthalmus maximus*) (7), zebrafish (*Danio rerio*) (8), and grass carp (*Ctenopharyngodon idella*) (9). In addition, long-term feeding of soybean meal also resulted in structural and functional changes in the distal intestine, including shortened mucosal fold height, widened lamina propria, impaired absorptive function, and a profound infiltration of inflammatory cells in the lamina propria (5), which were commonly referred to as "soybean meal-induced inflammation." This negative influence may be correlated with antinutritional factors in soybean meal such as β-conglycinin, glycinin, trypsin inhibitor, isoflavone, saponins, etc. (10).

Although the antinutritional factors responsible for the aforementioned adverse effects have not been specified, some alcohol-soluble components in full-fat soybean meal (11) are likely to be to blame. It was because soy protein concentrate obtained through ethanol extraction technology did not induce pathological changes in the distal intestine of Atlantic salmon, whereas full-fat soybean meal caused intestinal inflammation in Atlantic salmon (12). Among the alcohol-soluble components, soybean saponins are polycyclic triterpene glycosides, which have been proposed as inducers of intestinal inflammation in some fish, such as zebrafish (13) and turbot (14). Additionally, saponins had relatively good heat stability (15), making passivation by extrusion processing technology during aquafeed manufacture or other heat treatments difficult. Furthermore, when the soybean saponins enter the alimentary tract, their amphipathic might interfere with membrane homeostasis, increase the permeability of cell membranes, and result in the uptake of antigens and potential toxins (16, 17). They are divided into group A, group B, group E, and DDMP based on their linked sugar side chains, and the saponin Bb is the most abundant component among the saponin groups (18). Therefore, soybean saponin Bb could be used as a representative compound to study the biological effects on aquatic animals. Several studies have shown that soybean saponin reduced weight gain, changed intestinal morphology, increased the permeability of enterocyte membrane (13), and induced upregulation of proinflammatory factors (*IL-1β*, *IL-8*, *TNF-α*) in aquatic animals such as Japanese flounder (*Paralichthys olivaceus*) (19), Atlantic salmon (20, 21), turbot (7, 14, 22), rice field eel (*Monopterus albus*) (23), zebrafish (24), and gilthead sea bream (*Sparus aurata*) (11). However, further studies are needed to

investigate the effect of soybean saponin Bb on the absorption and transport of nutrients in aquatic animals and the variation tendency of intestinal key pattern recognition receptors, transcription factors, and cellular signaling pathways that regulate the inflammatory response.

The present work was designed to explore the effects of 2.46% soybean saponin Bb in the diet on growth, nutrient digestion and absorption, intestinal morphology, gut microbiota, and intestinal immune signaling pathway, and reveal the possible reasons for the reduction of growth performance and the underlying inflammatory process in Chinese soft-shelled turtle. This work will contribute to a more comprehensive understanding of the adverse effects of dietary soy saponin in aquaculture animals.

2 Materials and methods

2.1 Experimental diet and design

Fish meal, gelatin, and casein were used as main protein sources, and soybean oil and soybean lecithin were applied as primary lipid sources. By adding different levels of soybean saponin Bb (0%, 12%) purchased from Shaanxi Xiazhou Biotechnology Co. Ltd, two isonitrogenous (47% crude protein) and isoenergetic (5% crude lipid) experimental diets were prepared. The actual contents of saponin Bb in two diets measured by high-performance liquid chromatography (Shanghai Microspectrum Detection Technology Co. Ltd., Shanghai, China) were 0% (CON) and 2.46% (SAP), respectively (Table 1). Y_2O_3 was added as an external indicator to determine the apparent digestibility of each nutrient. Betaine was supplemented in two diets to increase diet palatability. All ingredients were step-by-step mixed, ground sufficiently, and sifted (80 mesh), then 4% of soybean oil was mixed thoroughly with the powder and passed through an 80-mesh sieve three times. The mixture was then homogenized thoroughly with distilled water (15% of the powder mass) in a blender to form a stiff dough. The dough was then extruded into soft pellets with a 2.0-mm diameter using a pelletizer (Youyi Machinery Factory), and the pellets were preserved in airtight plastic bags at −20°C until use. The diet formulation and its proximate chemical composition are shown in Table 1.

2.2 Turtles and feeding management

The flow chart of experimental design is shown in Figure 1. Chinese soft-shelled turtles, purchased from Tangshan, Hebei province, were acclimated for 10 days with juvenile turtle commercial feed in the Laboratory of Physiology and Ecology, Hebei Normal University. In total, 70 juvenile turtles with an initial body weight of 6.92 ± 0.03 g (mean carapace length: 26.77 mm) were evenly assigned to 10 tanks (250 L) with hanging nets for shelter 24 h after starvation, and each of the five tanks was randomly fed same experimental diet for 5 weeks. The juvenile turtles were fed twice a day (8:30, 17:30), and the residual pellets were siphoned out 40 min after each meal. Approximately one-third of the tank's water was renewed once a day in the morning. The aquaculture room was kept dark throughout the feeding trial except when the residual pellets/feces were removed and tank water was exchanged. The water temperature was maintained at 30°C ± 0.2°C using an independent temperature controller, and the pH was measured at 7.5 ± 0.1.

2.3 Sample collection

At the end of the feeding trial, juvenile turtles in each tank were bulk-weighed to determine the growth-related parameters. Two turtles were randomly selected, weighed, and stored at −20°C after 24 h of food deprivation to analyze the turtle body composition. The remaining turtles were anesthetized with 60 mg/L eugenol and weighed individually 3 h after the last meal. The visceral mass, liver, and limb fat of eight turtles per group were dissected and weighed to calculate the morphological parameters. Jugular venous blood was collected into an anticoagulant tube containing heparin sodium and centrifuged at 4,000×*g* at 4°C for 15 min to obtain plasma, and stored at −80°C for the determination of biochemical parameters.

The digestive tracts of the remaining 24 turtles in each group were dissected from the body cavity and stripped of mesentery. The intestinal contents from 10 turtles per group were collected in five cryopreserved tubes, which were quickly put into liquid nitrogen for freezing and stored at −80°C prior to the determination of the intestinal microbiota. Subsequently, the digestive tract is divided into the large and small intestines according to the division of the Chinese soft-shelled turtle intestine (26). The middle sections of the large and small intestines from eight turtles per group were taken at 1–2 cm and stored in 4% paraformaldehyde separately for histological evaluation. Finally, the large and small intestines of another 16 turtles were frozen in liquid nitrogen, then they were transferred to −80°C for subsequent intestine transcriptome and gene expression analysis.

2.4 Biochemical analysis

A previous study (25) was used to estimate the chemical composition of feed, turtle body, and feces. The contents of plasma glucose (A154-1-1), triglyceride (A110-1-1), total cholesterol (A111-1-1), total amino acid (A026-1-1), lysozyme (A050-1-1), complement 3 (E032-1-1), and IgM (E025-1-1) were determined by the colorimetric method using

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TABLE 1 Diet formulation and proximate chemical composition of the experimental diets (dry matter basis, g/kg).

Ingredients	CON	SAP
Russian fish meal	380.00	380.00
Casein	150.00	150.00
Gelatin	110.00	110.00
Squid liver meal	40.00	40.00
Soybean oil	40.00	40.00
Soybean lecithin	10.00	10.00
Microcrystalline cellulose	120.00	0.00
Soybean saponin Bb	0.00	120.00
Monocalcium phosphate	15.00	15.00
Limestone	8.00	8.00
Zeolite powder	50.00	50.00
Potassium chloride	2.00	2.00
Sodium chloride	1.00	1.00
Betaine	1.00	1.00
Choline chloride	2.00	2.00
Y_2O_3	1.00	1.00
Premix[a]	20.00	20.00
Carboxymethylcellulose sodium	20.00	20.00
Proximate composition		
Crude protein	47.91	47.09
Crude lipid	5.39	5.27
Crude ash	23.16	22.05
Gross energy (MJ/kg)	17.47	18.48
Soybean saponins Bb	0.00	2.46

[a]Premix was according to Zhang et al. (25).

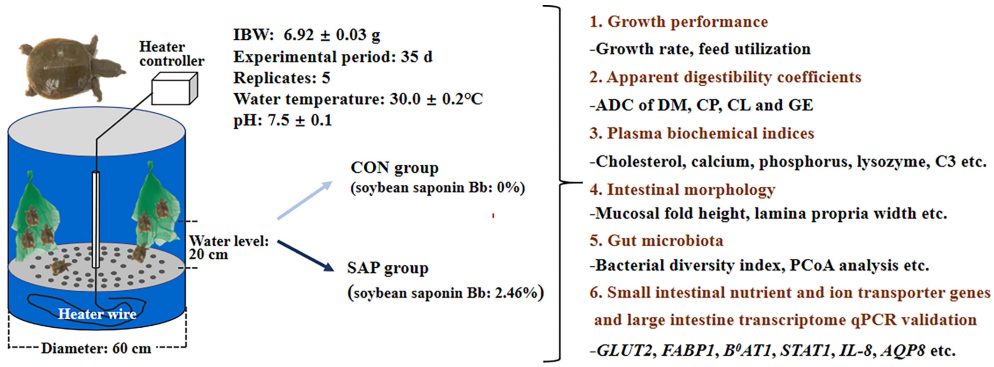

FIGURE 1
Flow chart of experimental design.

commercial diagnostic kits according to the manufacturer's protocols (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). The plasma phosphorus (BC1650), calcium (BC0720), potassium (BC2770), magnesium (BC2790), zinc (BC2810), and sodium (BC2800) were also determined colorimetrically by mercantile kits (Beijing Solarbio Science & Technology Co. Ltd., Beijing, China).

2.5 Intestinal histology

The fixed large and small intestines of one turtle from each tank were sent to Wuhan Sevell Technology Co. Ltd. and processed according to standard histological techniques, and stained with hematoxylin and eosin (H&E). Sections were examined with a Leica Microsystems CMS GmBH Ernst-Leitz-Str. 17-37, 35578 (Wetzlar, Germany).

2.6 Intestinal microbiota

2.6.1 DNA extraction and 16S rRNA gene sequencing

Intestinal chyme was collected from four turtles in each group. Microbial community genomic DNA was extracted from gut content samples using DNeasy PowerSoil Pro Kit (QIAGEN, USA) according to the manufacturer's instructions. The DNA extract was checked on 1% agarose gel, and DNA concentration and purity were determined with NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA).

The hypervariable region V3–V4 of the bacterial 16S rRNA gene was amplified with primer pairs 338F: ACTCCTACGGG AGGCAGCAG_806R: GGACTACHVGGGTWTCTAAT by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). PCR reactions were performed in triplicate. The PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions and quantified using Quantus™ Fluorometer (Promega, USA). Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq PE300 platform (Illumina, San Diego, CA, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

2.6.2 Bioinformatics analysis

The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered by fastp version 0.20.0, and merged by FLASH version 1.2.7 Operational taxonomic units (OTUs) with 97% similarity cutoff were clustered using UPARSE version 7.1, and chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analyzed by RDP Classifier version 2.2 against the 16S rRNA database using a confidence threshold of 0.7. PCoA analysis was evaluated by similarity analysis (ANOSIM) based on the Bray-Curtis distance. All-against-all (more strict) comparison of Discriminant Analysis Effect Size (LEfSe) was also utilized using rarefied 16S rRNA gene sequence data, the cut-off logarithmic Linear Discriminant Analysis (LDA) score was 2.0.

2.7 RNA-Seq

Total RNA was extracted from the large intestines of the CON and SAP groups using TRIzol® Reagent according to the manufacturer's instructions (Invitrogen), and genomic DNA was removed using DNase I (TaKara). RNA-seq transcriptome librarly was prepared and the paired-end RNA-seq sequencing library was sequenced with the Illumina HiSeqxten/NovaSeq 6000 sequencer (2×150 bp read length).

The raw data (raw reads) obtained by the Illumina platform were treated with a series of quality control processes to obtain high-quality quality control data (clean reads). The clean reads were then aligned with the *Pelodiscus sinensis* genome (GCF_000230535.1 https://www.ncbi.nlm.nih.gov/genome/?term=Pelodiscus+sinensis), and they were spliced and *de novo* assembled using StringTie (https://ccb.jhu.edu/software/stringtie/index.shtml?t=example).

Differentially expressed genes (DEGs), GO function enrichment, and KEGG pathway analysis were the same as Jiang et al. (27). To validate the RNA-Seq results, 20 DEGs were selected for QPCR. The method is described in Calculation formula and Statistical analysis.

2.8 RNA extraction and real-time PCR

Total RNA was extracted from the small and large intestines by the TransZol Up method. The quality control of RNA, reverse transcription of mRNA to cDNA, primer design, PCR amplification procedure, assessment of PCR product, and quantitative real-time PCR protocol were performed referring to a previous study (25). 18RPS was applied in the small intestine, and β-actin was applied in the large intestine as housekeeping genes to normalize expressions of target genes. The expression levels of these genes were computed based on the $2^{-\Delta\Delta Ct}$ method. Primer sequence, product length, annealing temperature, and accession number are presented in Supplementary Table S1.

2.9 Calculation formula and statistical analysis

The formulas of growth index, body index, and nutrient apparent digestibility are shown in Supplementary Table S2.

All data were analyzed using statistics 10.0 (Statsoft Inc., Tulsa, OK, USA). The *t*-test method was used to analyze the two groups of data and described as mean ± SD. $p < 0.05$ was considered a significant difference.

3 Results

3.1 Growth performance

The growth performance of juvenile turtles in the current feeding trial is shown in Table 2. Compared with the CON group, the final body weight (FBW), weight gain rate (WGR), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), protein deposition ratio (PDR), fat deposition ratio (FDR), and fatsomatic index (FSI) in the SAP group were significantly reduced, while feeding rate (FR) and FCR in the SAP group showed the opposite trend ($p < 0.05$). Survival rate (SR), viscerosomatic index (VSI), and hepatosomatic index (HSI) had no significant differences between the two groups ($p > 0.05$).

3.2 Proximate composition of the turtle's whole body, apparent digestibility coefficients, and plasma biochemical indexes

Figure 2A shows that the contents of moisture, crude protein, and crude lipid in the SAP group were significantly lower than those in the CON group ($p < 0.05$). Figure 2B indicates that the dietary soybean saponin Bb significantly decreased the apparent digestibility coefficients of dietary nutrients (dry matter, crude protein, and crude lipid) in juvenile turtles ($p < 0.05$). Figure 2C displays that the plasma cholesterol concentration of juvenile turtles in the SAP diet significantly decreased ($p < 0.05$), and glucose and triglyceride decreased but had no significant influence ($p > 0.05$). The concentration of serum calcium, phosphorus, and potassium in the SAP diet was significantly lower than those in the CON diet ($p < 0.05$). Figure 2D shows that lysozyme and C3 activities were significantly reduced in the SAP diet ($p < 0.05$).

3.3 Intestinal morphology

Table 3 shows that dietary soybean saponin Bb had remarkably decreased the mucosal fold height and increased lamina propria width of the small intestine ($p < 0.05$). However, in the large intestine, dietary soybean saponin Bb not only the

TABLE 2 Effects of dietary soybean saponin Bb on growth performance and morphology of juvenile *P. sinensis* ($n = 5$ for growth performance, $n = 8$ for morphological parameters).

Parameters	CON	SAP	*p*-value
SR (%)	100.00 ± 0.00	97.14 ± 0.39	0.347
IBW (g)	6.93 ± 0.03	6.92 ± 0.03	0.798
FBW (g)	17.49 ± 0.79	12.73 ± 1.57	< 0.001
FR (%/day)	3.66 ± 0.09	4.73 ± 0.28	< 0.001
WGR (%)	152.48 ± 10.48	83.95 ± 23.13	< 0.001
SGR (%/day)	2.64 ± 0.12	1.72 ± 0.35	0.001
FCR	1.48 ± 0.07	2.98 ± 0.46	< 0.001
PER	1.41 ± 0.07	0.73 ± 0.14	< 0.001
PDR (%)	67.62 ± 2.44	37.47 ± 6.13	< 0.001
FDR (%)	203.09 ± 6.34	127.94 ± 11.71	< 0.001
VSI (%)	10.93 ± 0.78	10.84 ± 1.77	0.855
HSI (%)	3.98 ± 0.39	4.00 ± 0.52	0.921
FSI (%)	3.40 ± 0.12	2.74 ± 0.35	< 0.001

SR, survival ratio; IBW, initial body weight; FBW, final body weight; FR, feeding ratio; WGR, weight gain ratio; SGR, specific growth ratio; FCR, feed conversion ratio; PER, protein efficiency ratio; PDR, protein deposition ratio; FDR, fat deposition ratio; VSI, viscerosomatic index; HSI, hepatosomatic index; FSI, fatsomatic index.

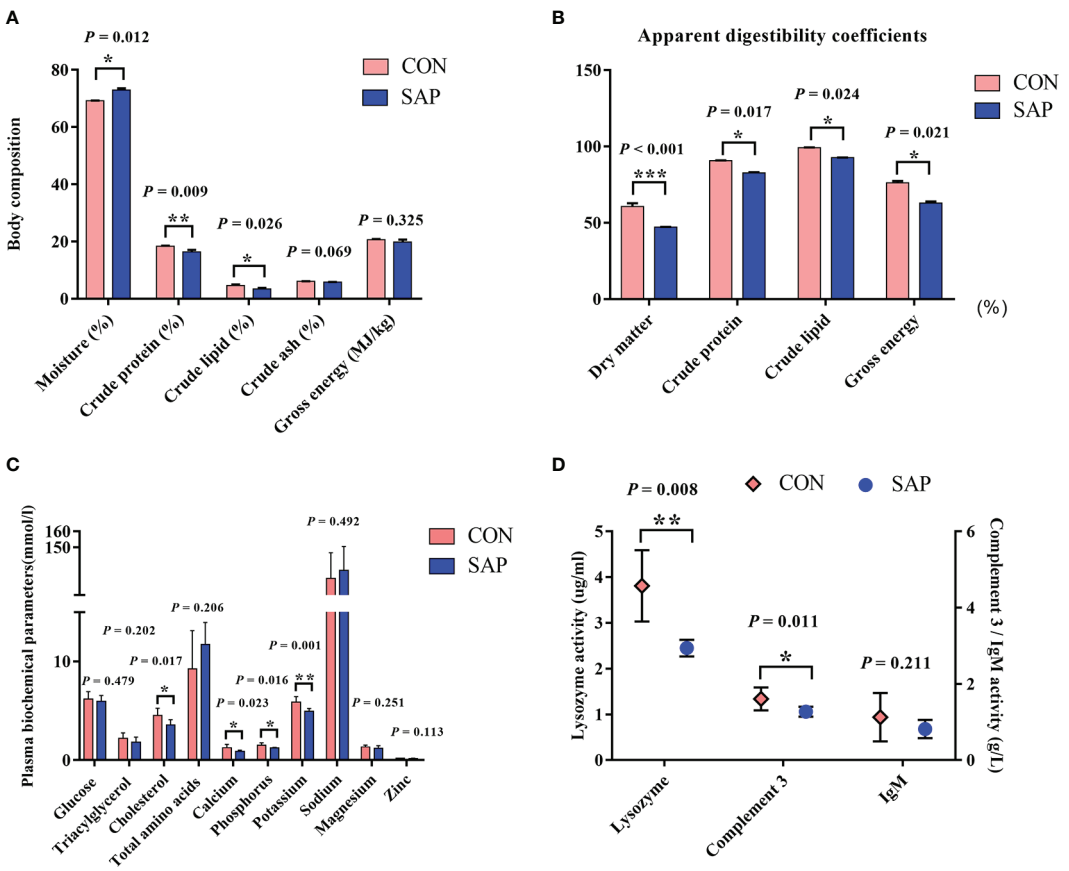

FIGURE 2
Effects of dietary soybean saponin Bb on body composition **(A)** (n = 3), apparent digestibility coefficients **(B)** (n = 3), plasma biochemical parameters **(C, D)** in juvenile *P. sinensis* (n = 6). *p < 0.05; **p < 0.01; ***p < 0.001; Student's t-test.

mucosal fold height and lamina propria width the same change as in the small intestine but also the muscularis thickness was decreased (p < 0.05). The measurement range of intestinal microstructure parameters is shown in Figure 3.

3.4 The mRNA relative expression levels of nutrient transport and ion transport/channel-related genes in the small intestine

As shown in Figure 4, the mRNA expression levels of glucose transporter *GLUT2* (A) and fatty acid transporter *FABP1* and *FABP2* (B) were markedly lower in the SAP group compared to the CON group (p < 0.05). The small peptide transporter *PEPT1* (C) was significantly upregulated, but the amino acid transporters *ASCT2* and B^0AT1 (C) were downregulated (p < 0.05), and the sodium–phosphorus co-transport *NaPi-IIb* (D) was also noticeably reduced (p < 0.05).

3.5 Composition and diversity of gut microbiota

A total of 396,239 sequences and an average of 46,154.875 sequences (ranging from 40,341 to 54,541) were acquired from eight intestinal content samples in the Chinese soft-shell turtles fed CON and SAP diets. The raw data of the intestinal microbiota analysis have been uploaded to the SRA database of the NCBI with the accession PRJNA870236. The rarefaction curves of all samples tended to be stable, indicating the accuracy of the sequencing results (Supplementary Figure S1). The coverage index showed that the coverage of the community's coverage reached more than 99%, which basically covered all microbial communities in the tested samples. The bacterial diversity index was assessed at the OTU level. Compared with the CON group, Shannon increased and Sobs, Simpson, Ace, and Chao decreased in the SAP group, but the differences were not significant (Supplementary Table S3). As shown in Figure 5, PCoA analysis showed that the CON and SAP groups were

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TABLE 3 Effect of dietary soybean saponin Bb on the intestinal microstructure of juvenile *P. sinensis* (n = 3).

Parameters	CON	SAP	*p*-value
Small intestine			
Mucosal fold height (μm)	344.38 ± 9.46	281.80 ± 24.45	0.014
Mucosal fold width (μm)	103.32 ± 20.36	115.10 ± 17.66	0.449
Lamin propria width (μm)	14.85 ± 2.29	19.04 ± 1.17	0.048
Muscularis (μm)	66.77 ± 2.86	70.67 ± 5.91	0.363
Submucosa (μm)	40.85 ± 2.08	43.11 ± 1.05	0.206
Large intestine			
Mucosal fold height (μm)	118.99 ± 5.76	91.82 ± 10.82	0.007
Lamin propria width (μm)	13.35 ± 1.67	19.85 ± 1.88	0.004
Muscularis (μm)	74.02 ± 6.57	46.34 ± 6.40	0.007
Submucosa (μm)	28.52 ± 4.23	34.71 ± 2.06	0.085

aggregated into two mutually disjoint groups, and Analysis of Similarities (ANSION) analysis showed that there was no significant difference between the two groups (R = 0.6146, p = 0.0650). A Venn diagram showed that the common OTUs of the CON and SAP groups were 156, and the number of unique OTUs in the CON and SAP groups were 119 and 33, respectively.

LEfSe multilevel species discriminant analysis and LDA discriminant result (Figure 6) showed that the SAP group significantly reduced the relative richness of Firmicutes, Actinobacteriota, and Acidobacteriota and increased Fusobacteriota and Campilobacterota at phylum level. At the genus level, *Streptococcus*, *Lactobacillus*, *Lactococcus*, *Pseudomonas*, *Enterococcus*, *Macrococcus*, *Bifidobacterium*, *Bacillus*, and *Anoxybacillus* in the CON group were significantly higher than the SAP group. *Helicobacter*, *Bacteroides*, and *Parabacteroides* were the opposite trend. At the species level, compared with the CON group, the relative abundance of *Lactobacillus_rossiae*, *Bacillus_anthracis*, *Lactobacillus_helveticus*, *Bacillus_shackletonii*, *Lactobacillus_kefiri*, *Macrococcus_caseolyticus*, and *Bifidobacterium_mongoliense* were decreased and *Bacteroides_ovatus_V975* was increased in the SAP group, significantly.

3.6 Transcriptome analysis

The raw data of transcriptome analysis have been uploaded to the SRA database of the NCBI with the accession number PRJNA895971. A total of 319,471,138 clean reads were obtained by transcriptome analysis of six samples on the Illumina sequencing platform, and the clean reads of each sample reached more than 50,341,908, and the percentage of Q30 base

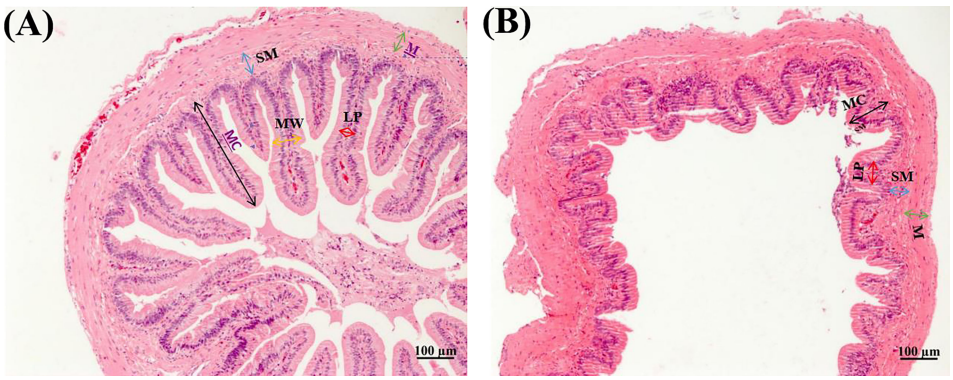

FIGURE 3
Morphology of small **(A)** and large **(B)** intestines of juvenile *P. sinensis* (×100).

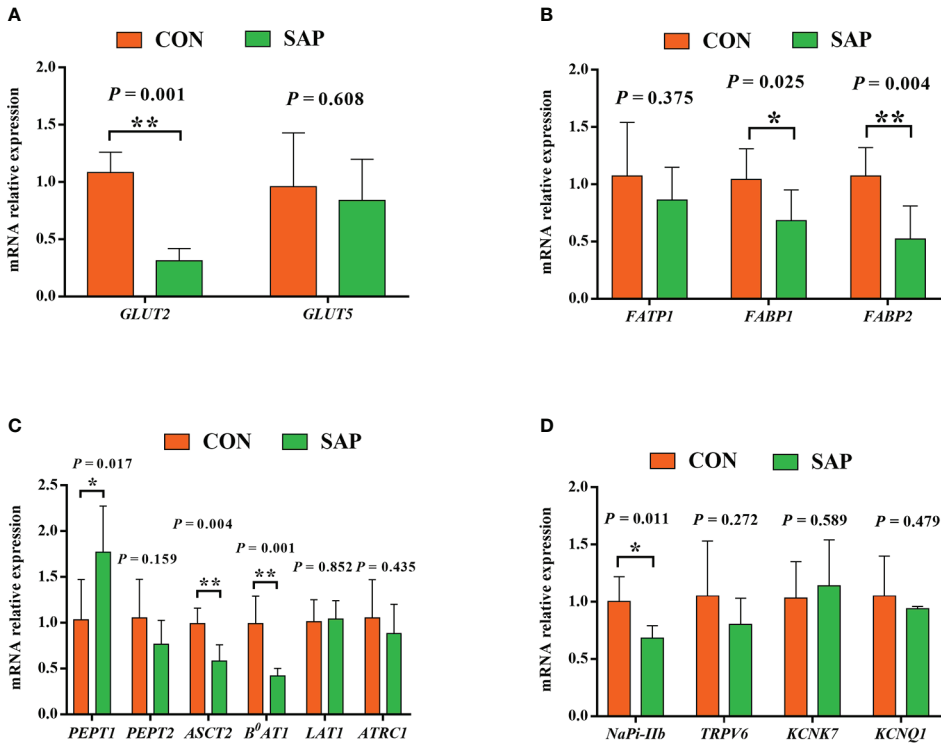

FIGURE 4
Effect of dietary soybean saponin Bb mRNA relative expression of small intestinal nutrient transporter genes **(A–C)** and ion transporters/channel genes **(D)** (n = 6). *p < 0.05; **p < 0.01; Student's t-test. *GLUT2*, glucose transporter 2; *GLUT5*, glucose transporter 5; *FATP1*, fatty acid transporter 1; *FABP1*, fatty acid binding protein 1; *FABP2*, fatty acid binding protein 2; *PEPT1*, polypeptide transporter 1; *PEPT2*, polypeptide transporter 2; *ASCT2*, amino acid transporter 2; B^0AT1, $B^{0,+}$-type amino acid transporter 1; *ATRC1*, cationic amino acid transporter 1; *LAT1*, large neutral amino acid transporter 1; *Napi-IIb*, sodium-dependent phosphate transport protein 2b; *TRPV6*, transient receptor potential cation channel subfamily V member 6; *KCNK7*, potassium two pore domain channel subfamily K member 7; *KCNQ1*, potassium voltage-gated channel subfamily Q member 1.

was more than 93.68% (Supplementary Table S4). These clean reads could be mapped to the reference genome of *Pelodiscus sinensis*, respectively (Supplementary Table S5). The CON and SAP groups expressed a total of 14,156 genes. A total of 13,210 genes were co-expressed, and the specific expression genes were 253 and 693, respectively (Supplementary Figure S2).

Compared with the CON diet, a total of 476 upregulated DEGs and 277 downregulated DEGs were identified in the SAP diet, respectively (Supplementary Figure S3). We conducted GO enrichment analysis for upregulated DEGs and downregulated DEGs, respectively. Figures 7A, B shows the top 20 items, respectively. We found that the SAP group significantly upregulated items that were mainly concentrated in response to the virus, ion binding, and immune response, and downregulated items that were predominantly enriched in hydrolase activity, acting on carbon–nitrogen (but not peptide) bonds, transporter activity, catalytic activity, and being an integral and intrinsic component of the membrane. We then focused our interest on the immune response. Through KEGG enrichment analysis, the genes with significantly upregulated differences were identified by "immune." Figure 8 shows the metabolic pathways of the top 20, and it was found that it was significantly enriched in coronavirus disease (COVID) 19, Fc gamma R-mediated phagocytosis, Nod-like receptor signaling pathway, etc.

3.7 QRT-PCR verification

In Figure 9, we selected 20 Chinese soft-shelled turtle large-intestine transcriptome differential genes for qPCR validation and found that the gene (*STAT1*, *TBX21*, *FOS*, *CCL3*, *AQP3*, *AQP3*, *AQP8*, *SLC30A1*, *SLC52A3*, *TMEM37*) validation results were consistent with the RNA-seq results with a significant difference. The genes (*IRF-7*, *JUN*, *TNF-α*, *IL-6*, *IL-8*, *IL-1β*,

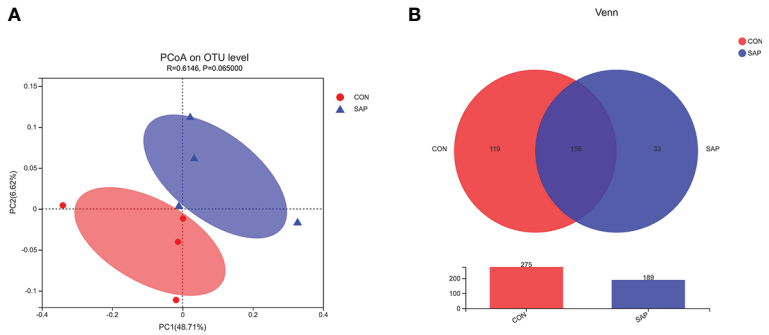

FIGURE 5
Principal coordinate analysis (PCoA) **(A)** and Venn diagram **(B)** of the intestinal bacterial community (n = 4). LEfSe multilevel species hierarchy tree **(A)** and LDA discriminant result table **(B)** (phylum species) (n = 4).

IL-10, *TGF-β2*, *SLC41A3*, *KCNJ16*) consistently matched the trend of RNA-seq results, indicating that the RNA-seq results were reliable.

4 Discussion

In the current study, dietary 2.46% soybean saponin Bb significantly reduced growth and feed utilization in juvenile turtles. Similarly, dietary supplementation of 5–10 g/kg soybean saponin markedly decreased the growth rate and feed efficiency in juvenile Japanese flounder (19) and zebrafish (28). Interestingly, in these two studies, the feed intake of Japanese flounder and zebrafish decreased concurrently with the addition of dietary saponin, whereas it was noticeably increased in the current study. It is speculated that the feed intake of the turtle was increased to meet body energy demands as a result of the overall decline in dietary nutrient utilization efficiency in the saponin-supplemented group. Therefore, the decreased growth performance of the turtles in the SAP group was attributed to the

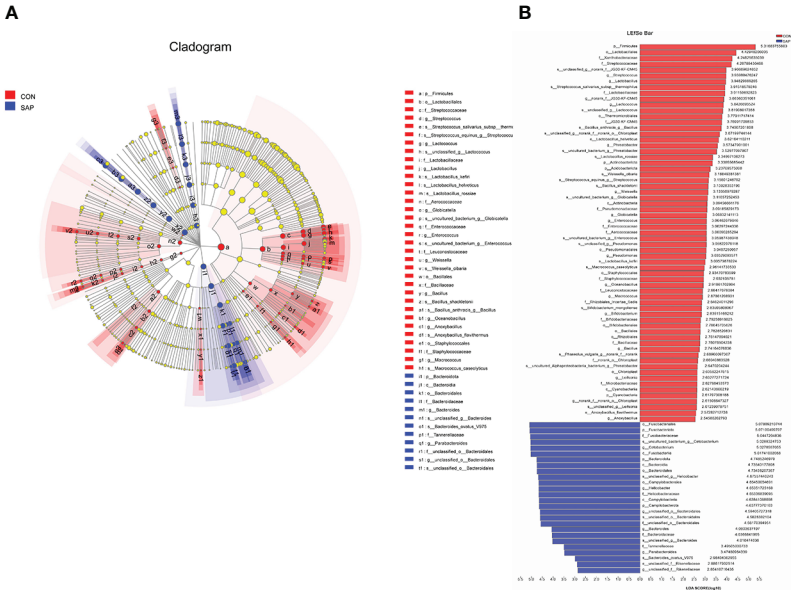

FIGURE 6
LEfSe multilevel species hierarchy tree **(A)** and LDA discriminant result table **(B)** (phylum-species) (n = 4). In the hierarchy tree diagram, red-colored nodes represent the microbial groups in the CON group are significantly higher than the SAP group. Blue-colored nodes represent the microbial groups in the SAP group are significantly higher than the CON group. The LDA discriminant column chart counts the microbial groups with significant effects in the two groups. The larger the LDA score is, the greater the impact of species abundance on the different effects.

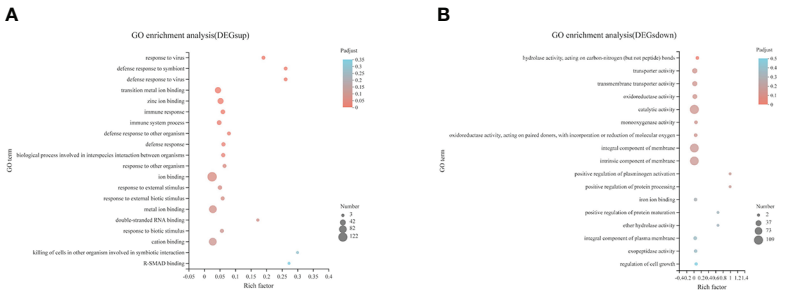

FIGURE 7
GO pathway enrichment analysis of the upregulated **(A)** and downregulated **(B)** DEGs in the comparison of CON vs. SAP.

decrease in feed utilization and not to the feed intake. Furthermore, the decrease in feed utilization may be related to the decline of nutrient digestion and absorption, which was evidenced by the decreased nutrient digestibility coefficients.

Digestibility *in vivo* is critical to evaluating nutrient availability in feed (29). We found that 2.46% soybean saponin Bb markedly decreased the apparent digestibility coefficients (ADCs) of dry matter, crude protein, crude lipid, and gross energy in juvenile turtles. Likewise, the ADCs of crude protein or/and crude lipid were decreased by the dietary addition of soybean saponin in Japanese flounder (19) and Atlantic salmon (30). This may be explained by the fact that the poorly digested soybean saponin increased the proportion of the nonabsorbable substances in the intestine, which negatively affected the digestion and absorption of other nutrients (31). More importantly, dietary soybean saponin gradually accumulated over time in the digestive tract and progressively damaged intestinal microstructure, and then the digestive function of the alimentary system was impaired, which was also corroborated by the adverse changes of the small intestine in the current study. It is widely believed that the height of the mucosal fold in the small intestine of aquaculture animals is positively correlated with the absorption and transport efficiency of dietary nutrients (32). Additionally, the width of the lamina propria was another parameter to evaluate intestinal health, and the degree of widening of the lamina propria was negatively related to intestinal health (33). In the current study, the height of the mucosal fold was shortened in the small intestine by dietary soybean saponin, and the width of the lamina propria was increased as well. Therefore, it is implied that the digestive function of the turtle was impaired by the dietary soybean saponin.

To further investigate which nutrient transports in the small intestine were hindered by the soybean saponin, we measured the key gene expressions related with glucose, fatty acid, peptide, amino acid, phosphorus, and calcium transports, as well as

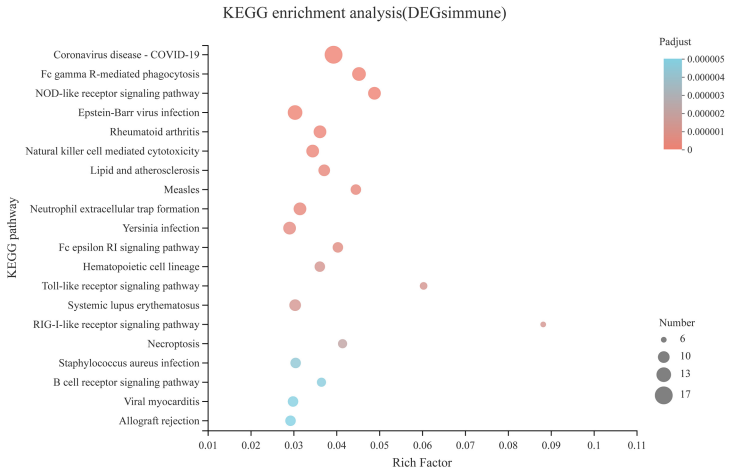

FIGURE 8
KEGG pathway enrichment analysis of the immune-related DEGs in the comparison of CON vs. SAP.

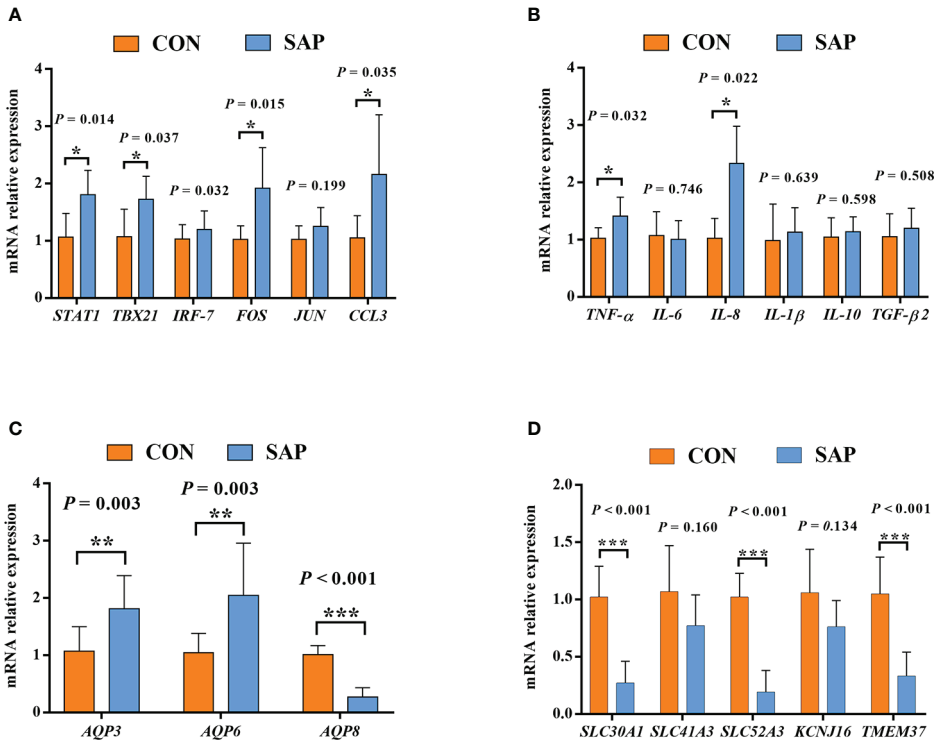

FIGURE 9
Effect of dietary soybean saponin Bb mRNA relative expression of large intestinal immune-related genes **(A, B)** and membrane transport/channels **(C, D)** (n = 6). *p < 0.05; **p < 0.01; ***p < 0.001; Student's t-test. *STAT1*, signal transducer and activator of transcription 1; *TBX21*, T-box transcription factor 21; *IRF7*, interferon regulatory factor 7; *FOS*, Fos proto-oncogene (AP-1 transcription factor subunit); *JUN*, Jun proto-oncogene (AP-1 transcription factor subunit); *CCL3*, C–C motif chemokine ligand 3; *TNF-α*, tumor necrosis factor alpha; *IL-6*, interleukin 6; *IL-8*, interleukin 8; *IL-1β*, interleukin 1 beta; *IL-10*, interleukin 10; *TGF-β2*, transforming growth factor beta 2; *AQP3*, aquaporin 3; *AQP6*, aquaporin 6; *AQP8*, aquaporin 8; *SLC41A3*, solute carrier family 41 member 3; *SLC30A1*, solute carrier family 30 member 1; *SLC52A3*, solute carrier family 52 member 3; *KCNJ16*, potassium inwardly rectifying channel subfamily J member 16; *TMEM37*, transmembrane protein 37.

relevant plasma metabolite and electrolyte concentrations. Most mammalian cells import glucose through a process of facilitated diffusion mediated by members of the membrane transporter GLUT (SLC2a) family (34). The facilitated-diffusion glucose transporter (*GLUT2*) on the basolateral surface of the enterocytes allows glucose to move from the small intestinal epithelial cells into the extracellular medium near blood capillaries (35). In this study, dietary 2.46% soybean saponin Bb significantly reduced the mRNA expression level of *GLUT2*, whereas the plasma glucose concentration was unaffected. It is probably because the plasma glucose levels in the turtle body should be regulated within a certain range through a dynamic balance mechanism to maintain body homeostasis. It is worth mentioning that the increase in feed intake in this study may be one of the strategies for maintaining blood glucose homeostasis. In terms of intestinal fatty acid absorption, the protein-mediated fatty acid uptake system is now thought to be the main pathway by which fatty acids are transported by membrane-associated fatty acid-binding proteins (FABPs) on the apical membrane of enterocytes (36). In this study, the relative expressions of *FABP1* and *FABP2* were reduced significantly by dietary soybean saponin Bb. This was in line with a previous study indicating that soy saponin significantly reduced *FABP2* expression level in the distal intestine of gilthead sea bream (11). This could also explain the decrease in turtle body lipids, apparent digestibility of crude lipid, and plasma cholesterol concentration in the soybean saponin Bb supplemented group. The terminal products of digested protein in the digestive tract of aquaculture animals were amino acids and small peptides, which were transported into/out of enterocytes *via* amino acid and di- and tripeptide transporters (37). The functional unit responsible for the absorption and transport of small peptides (dipeptides and tripeptides) in the small intestine of animals is *PEPT1*, which is located in intestinal epithelial cells (38). Dietary 2.46% soybean saponin Bb significantly increased the expression of *PEPT1* in juvenile turtles, which was consistent with the findings in zebrafish (24) and gilthead sea bream (11). This might be due to the fact that a higher proportion of dietary protein was digested in the form of small peptides in the intestine of turtles under the toxic effect of soybean saponin

Bb, which could be verified backward in the results of amino acid transporter expression level (*ASCT2* and B^0AT1). In addition, the transport of small peptides and free amino acids is carried out by two completely independent systems (39). In the current study, soybean saponin Bb decreased the intestinal expression of neutral amino acid transporters *ASCT2* and B^0AT1, but it had no significant effect on neutral amino acid transporter *LAT1* and basic amino acid transporter *CAT1*, this may have to do with the substrate specificity of amino acid transporters. Furthermore, the absorption and utilization of dietary calcium and phosphorus are also of great importance for the growth and health of animals (40). Therefore, the key genes related to calcium (*TRPV6*, *KCNK7*, *KCNQ1*) and phosphorus (*NaPi-IIb*) transport in the intestine and relevant plasma ion concentration were also analyzed in the current study. We found that *NaPi-IIb* expression level, as well as plasma phosphorus concentration, were significantly reduced by dietary saponin Bb, whereas the intestinal calcium transport-related gene expression was not affected, despite the fact that the plasma calcium level was reduced by saponin Bb. These results implied that dietary soybean saponin Bb negatively affects calcium and phosphorus utilization in the turtle body, which might be one of the reasons for decreased growth rate in this group. The downregulated expression levels of nutrient transporters (*GLUT2*, *FABP1*, *FABP2*, *ASCT2*, B^0AT1, and *NaPi-IIb*), as well as decreased plasma metabolites (cholesterol, calcium, phosphorus, and potassium) in the soybean saponin Bb group, may be attributed to impaired function of the apical membrane of enterocyte in turtles. It has been reported that the hydrophobic steroid backbone of saponins could intercalate into the hydrophobic interior of the phospholipid bilayer of enterocytes (41, 42), and then intestinal nutrient transport interfered.

It has been recognized that change in the intestinal microbial composition was closely correlated with intestinal health of the body (43, 44). On the genus level, dietary soybean saponin Bb significantly decreased the abundance of beneficial bacteria including *Lactobacillus*, *Bifidobacterium*, and *Bacillus*. It has been reported that *Lactobacillus* and *Bacillus* could promote nutrient digestion and absorption *via* secreting digestive enzymes (proteases, lipases) (45). Furthermore, *Lactobacillus* and *Bifidobacterium* exerted protective effects by counteracting enteropathogen infections (46). The decline of intestinal beneficial bacteria is also a good explanation for the decreased nutrient digestion and absorption in the saponin-supplemented group. Additionally, we found that dietary soybean saponin Bb increased the abundance of harmful bacteria with *Helicobacter* and *Bacteroides*. *Helicobacter* (47) and *Bacteroides* (48, 49) are the common opportunistic enteropathogens in aquatic animals, which might cause intestinal immune response and inflammation through generating endotoxins.

The dietary supplementation of soybean saponin Bb not only impaired the digestive function of the small intestine and disturbed intestinal microbiota composition but also induced inflammatory responses in the large intestine of this turtle. In the GO enrichment analysis of transcriptomics in the large intestine, the significantly upregulated genes between the treatment group and control group centered on the immune response and immune process. In order to further understand the inflammation-related metabolic pathway, we performed KEGG enrichment analysis for immune-related genes; the inflammatory response process starts from the recognition of foreign pathogenic microbial model molecules and self-injury model molecules by the cellular pattern recognition receptor PRRs to relevant transcription factors, chemokines, and cytokines. Also, the qPCR analysis proved that key transcription factors (*STAT1*, *TBX21*, *FOS*), chemokines (*CCL3*), and cytokines (*TNF*, *IL-8*) in inflammatory response were significantly upregulated in the large intestine of turtle by dietary soybean saponin Bb, which indicates that more active inflammatory responses were activated by soybean saponin Bb. In addition, in this study, soybean saponin Bb markedly upregulated the mRNA levels of *AQP3* and *AQP6* but downregulated *AQP8* in the large intestine of this turtle. Aquaporins are protein channels that promote the diffusion of water and small, uncharged molecules (such as glycerol or hydrogen peroxide) across the membrane, and they were dysregulated in response to immune and inflammatory stimulation (50). *AQP3* and *AQP8* are highly expressed in the colon, and some studies have shown that *AQP3* and *AQP8* were involved in the occurrence of intestinal inflammation (51, 52). Similarly, the intestinal *AQP8* expression level was also downregulated by the dietary addition of soybean saponin in *Sparus aurata* (11). Meanwhile, Lehmann et al. suggest that the decreased AQP8 expression is through a post-transcriptional mechanism mediated by tumor necrosis factor-α, resulting in the alterion of membrane homeostasis (53). We held the opinion that the increase of AQP3 and AQP6 may enhance the membrane permeability of H_20_2, cause oxidative stress of large intestine mucosa, and lead to intestinal inflammation. However, the decrease of AQP8 is a response mechanism after inflammation, which may be a defense mechanism against oxidative stress by proinflammatory factors. The signaling pathway of water channels in intestinal inflammation needs to be further studied.

5 Conclusions

In a word, this study indicates that the dietary soybean saponin Bb reduced the growth performance by decreasing the digestion and absorption of macronutrients and minerals in the small intestine. Meanwhile, dietary soybean saponin Bb damaged intestinal morphology and upregulated key transcription factors, chemokines, cytokines, and aquaporins in the inflammatory response in the large intestine, leading to

the intestinal inflammation of the turtle. In addition, dietary soybean saponin Bb reduced growth performance and induced intestinal inflammation, accompanied by the decreased abundance of beneficial bacteria and the increase of harmful bacteria in the digestive tract of Chinese soft-shelled turtles. This study provided a more comprehensive understanding of the adverse effects of dietary soybean saponin Bb on Chinese soft-shelled turtles, which was conducive to the development of nutritional strategies to relieve soybean meal-induced enteritis in other aquaculture animals.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA893835; https://www.ncbi.nlm.nih.gov/, PRJNA895971.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Hebei Normal University (198013, Shijiazhuang, Hebei, China).

Author contributions

YW: investigation, methodology, formal analysis, and writing—original draft. XJ and ZXG: investigation and data validation. LL and TL: data curation and validation. PZ: conceptualization, funding acquisition, supervision, validation, project administration, and writing—review. HL: funding acquisition, supervision, visualization, software, and writing—review. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Scientific Research Fund of Hebei Normal University (L2020B23), Hebei MATRT (HBCT2018180205, HBCT2018170205), the Science and Technology Project of Hebei Education Department (QN2022125), and the Natural Science Foundation of Hebei Province (C2022205034).

Acknowledgments

The authors thank Shijiazhuang Haitai Feed Factory for providing this experimental feed, Chengdu Efa Biotechnology Co. Ltd. for providing soybean saponin Bb standards, and Shanghai Meiji Biomedical Technology Co. Ltd. for providing sequencing analysis services.

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/fimmu.2022.1093567/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 30 January 2023
DOI 10.3389/fimmu.2023.1119902

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Comparative Immunology, a section of the journal Frontiers in Immunology

RECEIVED 09 December 2022
ACCEPTED 19 January 2023
PUBLISHED 30 January 2023

CITATION
Shen K, Bao L, Liu M, Lei W, Zhou Q, Ding J, Fang P, Hu B, Wen C, Kumar V, Peng M and Yang G (2023) Dietary supplementation of β-1, 3-glucan improves the intestinal health of white shrimp (*Litopenaeus vannamei*) by modulating intestinal microbiota and inhibiting inflammatory response.
Front. Immunol. 14:1119902.
doi: 10.3389/fimmu.2023.1119902

Dietary supplementation of β-1, 3-glucan improves the intestinal health of white shrimp (*Litopenaeus vannamei*) by modulating intestinal microbiota and inhibiting inflammatory response

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The phenomenon of intestinal dysfunction is widely observed in white shrimp (*Litopenaeus vannamei*) culture, and β-1,3-glucan has been confirmed to be beneficial in intestinal health with a lack understanding of its underlying mechanism. Proteobacteria, Firmicutes, and Actinobacteria served as the predominant phyla inhabiting the intestine of white shrimp, whilst a significant variation in their proportion was recorded in shrimp fed with basal and β-1,3-glucan supplementation diets in this study. Dietary supplementation of β-1,3-glucan could dramatically increase the microbial diversity and affect microbial composition, concurrent with a notable reduction in the ratio of opportunistic pathogen Aeromonas, gram-negative microbes, from Gammaproteobacteria compared to the basal diet group. The benefits for microbial diversity and composition by β-1,3-glucan improved the homeostasis of intestinal microbiota through the increase of specialists' number and inhibition of microbial competition caused by Aeromonas in ecological networks; afterward, the inhibition of Aeromonas by β-1,3-glucan diet dramatically suppressed microbial metabolism related to lipopolysaccharide biosynthesis, followed by a conspicuous decrease in the intestinal inflammatory response. The improvement of intestinal health referred to the elevation in intestinal immune and antioxidant capacity, ultimately contributing to the growth of shrimp fed β-1,3-glucan. These results suggested that β-1,3-glucan supplementation improved the intestinal health of white shrimp through the modulation of intestinal microbiota homeostasis, the suppression of intestinal inflammatory response, and the elevation of immune and antioxidant capacity, and subsequently promoted the growth of white shrimp.

KEYWORDS

***Litopenaeus vannamei*, β-1,3-glucan, intestinal microbiota, inflammatory response, immunity**

1 Introduction

White shrimp (*Litopenaeus vannamei*), one of the most widely farmed crustaceans in the world, can be cultured in fresh seawater due to its eurysalinity (1). As one of the high density breeding species in China, white shrimp farming has been plagued by intestinal dysfunction caused by an inflammatory response, which can be induced by the infection of pathogenic bacteria from *Aeromonas*, gram-negative microbes (2). The intestinal dysfunction could ulteriorly lead to metabolic dysfunction and trigger various diseases, followed by substantial economic losses to farmers. As we know, the intestine inhabits a complex micro-ecological system, and intestinal inflammation is confirmed to be closely related to microbial dysbiosis (3). The intestine is a crucial digestion organ, while it is also an essential immunity organ for animals. And thus, finding an effective way to solve the problem of intestinal dysfunction would promote the rapid development of shrimp culture.

β-glucan is a well-known immunostimulant, which can be extracted from cell walls of fungi, algae, and natural bioactive polysaccharides and contains three different specific glycoside linkage sources, including β- (1, 3)/β- (1, 4)/β- (1, 5) (4). The β-glucan has been widely applied to the aquaculture industry due to its positive impact on the health of aquatic animals (5, 6). Numerous studies in β-1,3-glucan have confirmed its property of anti-inflammatory, immune-enhancing, and anti-oxidation in aquatic animals such as white shrimp (7–9), marine swimming crab (*Portunus trituberculatus*), hybrid striped bass (*Morone chrysops* × *M. saxatilis*) (10), and large yellow croaker (*Pseudosciaena crocea*) (11). Evidence from human hepatocytes suggested that β-1,3-glucan could attenuate inflammation and accumulation of reactive oxygen species (ROS) induced by lipopolysaccharide (12). Similarly, the results in rainbow trout (*Oncorhynchus mykiss*) (13), silver catfish (*Rhamdia quelen*) (14), and common carp (*Cyprinus carpio* L) (15) suggested that β-glucan (including β-1,3-glucan) could reduce the intestinal inflammatory response and oxidative stress induced by the challenge of *Aeromonas hydrophila* or tissue damage. Additionally, the study in Indian major carp (*Labeo rohita*) suggested that β-1,3-glucan supplementation could dramatically elevate antibody response after being challenged with the *Edwardsiella tarda* vaccine (16).

Multitudinous evidence has confirmed that a vital role of intestinal microbiota in the intestinal health of the host, and β-glucan, a complex polysaccharide, is authorized to possess various health-promoting properties through intestinal microbiota regulation (17–19). Several studies explored the effects of β-glucan on the intestinal microbiota of aquatic animals including common carp (20), tilapia (21), rainbow trout (1), and white shrimp (22), mainly focusing on microbial composition. Undoubtedly, the number and richness of species are essential elements for a microbial ecosystem, while complex interspecific interactions are crucial to maintaining intestinal microbiota homeostasis (23). And thus, β-glucan supplementation altered the composition of intestinal microbial community and would further affect interactions between different species within the microbial community. It is well accepted that the microbes perform different roles due to their ecological niches, and the critical microbes were considered essential in maintaining intestinal microbiota homeostasis (24). Since 100-fold more genes than hosts possessed by microbes could synthesize many enzymes and other products, these symbiotic microbes inhabiting the intestine could participate in host nutrition metabolism and affect intestinal health through their metabolites (25–27). Hence, the intestinal microbiota is considered as an auxiliary metabolic organ involved in the metabolic process of the host (28).

Although β-1,3-glucan displayed positive effects on intestinal health, mechanisms explaining observed immunomodulatory in white shrimp have mainly remained obscure; hence, the purpose of the current study was to explore the underlying mechanism of β-1,3-glucan improving intestinal health of white shrimp through the investigation of its effects on the intestinal microbiota, inflammatory response, immunity, and antioxidant capacity. The results from this study would provide a better understanding of the mechanism of β-1,3-glucan ameliorating the intestinal health of white shrimp and further promote the application of β-1,3-glucan in the shrimp culture.

2 Materials and methods

2.1 Experimental procedure and sample collection

Healthy juvenile freshwater-acclimated *Litopenaeus vannamei* were provided by Huachuang Ecological Agriculture Development Co., LTD (Fuzhou, China). Before the experiment, shrimp were acclimatized in a filtered, aerated freshwater cage (2.0 × 2.0 × 1.5 m) to adapt to experimental conditions for two-week. In this study, a commercial feed (Charoen Pokphand) serves as a basal diet, and its ingredients are shown in Table 1. After being fasted for 24 h, shrimps with an average weight of 1.21 g were randomly distributed into six cages (1.0 × 1.0 × 1.5 m) in an outdoor pond at a density of sixty per cage. The β-1,3-glucan (65% purity), extracted from *Euglena gracilis*, was provided by Yunnan Shangri-la Zeyuan Algae Industry Health Technology Co., LTD. The glucan diet adds 0.325g/kg β-1,3-glucan to the basal diet. And then, the shrimp were fed satiated with basal diet (Control group, three replicates) and glucan diet (GL group, three copies) at 06: 00, 12: 00, and 18: 00 per day for two months, respectively. Environmental conditions were monitored during the experiment: dissolved oxygen > 5.0 mg L^{-1}; $NH4^{+}$-N< 0.2 mg L^{-1}; NO^{2-}N< 0.1mg L^{-1}.

At the termination of the feeding trial and following a 24 h starvation period, the total number and weight of the shrimps per cage were recorded. Five shrimps per cage were weighed and measured for condition factor (CF). After sterilization of surface skin with 70% ethanol, hemolymph samples were obtained from the

TABLE 1 Nutritional compositions of basal diets.

Ingredients	Content (%)	Ingredients	Content (%)
Crude protein	≥ 41.0	Moisture	≤ 12.0
Crude fat	≥ 4.0	Lysine	≥ 2.1
Crude fiber	≤ 3.0	Total phosphorus	≥ 1.0
Crude ash	≤ 16.0		

ventral sinus of three shrimp. The serum was separated from hemolymph and then stored at −80°C for subsequent analysis. Additionally, midgut tissues from three shrimp per cage were stored at −80 °C. Additionally, the whole intestine containing digest from two shrimp per cage (6 samples in each group) was stored in sterile freezing tubes under −80°C.

2.2 16S rRNA sequencing and intestinal microbiota analysis

According to previous studies (29), the genomic DNA of microorganisms was extracted from intestinal samples and performed High-throughput sequencing after amplifying the 16S rRNA V3-V4 region using the Illumina HiSeq platform. After the quality control, a total of 1,038,520 clean reads were clustered into operational taxonomic units (OTUs) based on the Ribosomal Database (rdp_16s_v16_sp), and then performed α-diversity (Observed OTUs, Chao1, AEC, Shannon, Simpson, and invSimpson), Treemap, and Principal coordinates analysis (PCoA) based on the Bray-Curtis dissimilarity using Rstudio (30). The ecological network was constructed with a random matrix theory (RMT)-based approach (31) and then was visualized using Circos (30) and Cytoscape 3.9.0 (32). The KEGG pathway of OTUs was analyzed by PICRUSt2 software (33) and visualized by Rstudio and software STAMP (34).

2.3 Intestinal gene expression

Total RNA samples were extracted from midgut tissue using the Trizol reagent (Takara, Dalian, China), and cDNA was synthesized using a SMART cDNA Synthesis Kit (Clontech Laboratories, Palo Alto, CA). For quantitative real-time PCR, the specific primer pairs were shown in Table 2. qRT-PCR reactions were carried out in a BIO-RAD CFX96 touch q-PCR system (Applied Biosystems Inc., USA). The $2^{-\Delta\Delta CT}$ method was used to compute relative gene expression levels.

2.4 Immune and antioxidant capacity analysis

The intestine and serum samples were used for measurement of the activities of acid phosphatase (ACP), alkaline phosphatase (AKP), lysozyme (LMZ), total superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), and the contents of reduced glutathione (GSH) and malondialdehyde (MDA) using commercial diagnostic kits (Nanjing Jiancheng Bioengineering Institute, China). The phenoloxidase (PO) parameter in the intestine and serum was assayed by a competitive ELISA kit produced by Nanjing Camilo.

2.5 Statistical analysis

The growth performance of shrimp was determined by final weight, Length gain rate (%), Specific growth rate (%/d) and Condition factor (g/cm^3). The formulas were defined as follows:

$$Length\ gain\ rate\ (\%) = 100 \times (final\ length - initial\ length)/initial\ size \quad ;$$

$$Specific\ growth\ rate\ (\%/d) = 100 \times (ln\ final\ weight - ln\ initial\ weight)/days \quad ;$$

Condition factor (g/cm^3) = final weight/final length^3.

The data for gene expression, immune and antioxidant parameters, and growth were analyzed by student's t-test using SPSS version 26 software. Data are presented as mean ± standard error of the mean (SE). Statistical significance was determined at $P< 0.05$.

TABLE 2 The primers for quantitative real-time PCR.

Primer name	Forward primers (5′ - 3′)	Reverse primers (5′ - 3′)
β-actin	GAGCAACACGGAGTTCGTTGT	CATCACCAACTGGGACGACATGGA
Crustin	GAGGGTCAAGCCTACTGCTG	ACTTATCGAGGCCAGCACAC
Penaiedin 3a	CACCCTTCGTGAGACCTTTG	AATATCCCTTTCCCACGTGAC
LZM	TGTTCCGATCTGATGTCC	GCTGTTGTAAGCCACCC
LGBP	CATGTCCAACTTCGCTTTCAGA	ATCACCGCGTGGCATCTT
Relish	CCTGTGAAGACATTAGGAGGAGTA	CCAGTTGTGGCATTCTTTAGG
Imd	TCACATTGGCCCCGTTATCC	ATCTCGCGACTGCACTTCAA
HSP70	AACGATTCTCAGCGTCAGG	AGGTGCCACGGAACAGAT
TGF-β	AACCATGCCCTTGTGCAAAC	CTTTGGGGGAACCTCGGTC
IL-1β	TGTGACCACCATCCACCAGAAC	GATCCCGCAGTAACCGAATAAG
TNF	AAAGAGGAACGTGGTCATGG	CACTCCTTTCCCCACTGTGT
IFN-γ	GACTTCGGTGCCACGGAACAAG	GACGCTCACTTTCACGCGGTCT
AIF	GCTGACATCATCCCCAACT	CTGGAATGTGCTATGGTG

LZM, lysozyme; LGBP, lipopolysaccharide and β-1,3-glucan-binding protein; HSP70, heat shock protein 70; IMD, innate immune deficiency; TGF-β, transforming growth factor-β; IL-1β, Interleukin-1β; TNF, tumor necrosis factor; IFN-γ, interferon-γ; AIF, apoptosis inducing factor.

3 Results

3.1 The intestinal microbiota of white shrimp

3.1.1 The composition of the intestinal microbial community in white shrimp

Compared to the Control group, the shrimp in the GL group exhibited a significantly higher microbial α-diversity in terms of a higher value in Observed OTUs, Chao1, AEC, Shannon, Simpson, and in InvSimpson ($P< 0.05$, Figure 1A). As shown in Figure 1B, Proteobacteria (Control: 46.43, GL: 50.12%), Firmicutes (Control: 50.80%, GL: 17.47%), and Actinobacteria (Control: 1.35%, GL: 12.47%) took dominate in the intestinal microbial community. Mainly, Gammaproteobacteria (43.25%) and Bacilli (47.80%) were observed as the predominant classes in the shrimp-fed basal diet. In contrast, shrimp in the GL group was predominated by Alphaproteobacteria (28.58%), Clostridia (12.81%), Actinobacteria (12.46%), Gammaproteobacteria (9.96%), and Betaproteobacteria (7.58%, Figure 1C). Shrimp fed basal diet possessed a significantly higher proportion of Photobacterium, Enterobacteriales and Aeromonadales from Gammaproteobacteria, and Bacillales and Unassigned microbes from Bacilli ($P< 0.05$). In contrast,

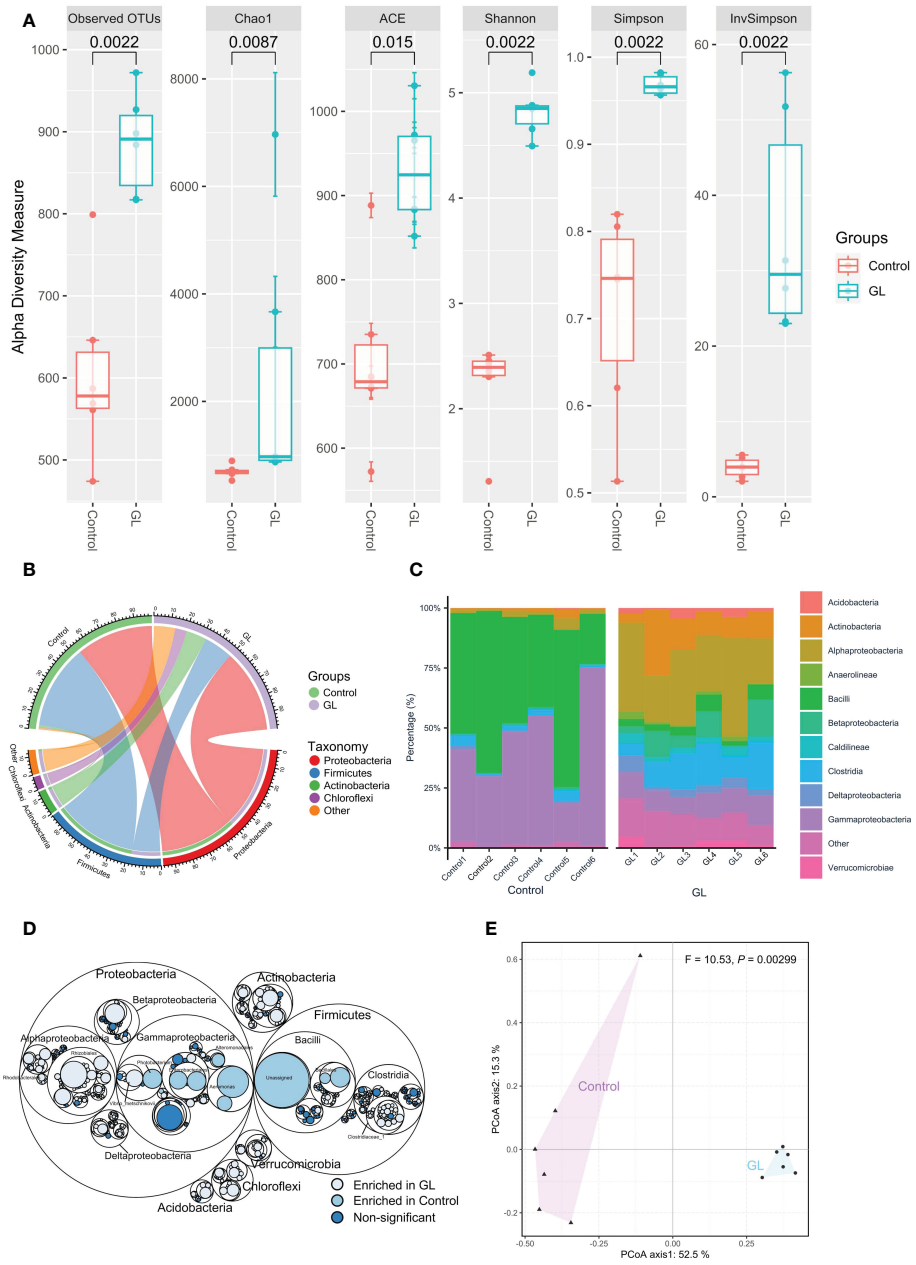

FIGURE 1
The effects of β-1,3-glucan on the composition of intestinal microbial community of *Litopenaeus vannamei*. **(A)** The α-diversity of the intestinal microbiota; **(B)** The relative abundance of species above ≥ a cutoff value of 2%; **(C)**The close lots of the top 10 classes; **(D)** Maptree plot descriptions of the taxonomic differences. The giant circles represent species level; the inner circles represent class, order, family, and genus for the panel; **(E)** PCoA analysis visualizing dissimilarities in the intestinal microbial community through permutational analysis of variance (PERMANOVA) based on the Bray–Curtis distance.

Rhodobacterales and Rhizobiales from Alphaproteobacteria and Clostridiaceae_1 from Clostridia were detected dramatically higher in the GL group, with a notably higher percentage of microbes from Acidobacteria, Verrucomicrobia, Chloroflexi, and Actinobacteria ($P< 0.05$, Figure 1D). Meanwhile, the PCoA analysis further confirmed the significant separation in the microbial community between Control and GL groups ($P = 0.00299$, Figure 1E).

3.1.2 The microbial interactions within the intestinal microbial community of white shrimp

Except for the notable variation in the microbial community, the circus plot exhibited the species-species interactions (red edges: negative interactions, gray edges: positive interactions) across different OTUs (Control: 558 OTUs, GL: 813 OTUs) from 32 bacterial classes within the intestinal microbial community (Figure 2A). The predominate classes, including Clostridia, Alphaproteobacteria, Actinobacteria, Gammaproteobacteria, and Bacilli, were observed to dominate the ecological networks, in which 10 and 11 modules (≥ 10 OTUs) were respectively attended in Control and GL networks as shown in Figure 2B. GL network was dominated by positive interactions, whereas opposite results were followed in the Control network with submodules C2, C3, C5, C6, C8, and C9 predominated by negative interactions. Within the network, the Control group has 10 module hubs and 3 connectors, while 11 module hubs and 3 connectors were found in the GL group (Figure 2C and Table 3).

3.1.3 The metabolic function of intestinal microbial community of white shrimp

The microbial function in the GL group dramatically differed from that in the Control group at KO levels (P = 0.005, Figure 3A). To explore the response of microbial metabolism to the diets, the KEGG functional categories were further analyzed, mainly focusing on amino acid metabolism, carbohydrate metabolism, lipid metabolism, and protein families: metabolism (Figure 3B). Compared to the shrimp-fed basal diet, much more metabolic

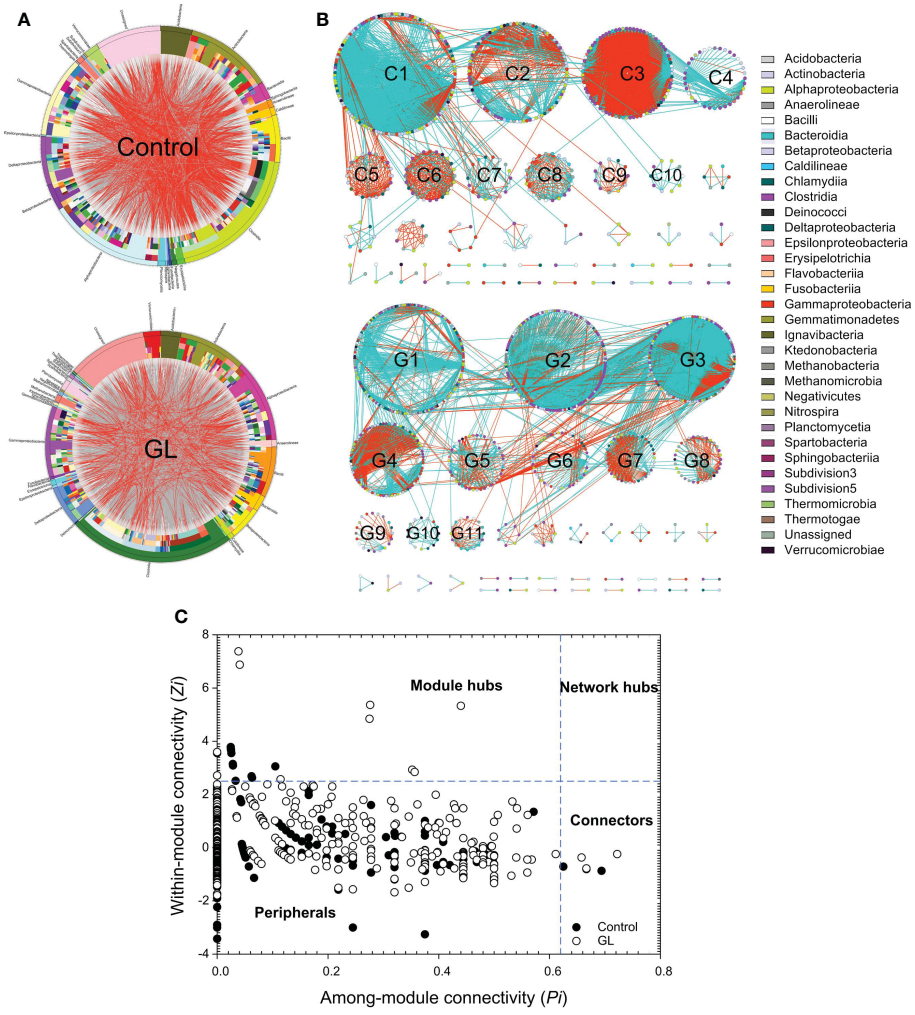

FIGURE 2
The effects of β-1,3-glucan on the interspecific interactions within the intestinal microbial community of *Litopenaeus vannamei*. **(A)** Circular plot descriptions of the interaction between different species. The taxonomic levels were class, order, family, genera, and species from the outside to the inside of the circle; **(B)** Ecological network within the microbial community. The edges (gray edge = positive interaction and red edge = negative interaction) inside the circle and ecological network represent the interactions between species. **(C)** Z-P plot showing the distribution of OTUs based on their topological roles.

TABLE 3 The composition of the ecological network.

Index	Control	GL
Acidobacteria	24	23
Actinobacteria	66	78
Alphaproteobacteria	88	95
Anaerolineae	6	8
Bacilli	36	58
Bacteroidia	13	18
Betaproteobacteria	20	38
Caldilineae	6	6
Chlamydiia	0	1
Clostridia	109	191
Deinococci	0	1
Deltaproteobacteria	37	46
Epsilonproteobacteria	1	3
Erysipelotrichia	6	6
Flavobacteriia	0	1
Fusobacteriia	2	3
Gammaproteobacteria	61	81
Gemmatimonadetes	0	2
Ignavibacteria	1	3
Ktedonobacteria	0	1
Methanobacteria	0	8
Methanomicrobia	0	5
Negativicutes	4	1
Nitrospira	1	1
Planctomycetia	6	12
Spartobacteria	5	4
Sphingobacteriia	1	4
Subdivision3	2	1
Subdivision5	1	1
Thermomicrobia	0	1
Thermotogae	1	1
Verrucomicrobiae	11	20
Unassigned	50	91
Total number of OTUs	558	813
The number of modules (≥10 OTUs)	10	11
The number of connectors	3	3
The number of gray edges	1880	3524
The number of red edges	1967	1219
Total number of edges	3847	4743

pathways related to the amino acid and carbohydrate metabolisms of intestinal microbiota were notably increased in the shrimp-fed β-1,3-glucan supplementation diet ($P< 0.05$). The microbial lipopolysaccharide biosynthesis and lipid metabolism were more active in the Control group together with more proteins related to metabolism ($P< 0.05$), especially the lipopolysaccharide biosynthesis proteins.

3.2 Gene expression related to inflammation and immune in the intestinal tissue of white shrimp

Compared to the shrimp fed basal diet, a significant reduction in the mRNA transcription level of inflammatory factor genes, including TGF-β, IL-1β, TNF, IFN-γ, and AIF, was detected in the intestinal tissue of shrimp in glucan diet ($P< 0.05$, Figure 4A). The β-1,3-glucan significantly induced the overexpression of immunity genes, including Cristin, Penaiedin 3a, LMZ, LGBP, and Imd in the intestinal tissue of shrimp ($P< 0.05$), while it unaffected the gene expression of Relish (Figure 4A); meanwhile, the expression of heat shock protein gene HSP70 in the intestinal tissue was dramatically suppressed in GL group ($P< 0.05$).

3.3 The immunity, antioxidant capacity, and growth performance of white shrimp

The β-1,3-glucan supplementation enhanced the concentration of PO and the activities of AKP, ACP, and LMZ in intestinal tissue. Significant differences in the value of activities of AKP, ACP, and LMZ were found between GL and Control groups ($P< 0.05$, Table 4). Similarly, significantly higher activities of serum AKP, ACP, and PO have been detected in shrimp from glucan group ($P< 0.05$). Compared to the Control group, the actions of CAT, TSOD, and GPx were notably elevated in the intestine tissue of shrimp from the GL group together with a reduction in MDA content ($P< 0.05$, Table 5); meanwhile, the GL group recorded higher activities of serum CAT and GPx, followed by a notably lower MDA content. Additionally, compared to the Control group, shrimp fed β-1,3-glucan diet exhibited significantly higher values in Final weight, Final length, Length gain, and Specific growth rate ($P< 0.05$), concurrent with a notable reduction in Condition factor ($P< 0.05$, Table 6).

4 Discussion

Multitudinous pieces of evidences from the animal model and human studies have verified the critical role played by intestinal microbiota in intestinal health, and the diet consumed by the host could shape the microbial composition (25, 28, 35, 36). β-glucan serves as a prebiotic and is confirmed to positively impact the intestinal microbiota and the host's health (19). Here, β-1,3-glucan supplementation did affect the intestinal microbiota and significantly increased the microbial diversity in white shrimp. Similar results were

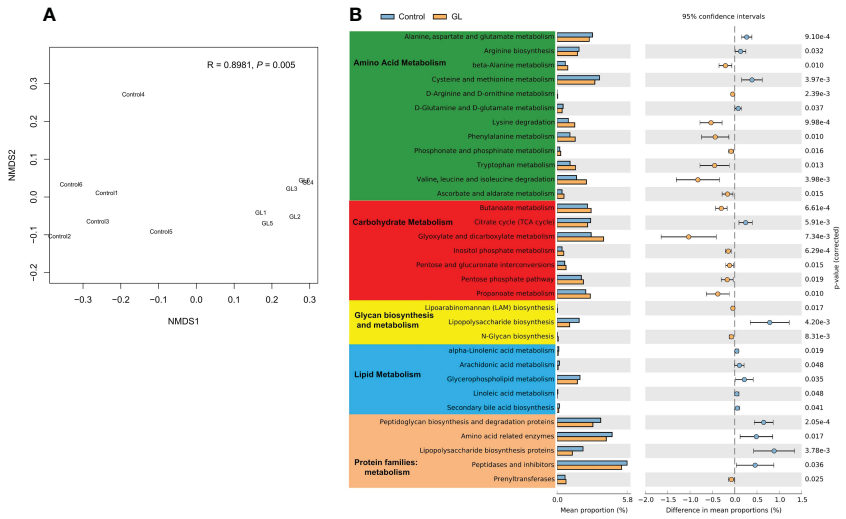

FIGURE 3
The effects of β-1,3-glucan on the microbial function. **(A)** NMDS plot visualizing microbial function dissimilarities using Bray–Curtis distance at KO level, and the test of difference was calculated by analysis of similarity (ANOSIM); **(B)** A two-sided Welch's t test analysis of the microbial metabolic function using the response ratio method at a 95% confidence interval (CI).

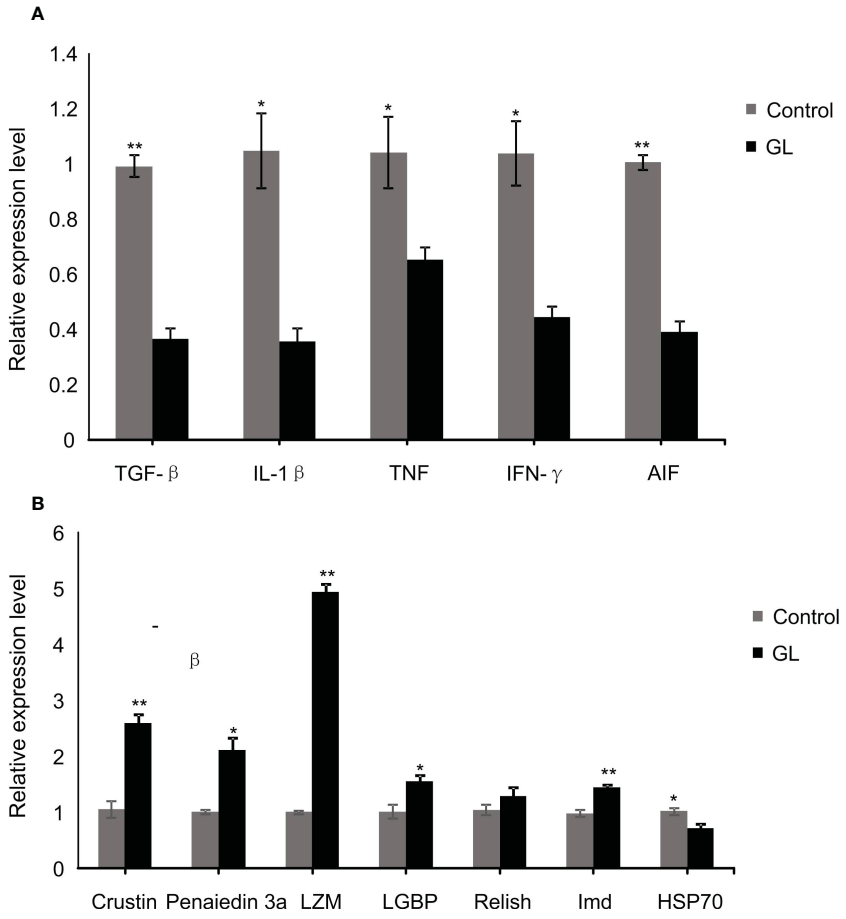

FIGURE 4
The relative mRNA levels of immune **(A)** and inflammation **(B)** related genes in intestinal tissue of *Litopenaeus vannamei*. *P< 0.05, **P< 0.01.

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TABLE 4 The effects of β-1,3-glucan on the immunity of *Litopenaeus vannamei*.

Parameters	Control	GL	P value
Intestine			
AKP (King unit/gprot)	50.12 ± 4.46	100.82 ± 10.62	0.006
ACP (King unit/gprot)	160.05 ± 8.19	204.13 ± 14.5	0.021
LZM (U/mgprot)	29.93 ± 1.9	37.87 ± 1.46	0.011
PO (ng/gport)	22.3 ± 3.15	46.89 ± 11.65	0.09
Serum			
AKP (King unit/100ml)	5.41 ± 0.62	12.29 ± 1.7	0.004
ACP (King unit/100ml)	3.40 ± 0.3	9.91 ± 2.23	0.033
LZM (U/ml)	25.00 ± 4.3	36.11 ± 6.33	0.177
PO (U/L)	85.99 ± 4.67	132.78 ± 6.55	<0.001

TABLE 5 The effects of β-1,3-glucan on the antioxidant capacity of *Litopenaeus vannamei*.

Parameters	Control	GL	P value
Intestine			
CAT (U/mgprot)	6.85 ± 0.91	10.39 ± 1.58	0.089
TSOD (U/mgprot)	24.92 ± 2.56	33.98 ± 2.32	0.025
GPx (U/mgprot)	15.74 ± 1.59	39.43 ± 3.18	<0.001
GSH (mg/gprot)	11.93 ± 1.52	15.06 ± 1.25	0.143
MDA (nmol/gport)	1861.11 ± 202.84	959.56 ± 73.84	0.005
Serum			
CAT (U/mL)	23.84 ± 2.31	31.67 ± 1.43	0.015
TSOD (U/mL)	704.23 ± 16.51	751.51 ± 14.21	0.055
GPx (U/mL)	1109.38 ± 48.99	1296.88 ± 19.62	0.005
GSH (mg/mL)	26.14 ± 3.95	59.24 ± 6.45	0.005
MDA (nmol/ml)	22.37 ± 1.4	15.79 ± 1.92	0.02

TABLE 6 The effects of β-1,3-glucan on the growth performance of *Litopenaeus vannamei*.

Parameters	Control	GL	P value
Initial weight (g)	1.19 ± 0.04	1.23 ± 0.01	0.402
Final weight (g)	10.62 ± 0.36	12.21 ± 0.22	<0.001
Final length (cm)	11.7 ± 0.1	12.53 ± 0.08	<0.001
Length gain rate (%)	1069.52 ± 10.34	1153.04 ± 8.35	<0.001
Specific growth rate (%/d)	8.74 ± 0.04	8.93 ± 0.02	0.001
Condition factor (g/cm^3)	0.66 ± 0.01	0.62 ± 0.01	0.007

also observed in tilapia (21), carp (*Cyprinus carpio*) (37) and white shrimp from a recent study (22). Although the composition of the intestinal microbial community can be dramatically affected by the diets, the core microbiota is essentially stable in the host (38). Here, results indicated that white shrimp fed basal and β-1,3-glucan diet also shared the same core microbiota from Proteobacteria and Firmicutes, even though intestinal microbiota structure was notably altered by dietary supplementation of β-1,3-glucan. The current results suggested that shrimp fed with commercial feed displayed a significant increase in the percentage of Aeromonas species from Gammaproteobacteria, which were confirmed to be opportunistic pathogens in freshwater-farmed white shrimp (2); β-1,3-glucan diet was found to inhibit these Aeromonas species significantly, and dramatically increase the proportion of microbes from Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Clostridiaceae_1, and scarce class groups like Actinobacteria, Chloroflexi, Verrucomicrobia, and Acidobacteria. The increased ratio of these low-class groups led to the rise of the microbial diversity in shrimp.

The interspecific interactions capacitate the microbial community to form an ecological network through which the micro-ecological ecosystem maintains its dynamic balance in intestine (23). In the present study, shrimp fed basal and β-1,3-glucan diets displayed two completely different patterns of microbial interactions referred to as competition and cooperation dominated in submodules of the ecological network, respectively. Based on the r/K selection theory in ecology, the r-strategy species would be the representative community appearing in nutrient-rich environments with characteristics of low competition, high nutrient utilization capacity, and high growth rate (39). Although artificial feeding provided a nutrient-rich environment for the intestinal microbiota, the proliferation of Aeromonas species, the pathogens, intensified the competition within the microbial community for the nutrients in this study. In spatial conditions, the cooperation-dominated communities are more stable because the cooperative interactions are more robust to population disturbance, while the competitive interactions are susceptible to perturbations (40, 41). And thus, the β-1,3-glucan diet promoted intestinal microbiota homeostasis by inhibiting the competition within the microbial community through the suppression of Aeromonas. The dominant microbiota is the main component of the ecological network, in which the generalists referred to connectors and module hubs serve as structural and functional keystones and execute a key role in maintaining the property of the web (42). Therefore, β-1,3-glucan promoted the stability of ecological network by increasing the number of module hubs. Taken together, dietary supplementation of β-1,3-glucan could improve the homeostasis of intestinal microbiota in white shrimp through the inhibition of composition caused by Aeromonas and the increase in number of module hubs.

The intestinal microbiota plays a vital role in host nutrient metabolism since microbial fermentation and nutrient synthesis provide numerous metabolites (25–27). Undoubtedly, the variation in microbial composition would further cause a change in microbial metabolism. The microbial carbohydrate metabolism was more active in the white shrimp fed a β-1,3-glucan diet in response to polysaccharide supplementation. Meanwhile, due to the significant reduction in the ratio of Aeromonas, the microbial lipopolysaccharide biosynthesis was significantly inhibited in shrimp fed β-1,3-glucan diet in this study. As we know, lipopolysaccharide could induce intestinal inflammation by triggering TLR4-mediated inflammatory

pathway (43, 44), and this may explain why β-1,3-glucan diet displayed an anti-inflammatory capacity in white shrimp in terms of the significant decrease in expression of inflammatory factor genes in this study. Additionally, β-1,3-glucan also could suppress intestinal inflammation by elevating the activity of intestinal AKP in the present study since the AKP can remove the phosphoric acid groups from lipopolysaccharide, thereby reducing its inflammatory effects (45, 46). Therefore, dietary supplementation of β-1,3-glucan could benefit intestinal health by recovering intestinal dysfunction induced by an inflammatory response.

The β-glucan, as an ideal immunostimulant, is widely applicated in aquaculture and positively impacts the immunity and antioxidant system of aquatic animals. Pieces of evidence from previous studies in white shrimp have confirmed the immune-enhancing effect of β-1,3-glucan (8, 47). The lipopolysaccharide and β-1,3-glucan binding protein (LGBP), vital pattern recognition proteins, can recognize lipopolysaccharide and β-1,3-glucan and subsequently trigger innate immunity (48). Indeed, the present results suggested that the expression of intestinal LGBP was significantly increased in shrimp fed β-1,3-glucan diet, together with an enhancement in immunity parameters in intestinal tissue and serum. Additionally, β-1,3-glucan supplementation enhanced the antioxidant capacity of shrimp, and similar results were also observed in white shrimp fed β-1,3-glucan (7). HSP70 serves as an important oxidative stress biomarker (49), and higher expression of HSP70 in the intestinal tissue shrimp fed β-1,3-glucan confirmed the antioxidant property of β-glucan again. Along with the improvement of intestinal function, the growth performance of white shrimp was also dramatically promoted by β-1,3-glucan finally.

In conclusion, β-1,3-glucan supplementation significantly altered the microbial composition and promoted the homeostasis of intestinal microbiota, and inhibited intestinal inflammatory through the suppression of pathogenic bacteria Aeromonas, concurrent with an enhancement in the immunity, antioxidant capacity, and growth performance of white shrimp.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA908810.

Ethics statement

The animal study was reviewed and approved by Nanchang University.

Author contributions

GY and MP designed the experiments and supervised the manuscript. KS and LB carried out the animal experiment and sample analysis with the help of ML, WL, QZ, JD, PF, BH, CW, and wrote the manuscript. VK revised the manuscript. All authors read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by the Double Thousand Program of Jiangxi Province (2019) and the Excellent Youth Cultivation Project of the National Natural Science Foundation of China (20202ZDB01010).

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 01 March 2023
DOI 10.3389/fimmu.2023.1110696

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Comparative Immunology, a section of the journal Frontiers in Immunology

RECEIVED 29 November 2022
ACCEPTED 13 February 2023
PUBLISHED 01 March 2023

CITATION
Li L, Wang Y, Huang Y and Wang C (2023) Multi-omics approach to study the dual effects of novel proteins on the intestinal health of juvenile largemouth bass (*Micropterus salmoides*) under an alternate feeding strategy. *Front. Immunol.* 14:1110696. doi: 10.3389/fimmu.2023.1110696

Multi-omics approach to study the dual effects of novel proteins on the intestinal health of juvenile largemouth bass (*Micropterus salmoides*) under an alternate feeding strategy

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Introduction: In an effort to minimize the usage of fishmeal in aquaculture, novel protein diets, including Tenebrio molitor, cottonseed protein concentrate, *Clostridium autoethanogenum*, and *Chlorella vulgaris* were evaluated for their potential to replace fishmeal. Nevertheless, comprehensive examinations on the gut health of aquatic animals under an alternate feeding strategy when fed novel protein diets are vacant.

Methods: Five isonitrogenous and isolipidic diets containing various proteins were manufactured, with a diet consisting of whole fishmeal serving as the control and diets containing novel proteins serving as the experimental diets. Largemouth bass (Micropterus salmoides) with an initial body weight of 4.73 ± 0.04g employed as an experimental animal and given these five diets for the first 29 days followed by a fishmeal diet for the next 29 days.

Results: The results of this study demonstrated that the growth performance of novel protein diets in the second stage was better than in the first stage, even though only the *C. vulgaris* diet increased antioxidant capacity and the cottonseed protein concentrate diet decreased it. Concerning the intestinal barriers, the *C. autoethanogenum* diet lowered intestinal permeability and plasma IL-1β/TNF-α. In addition, the contents of intestinal immunological factors, namely LYS and sIgA-like, were greater in *C. vulgaris* than in fishmeal. From the data analysis of microbiome and metabolome, the levels of short chain fatty acids (SCFAs), anaerobic bacteria, *Lactococcus*, and Firmicutes were significantly higher in the *C. autoethanogenum* diet than in the whole fishmeal diet, while the abundance of *Pseudomonas*, aerobic bacteria, *Streptococcus*, and Proteobacteria was lowest. However, no extremely large differences in microbiota or short chain fatty acids were observed between the other novel protein diets and the whole fishmeal diet. In addition, the microbiota were strongly connected with intestinal SCFAs, lipase activity, and tight junctions, as shown by the Mantel test and Pearson's correlation.

Discussion: Taken together, according to Z-score, the ranking of advantageous functions among these protein diets was *C. autoethanogenum* diet > *C. vulgaris*

10.3389/fimmu.2023.1110696

diet > whole fishmeal diet > cottonseed protein concentrate > *T. molitor* diet. This study provides comprehensive data illustrating a mixed blessing effect of novel protein diets on the gut health of juvenile largemouth bass under an alternate feeding strategy.

KEYWORDS

intestinal health, alternate feeding strategy, intestinal barrier, novel protein diets, largemouth bass, SCFAs (short chain fatty acids)

1 Introduction

Aquaculture provides humans with enormous amounts of high-quality proteins. Both oceanic and freshwater aquaculture need vast quantities of fishmeal, a high-quality protein for aquaculture (1). However, fishmeal is unsustainable, from 2020 FAO report, the yearly output of fishmeal in the next decade may rise by no more than 1% compared to 2018, while the price of fishmeal will climb by 30%, putting a significant strain on diets prices and worldwide fishmeal stock capacity (2). Consequently, it is crucial to explore high-quality fishmeal substitutes in aquatic practices.

Chlorella vulgaris diet, *Clostridium autoethanogenum* protein diet, cottonseed protein concentrate diet, and *Tenebrio molitor* diet are developed to ease the shortage of fishmeal in feed industry. In recent years, researchers have concentrated on these novel proteins owing to their abundant fat/fatty acids, low price, high content of protein, mineral, balanced amino acid profiles, and vitamin content (3–6). Earlier studies have examined the impacts of these proteins on *Cyprinus carpio* var. *specularis*, *Litopenaeus vannamei*, and *Micropterus salmoides* (4, 7–9). Nonetheless, these studied mainly focus on partial substitution strategy of fishmeal, and explore their functions on muscle quality, growth performance and intestinal health. Few studies have been carried out to investigate in depth the impacts of novel proteins on fish growth performance and intestinal immunity from the perspective of alternate feeding strategy while comparing the relative substitution potential of each novel protein diet.

Alternate feeding strategy is an effective method to conserve high-value or resource-limited feed ingredients. Some studies have extensively conducted for fish oil based diet substitution with terrestrially derived oil or plant-sourced oil diets without compromising fish fatty acid composition (10), fish growth performance (11), or their physio-biochemical performance (12, 13) in *Scophthalmus maximus*, *Gadus morhua*, *Dicentrarchus labrax*, and *Acanthopagrus schlegelii*. These studies showed that using an alternate feeding strategy could reduce the use of fish oil. However, few studies have focused on replacing fishmeal with novel proteins under an alternate feeding strategy. The intestine is the primary location for the absorption and digestion of fish nutrients, and its health is favorable to the correct execution of fish physiological processes (14). It is commonly accepted that the intestinal barriers include of immunological, microbial, physical, and chemical barriers (15), which play key roles in fish growth, nutritional status, immunology, and resistance to illness.

Largemouth bass (*M. salmoides*), which is one of the most cultivated species in China, was selected as a research model owing to its high fishmeal consumption in commercial diets, which ranges between 35 and 50 percent (16). In order to explore novel protein diets that might potentially replace fishmeal in fish diets, this research aims to evaluate the effects of novel protein diets on the gut health and growth performance of juvenile largemouth bass under an alternate feeding approach. Compared to previously published research on these proteins, the present study displayed a comprehensive view of the interaction between novel proteins and gut health and growth performance, with a focus on the connection between gut microbiota, their metabolites, and physiochemistry biomarkers under an alternate feeding strategy, as illustrated in Figure 1.

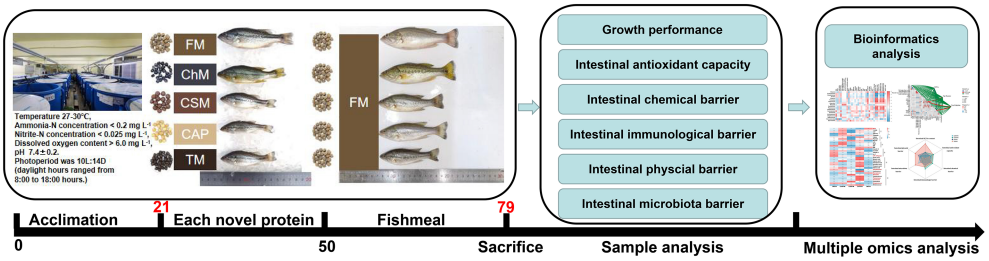

FIGURE 1
The work flow of this study. Whole fishmeal diet, *C. vulgaris* diet, cottonseed protein concentrate, *C. autoethanogenum* diet, and *T. molitor* diet, referred as FM/FM, ChM/FM, CSM/FM, CAP/FM, and TM/FM, respectively.

2 Materials and methods

2.1 Experimental design

The methodologies employed in this research properly adhered to the management Rule of Laboratory Animals (Chinese order no. 676 of the State Council, revised 1 March, 2017). The largemouth bass were provided by Hubei Zhenghao Fish Fry farm. Cottonseed protein concentrate, Fishmeal, *C. autoethanogenum*, *T. molitor* and *C. vulgaris* were formulated to five isonitrogenous and isolipidic expanded pellet diets. The approximate composition was shown in Supplementary Table 1. Each feed was molded into 2 mm floating pellets and air-dried for two days at room temperature. All experimental diets were kept at a temperature of -20°C. Anterior to the start of the growth trial, the five diets were pooled and supplied to juvenile largemouth bass for 21 days to prevent the unpalatability and to acclimatize them to the experimental surroundings. Five groups of largemouth bass (starting body weight: 4.73 ± 0.04g) with three replicates per treatment were allocated to 15 blue cylindrical acrylic tanks (Water volume: 400 L) at random, containing 35 fish per tank in a recirculation system. The experiment consisted of two stages, in which fish were initially given novel proteins for 29 days, then fishmeal for another 29 days, and all were fed twice daily at 9:00-9:30 am and 5:00-5:30 pm until a level of apparent satiety. The bulk body weight of fish was recorded every week. Throughout the duration of the growth trial, about 1/7 of the water was replenished every day to remove faeces buildup. During the growth trial, the water quality was maintained as follows: 27-30°C water temperature, ammonia-N<0.2 mg L^{-1}, nitrite-N<0.025 mg L^{-1}, and dissolved oxygen>5.0 mg L^{-1}, pH at approximately 7.5, and 10L:14D photoperiod with illumination from 8:00 am to 6:00 pm.

2.2 Sample collection

In order to examine the gut microbiota and short chain fatty acids (SCFAs), seven fish from each treatment were randomly taken after a 6-hour fast on day 58 and anesthesia with 75 mg L^{-1} MS-222 (Aladdin, Shanghai, China). The method for sacrificing fish was previously described (17). The entire contents of the intestine were collected in sterile centrifuge tubes, which were immediately frozen in liquid nitrogen and stored at -80°C. Three samples from each treatment, with similar bacterial structures and compositions, were analyzed for their SCFAs contents. On day 58, additional fish were starved for 24 hours, anesthetized with the same amount of MS-222, slaughtered, and dissected as described above. The middle intestine of nine fish from each treatment was rapidly frozen in liquid nitrogen and stored at -80°C for investigation of intestinal biochemical indexes and associated gene expression. For morphological study, the distal intestines of nine fish from each treatment group were preserved in 4% paraformaldehyde. For intestinal permeability investigations, serum was taken from nine fish per treatment, allowed to rest at 4°C for overnight, and the supernatant was collected. The initial body weight (IBW), final body weight (FBW), average daily gain (ADC), Feed conversion ratio (FCR), protein efficiency ratio (PER), specific growth rate (SGR), and survival rate (SR) were calculated using the following formulae:

$$\mathrm{ADC} = (\text{Final fish weight} - \text{Initial fish weight})/\text{Number of days}$$

$$\mathrm{FCR} = (\text{Feed intake}/\text{Weight gain})$$

$$\mathrm{PER}(\%) = 100 \times (\text{Fresh body weight gain})/(\text{Dry feed intake} \times \text{Protein of feed})$$

$$\mathrm{SGR}(\%) = \left\{ [\ln(\text{Final body weight}) - \ln(\text{Initial body weight})]/\text{Number of days} \right\}$$

$$\mathrm{SR}(\%) = 100 \times (\text{Final fish number}/\text{Initial fish number})$$

2.3 Examining the intestinal morphology and hematological parameters

The intestinal tissue slice slides were made by Service bio-Company (Wuhan, China). Briefly, the middle gut was washed in sterile saline, fixed for 48 hours in 4% paraformaldehyde, and then rinsed in 70% ethanol. 5μm thick slices of paraffin-embedded, aematoxylin-eosin-stained intestines. Using an Olympus DP72 microscope paired with a Nikon E600 camera and Olympus CellSens Standard software to acquire images. At 10 sites on a single slide, the height/width of each villi, the muscle layer thickness and the number of goblet cells, were assessed. In addition, in order to eliminate the effects from body length, the quantification results from morphology were calibrated. Using commercial Elisa kits, the serum concentrations of lipopolysaccharide (LPS), lysozyme (LYS), Interleukin 1 beta (IL-1β), and tumor necrosis factor (TNF-α) were measured (Enzyme-linked Biotechnology, Shanghai, China). The required operation was conducted in accordance with the instructions.

2.4 Evaluation of the intestinal biochemical parameters

Approximately 100 mg of intestinal tissue was mixed with cold saline to generate a 10% homogenate (Tissue: Saline=1:9). After centrifugation (4500 rpm/min) for 15 minutes, the supernatant was collected and analyzed. Employing commercial test kits (Nanjing jiancheng Bioengineering Institute, Nanjing, China), the activities of superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) were determined. Utilizing commercial Elisa kits, the level of glutathione peroxidase (GSH-Px) and mucin 2 (MUC2), lysozyme (LYS), immunoglobulin M (IgM), immunoglobulin G-like (IgG-like), and secretory immunoglobulin-like (sIgA-like) were determined (Enzyme-

linked Biotechnology, Shanghai, China). The required operation was conducted in conformity with the instructions.

2.5 Real-time fluorescent quantitative PCR

RT-qPCR was conducted as previously described (17). Briefly, total RNA of middle intestine was extracted, and RNA templates were used to transcribe cDNAs. The cDNAs were amplified by PCR using the PrimeScript RT reagent Kit (Takara, Japan). With the primers provided in Supplementary Table 2, the RT-qPCR was conducted on a CFX96^{TM} Real-Time System (bio-RAD, USA). *β-actin* was chosen as a housekeeping gene due to its stable expression. The primers, i.e., *occludin*, *claudin-1*, *zo-1*, *claudin-4*, *sod*, *cat*, *gpx*, *keap-1*, and *nrf-2*, were selected and exhibited amplification efficiencies ranging from 91% to 115%. The results of real-time RT-qPCR were analyzed by the $2^{-\Delta\Delta CT}$ method.

2.6 Microbiome study based on sequencing of the 16S rRNA gene

Following the manufacturer's instructions, total bacterial DNA was extracted from the intestinal contents using an OMEGA DNA Kit (D5625-01) (Omega Bio-Tek, Norcross, GA, USA). PCR amplification and Illumina MiSeq sequencing (Shanghai, China) were used to study the V3-V4 region of the bacterial 16S rRNA gene. Refer to the additional materials for more information.

2.7 Measurement of short chain fatty acids

The detection of intestinal contents short chain fatty acids was accomplished by the previously described technique of gas chromatography-mass spectrometry (GC-MS) (17). See supplementary materials for details.

2.8 Data analysis

SPSS 25.0 (IBM, Armonk, NY, USA) were used to analyze the data. For data not normally distributed, the nonparametric Krustal-Wallis test followed by the Dunn's multiple comparison test was applied. One-way ANOVA was used to evaluate normally distributed data, following with least significant differences for multiple treatments comparisons. $P<0.05$ was considered statistical differences. Illustrations were created using R 3.6.1 ggplot2 package as well as GraphPad Prism 8.3.0. In addition, we supported the idea that statistical differences should not serve as the exclusive criteria for assessing data discrepancies (18), and we wish to apply multiple criterion to study data discrepancies among experimental treatments fully. Consequently, z-score value was employed to standardize the data and it was used to assess the complex data and their complicated association owing to the huge amount of differently classified physiological indicators utilized in our study. In addition, Z value is negative once the original value is below the mean and positive when the original value is above the mean. This Z value is independent of the original unit of measurement.

3 Results

3.1 Growth performance, feed utilization and intestinal histological analysis of juvenile largemouth bass under an alternate feeding strategy

As demonstrated in Figures 2A, B, FBW, ADC, SGR, and PER were reduced in diets containing novel proteins, although the SR, intestinal morphology, villus structure, muscular layer thickness, and goblet cells were unaltered. However, significant changes in FCR were not seen in ChM/FM, CSM/FM, and CAP/FM. Figure 2C demonstrates that the slopes of K of 0-4 weeks were less than the 4-8 weeks, which showed that growth performance of treatments containing novel protein diets in the second stage outperformed the first stage.

3.2 The oxidative stress of juvenile largemouth bass under an alternate feeding strategy

The levels of CAT, T-SOD, MDA, GSH-Px, *cat*, *gpx*, *sod*, *nrf-2*, and *keap-1* were evaluated in the intestine (Figures 3A–J). With the greatest GSH-Px activity in the ChM/FM group and the lowest CAT activity in the CSM/FM group, there were no dramatically discrepancies in the levels of MDA and T-SOD between diets containing novel proteins and FM/FM. In comparison to other diets, the levels of *gpx* and *sod* were significantly reduced in the ChM/FM and CSM/FM groups. Although the expression patterns of *nrf-2* and *keap-1* were distinct, the ratio of *keap-1/nrf-2* did not alter substantially between diets containing novel proteins and FM/FM. These non-significant variations were also discovered in the level of *cat*.

3.3 The intestinal chemical barriers and immunologic barriers of juvenile largemouth bass under alternate feeding strategy

The contents of LYS, sIgA-like, IgG-like, IgM, TNF-α, IL-1β, amylopsin, lipase, pepsase, and mucin 2 in the gut were examined to further investigate the impacts of alternate feeding of diets containing novel proteins on immunochemistry barriers (Figures 4A–K). Except for sIgA-like, no statistically significant alterations were seen in these biochemical indices. However, the plasma concentrations of TNF-α and IL-1β were lower in CAP/FM than in FM/FM (P=0.074). Moreover, the greatest levels of two

10.3389/fimmu.2023.1110696

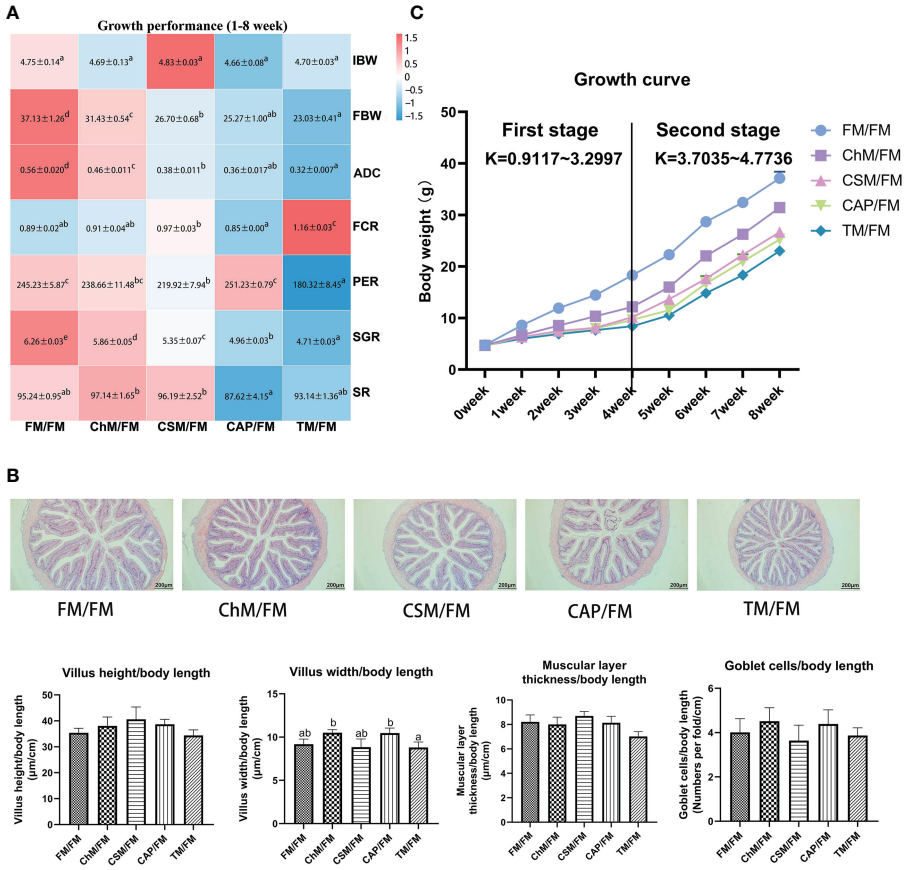

FIGURE 2
The growth performance and intestinal morphology of juvenile largemouth bass under an alternate feeding strategy. Growth performance heatmap **(A)**;The distal intestines of FM/FM, ChM/FM, CSM/FM, CAP/FM, and TM/FM protein diet under 100 x magnification and the intestinal quantitative data. **(B)**; Wight gain growth curve **(C)**. Data are also expressed as mean (S.E.M). Values on each column/heatmap with different superscripts typified statistical differences (*P<0.05*).

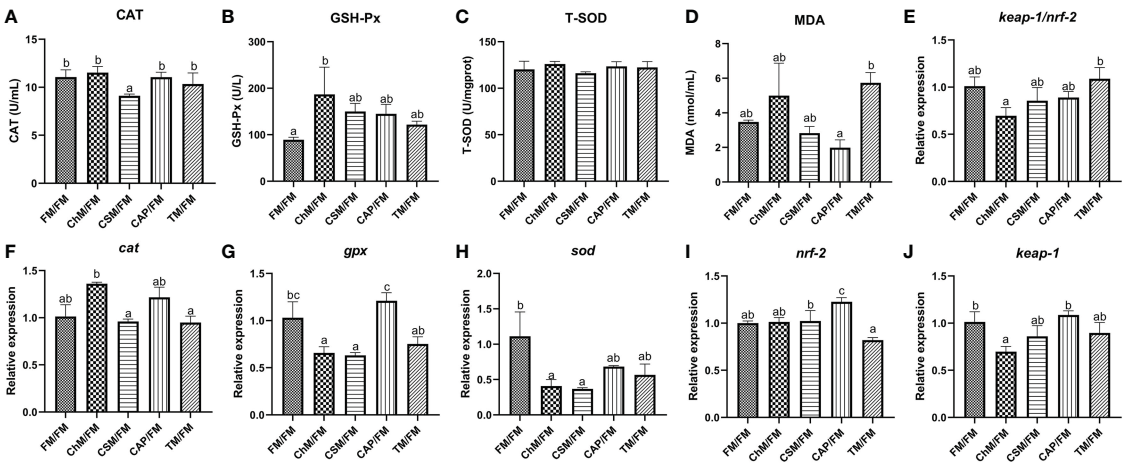

FIGURE 3
The antioxidant enzymes and genes expression of juvenile largemouth bass intestines under an alternate feeding strategy were shown as follows: CAT **(A)**, GSH-Px **(B)**, T-SOD **(C)**, MDA **(D)**, *keap-1/nrf-2* **(E)**, *cat* **(F)**, *gpx* **(G)**, *sod* **(H)**, *nrf-2* **(I)**, and *keap-1* **(J)**. Data are expressed as mean (S.E.M). Values on each column with different superscripts typified statistical differences (*P<0.05*).

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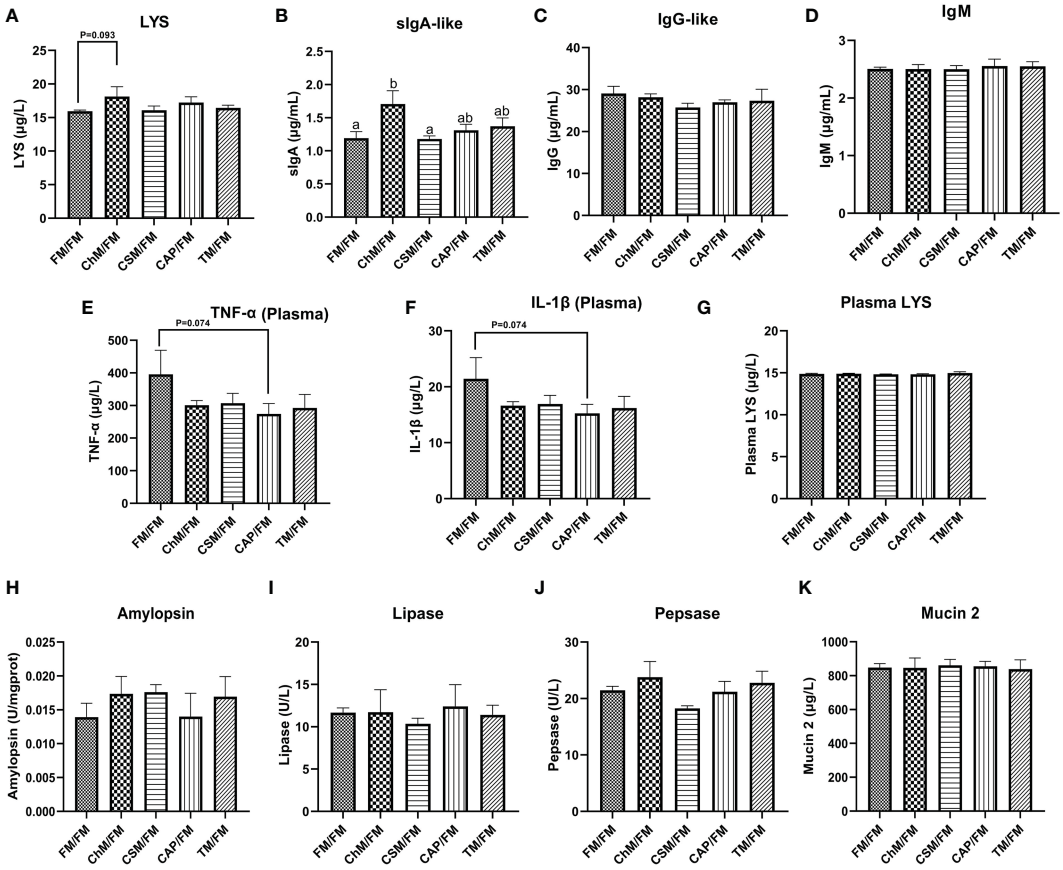

FIGURE 4
The immunological and chemical barriers of juvenile largemouth bass under an alternate feeding strategy were shown as follows: LYS **(A)**, sIgA-like **(B)**, IgG-like **(C)**, IgM **(D)**, plasma TNF-α **(E)**, plasma IL-1β **(F)**, LYS **(G)**, amylopsin **(H)**, lipase **(I)**, pepsase **(J)**, and mucin 2 **(K)**. Data are expressed as mean (S.E.M). Values on each column with different superscripts typified statistical differences (*P<0.05*).

crucial immunological markers, LYS and sIgA-like, were seen in the ChM/FM group.

3.4 The intestinal permeability of juvenile largemouth bass under an alternate feeding strategy

The intestinal permeability was measured to assess the intestinal physical barrier's integrity (Figures 5A–F). The level of *claudin-1* and *zo-1* did not differ statistically between CAP/FM and FM/FM, however the expression of these genes in other protein diets was considerably reduced in comparison to FM/FM (*P<0.05*). Intriguingly, the level of *claudin-4* was substantially greater in CAP/FM than in FM/FM (*P<0.05*), and the transcription of *occludin* was significantly lower in TM/FM (*P<0.05*). In addition, the plasma LPS concentrations of all diets containing novel proteins were lower than FM/FM, and the intestinal LPS content of CAP/FM was lower than CSM/FM (*P=0.057*) and ChM/FM (*P=0.051*), while there were no significant differences amongst the five protein diets.

3.5 Intestinal microbiome analysis of juvenile largemouth bass under an alternate feeding strategy

To better comprehend the impact of new protein diets on the intestinal microbiome of juvenile largemouth bass under an alternative feeding strategy, the microbial diversity, functions, and compositions were investigated. Figures 6A–C demonstrate that the β-diversity of CAP/FM is substantially greater than that of other diets (*P<0.05*). Similarly, the α-diversity of CAP/FM exhibited a greater Simpson index than FM/FM (*P<0.05*). The bugbase function and phenotypic predictions (Figure 6D) revealed that ChM/FM had more stress tolerance than all other diets. Additionally, the quantity of anaerobic bacteria in CAP/FM exceeded that of the other protein diets, but the abundance of aerobic bacteria was the lowest. Figures 6E, F illustrate the intestinal composition at both the phylum and genus levels. Firmicutes, a SCFAs-producing phylum, and Proteobacteria, a phylum of opportunistic pathogens, changed significantly. Accordingly, the quantity of probiotics (*Lactococcus*) and pathogens (*Pseudomonas*) in the five protein diets was highly

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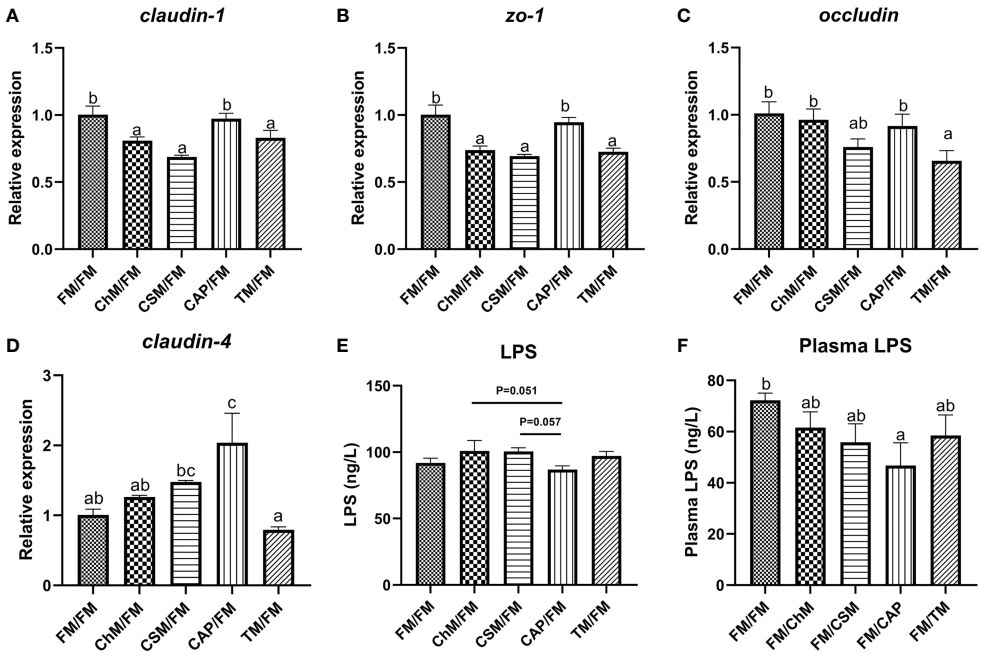

FIGURE 5
The intestinal physical barrier of juvenile largemouth bass under an alternate feeding strategy were shown as follows: *claudin-1* **(A)**, *zo-1* **(B)**, *occludin* **(C)**, *claudin-4* **(D)**, intestinal LPS **(E)**, and plasma LPS **(F)**. Data are expressed as mean (S.E.M). Values on each column with different superscripts typified statistical differences (*P<0.05*).

variable at the genus level. Specifically, Figures 6G, H depict the comparison of abundance of microbiota. The CAP/FM diet included the greatest abundance of *Lactococcus* and Firmicutes and the lowest abundance of *Pseudomonas* and Proteobacteria. In addition, CAP/FM showed a greater abundance of *Plesiomonas* than both ChM/FM and FM/FM (*P<0.05*). In contrast, these two protein diets included a greater number of Actinobacteria, *Streptococcus*, and Cyanobacteria than the CAP/FM diet. However, other microbiota did not differ considerably between diets containing novel proteins and FM/FM.

3.6 The contents of SCFAs in juvenile largemouth bass under an alternate feeding strategy

SCFAs have crucial roles in maintaining intestinal health and preventing occurrence of disease; thus, their presence in the five protein diets were considered. Figure 7A is a chord diagram depicting the contents of intestinal SCFAs in each protein diet. Seven SCFAs were discovered, with the greatest acetic acid concentration and the lowest isobutyric acid level. The heatmap in Figure 7B reveals that the CAP/FM diet included a greater proportion of SCFAs than other protein diets. In contrast, the concentration of SCFAs in TM/FM was the lowest. The bar graphs clearly (Figures 7C–J) demonstrate the considerable variation in SCFAs levels across all protein diets (Figure 7C). The lowest levels of total SCFAs, acetic acid, butyric acid, and propionic acid were found in the TM/FM diet, whereas the highest levels were found in the CAP/FM diet. Comparatively, the concentrations of isovaleric acid and isobutyric acid in the CAP/FM diet were considerably greater than in the FM/FM diet (*P<0.05*), although these two acids were significantly lower in other diets containing novel proteins (*P<0.05*). Additionally, no considerable differences were identified between diets containing novel proteins and FM/FM for caproic acid and valeric acid.

3.7 The integrity analysis of juvenile largemouth bass under an alternate feeding strategy

Pearson's correlation was used to further study the link between microbiota and physiological indicators (Figure 8A). Shannon index, Simpson index, Firmicutes, *Lactococcus*, Anaerobic bacteria, Fusobacteriam, *Plesiomonas*, *Cetobacterium*, and *Aeromonas* were positively correlated with SCFAs (acetic acid, butyric acid, isovaleric acid, isovaleric acid, and propionic acid) and *claudin-4*. In contrast, these two categories of physiological indicators were adversely associated with pathogens, including Proteobacteria, *Pseudomonas*, and Aerobic bacteria. Similarly, Cyanobacteria, Actinobacteria, and *Streptococcus* also had significant positive correlations with ADC, SGR, and *occludin* (*P<0.05*). Inversely, the Verrucomicrobia had adverse correlations with ADC, SGR, and PER (*P<0.05*) although a positive correlation with FCR was observed (*P<0.05*). Surprisingly, the intestinal structure biomarkers were associated with *Weissella* (*P<0.05*).

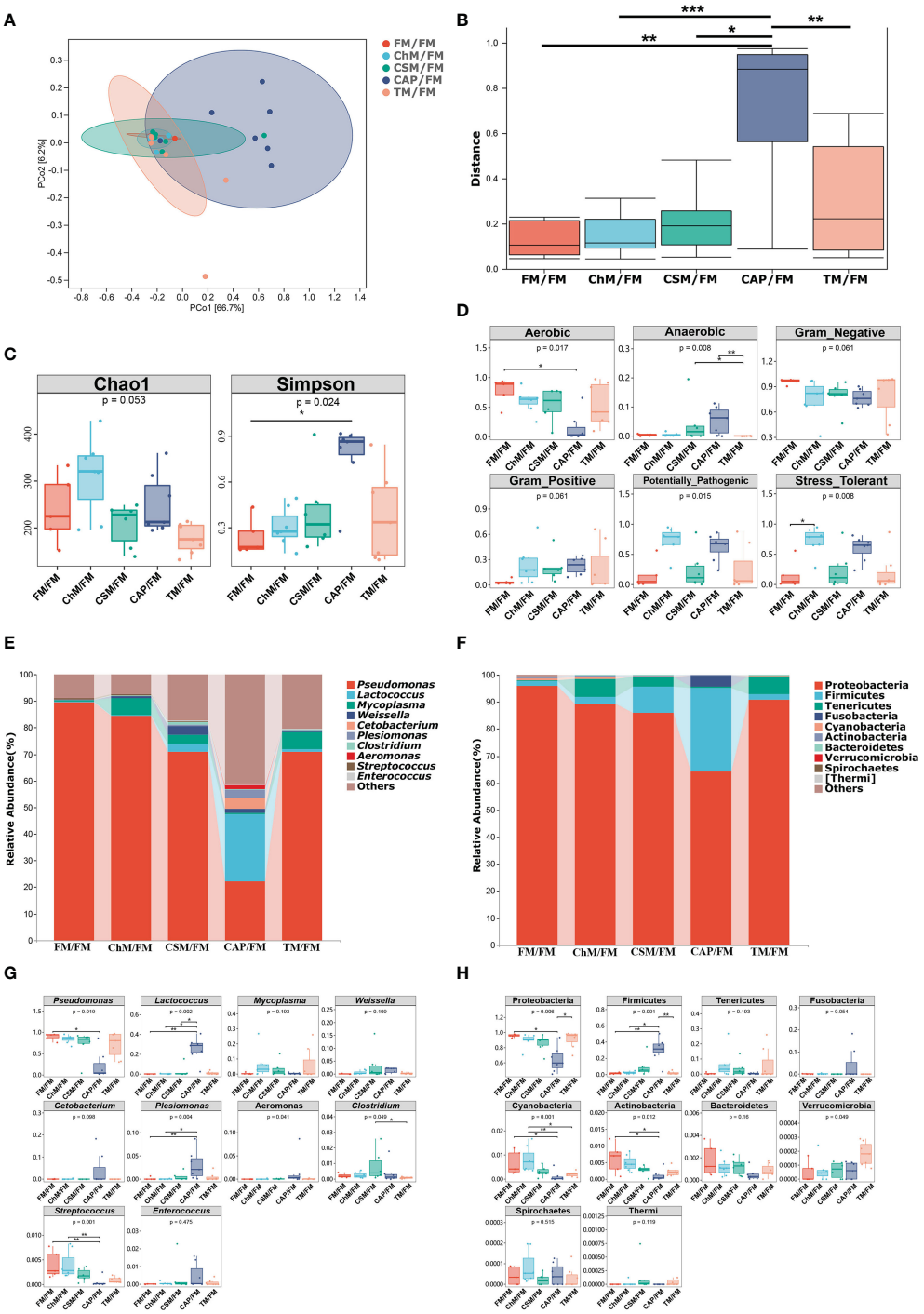

FIGURE 6
The intestinal microbiome of juvenile largemouth bass intestinal contents under an alternate feeding strategy were shown as follows: β-diversity **(A)**, abundance comparison of β-diversity **(B)**, α-diversity **(C)**, bugbase analysis **(D)**, top 10 microbiota at genus level **(E)**, Top 10 microbiota at phylum level **(F)**, abundance comparison of top ten microbiota at genus level **(G)**, and abundance comparison of top 10 microbiota at phylum level **(H)**.The statistical differences were presented as *$P<0.05$,**$P<0.01$, and ***$P<0.001$.

The Mantel test revealed the connections between microbiota/SCFAs, and physiological indicators (Figure 8B). Outside of the matrix, *claudin-4* had a significant influence on the abundance of SCFAs and the top ten bacteria at the phylum level ($P<0.05$). In addition, the lipase activity may have a major impact on the top ten microbiota at the genus or phylum level. Within the matrix, the Mantel test revealed the negative association between CAT and villus structure/muscular layer thickness ($P<0.05$), FCR and PER/SGR/occludin ($P<0.05$), lipase and MDA, trypsin and *claudin-1*, LYS and mucin 2 ($P<0.05$). On the other hand, there were positive correlations between villus structure and muscular layer thickness, ADC and *zo-1*/*occludin*/SGR/PER/LPS ($P<0.05$), PER and SGR/*zo-*

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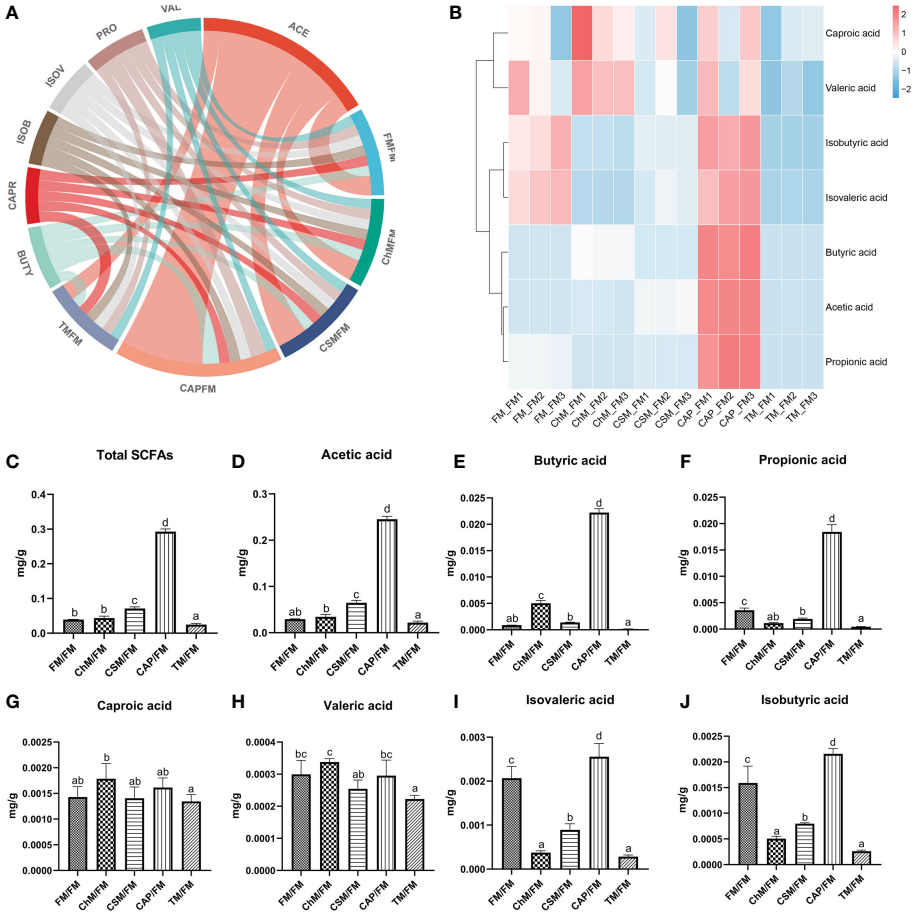

FIGURE 7
The intestinal metabolome of juvenile largemouth bass intestinal contents under an alternate feeding strategy were shown as follows: Distribution of SCFAs in each diet **(A)**, relative abundance of SCFAs in each diet **(B)**, total SCFAs contents **(C)**, acetic acid content **(D)**, butyric acid content **(E)**, propionic acid content **(F)**, caproic acid content **(G)**, valeric acid content **(H)**, isovaleric acid content **(I)**, and isobutyric acid content **(J)**. Data are expressed as mean (S.E.M). Values on each column with different superscripts typified statistical differences (*P<0.05*).

1/occludin (*P<0.05*), goblet cells and LYS/digestive enzymes, CAT and immune globulin/digestive enzymes, GSH-Px and sIgA-like/LYS/digestive enzymes, sIgA-like and digestive enzymes, pepsase and immune globulin/LYS (*P<0.05*), sIgA-like and LYS (*P<0.05*), *claudin-1* and *zo-1* (*P<0.05*).

Next, the Z-score was then computed in Figure 8C and presented in Figure 8D for a full understanding of the rank of functions for each diet. Briefly, the Z-score value were **microbiota barrier:** CAP/FM (4.87) > CSM/FM (0.89) > TM/FM (-1.29) > FM/FM (-1.36) > ChM/FM (-3.14); **physical barrier:** CAP/FM (4.95) > FM/FM (1.29) > CSM/FM (-0.48) > ChM/FM(-0.68)> TM/FM (-3.3); **immunological barrier:** ChM/FM (2.98)> CAP/FM (1.11) > TM/FM (0.66) > FM/FM (-0.98) > CSM/FM (-3.76); **chemical barrier** ChM/FM (1.68) > CAP/FM (0.62) > TM/FM (-0.27) > CSM/FM (0.91) > FM/FM (-1.11); **antioxidant capacity:** ChM/FM (2.69) > CAP/FM (2.32) > CSM/FM (1.06) > FM/FM (-1.05) > TM/FM (-1.83); **SCFAs contents:** CAP/FM (8.8) > FM/FM (0.12) > ChM/FM(0.11)> CSM/FM (-2.97) > TM/FM (-6.08).

4 Discussion

4.1 Growth performance, feed utilization and intestinal histological analysis of juvenile largemouth bass under an alternate feeding strategy

It is well known that growth performance is an essential criteria for evaluating fishmeal alternatives. Even though a great deal of research has been conducted on alternate feeding strategy in fish (10, 11, 19), the impacts of new proteins on the growth performance of fish under alternate feeding strategy have not been investigated. In the present research, a substantial decrease in growth performance was detected when comparing diets containing novel proteins to whole fish meal diet; nevertheless, the most surprising discovery was that the growth rate in the second 29 days outperformed the first 29 days. This might be linked to fish-compensatory growth, in which fish that have

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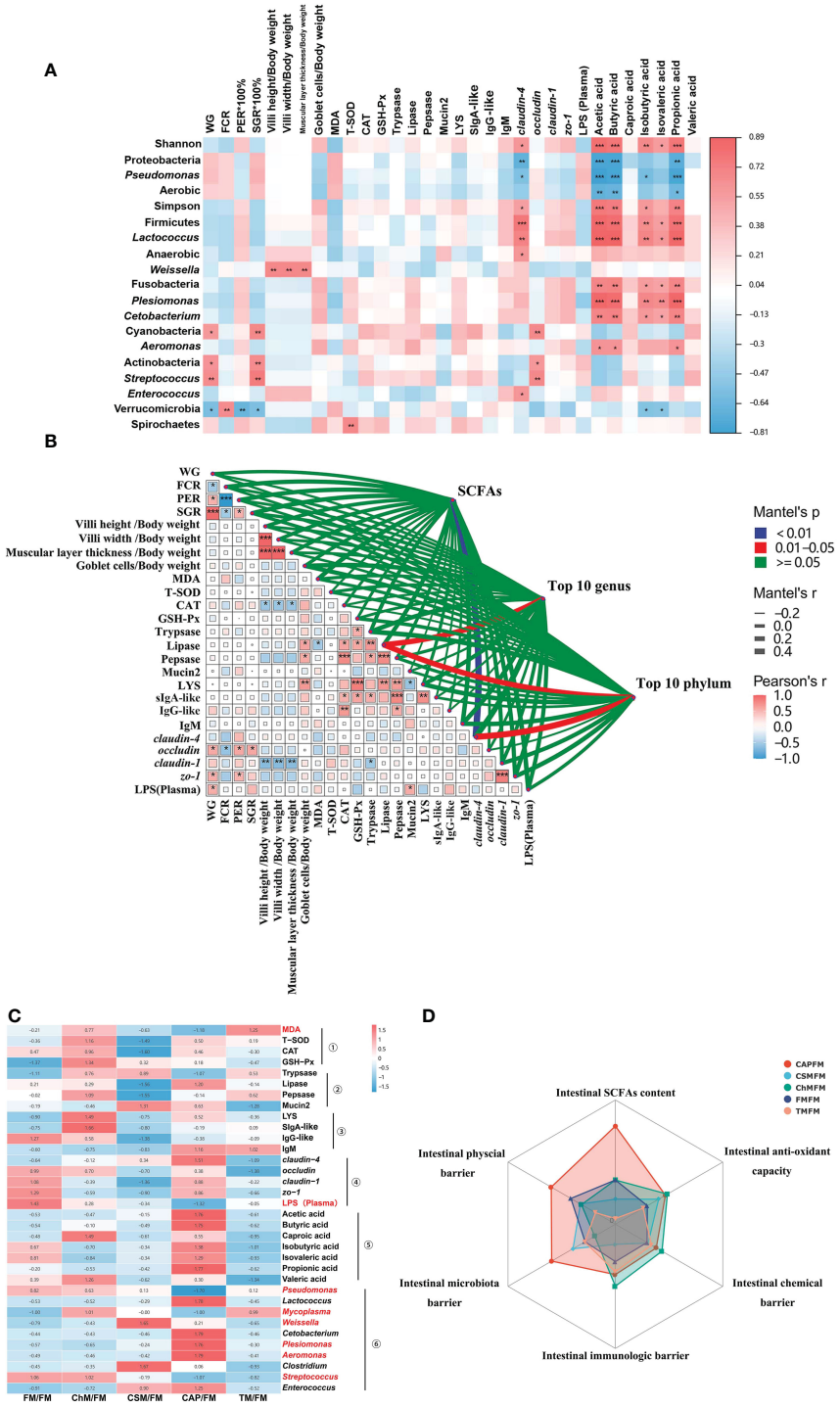

FIGURE 8
Combined analysis and scoring of all data obtained from various protein treatments: **(A)** Heat map of Pearson's correlation analysis (|r|>0.6, P 0.05) of bacterial phenotype, abundance at the genus/phylum level, SCFAs, and physiological biomarkers. On the heat map, the red square indicates a positive association, while the blue square indicates a negative correlation. **(B)** The Mantel test was performed on the core microbiota, intestinal SCFAs, and physiological indicators. The size of the square in the matrix symbolizes the coefficient value, with red squares representing positive correlations and blue squares negative correlations. The lines outside of the matrix reveal the relationship between the core microbiota as well as intestinal SCFAs and physiological markers, with thicker lines indicating stronger associations. **(C)** Heat map of the z-score analysis was clustered as the intestinal antioxidant①, intestinal chemical barrier②, intestinal immunological barrier③, intestinal physical barrier④, intestinal SCFAs contents⑤, and intestinal microbiota barrier⑥. The red-highlighted indexes indicated their negative relationship with fish intestinal health, whereas the black-highlighted indicated their positive relationship with fish intestinal health. **(D)** On the basis of the Z-score calculated from panel **(C)**, a multidimensional radar map analysis of the effects of five protein treatments on the intestinal health of juvenile largemouth bass was presented. The statistical differences were presented as *$P<0.05$,**$P<0.01$, and ***$P<0.001$..

suffered nutritional deficits instantly undergo a high rate of synthesis upon re-feeding, rapidly raising metabolic levels and enhancing food conversion rates (20). Therefore, the improved growth performance in the second stage of the current study was enhanced, and this was similar to studies done by Zhu et al., 2022, Bi et al., 2021, and Yılmaz et al., 2016 (10, 12, 13), which found the re-feeding of fish oil can also improve the decreased growth performance as well as n-3 ΣPUFA caused by linseed oil, beef tallow-based diets, and canola oil in *A. schlegelii*, *S. maximus*, and *D. labrax* respectively. Moreover, growth performance is possibly related with intestinal bacteria. On the basis of Pearson's correlation analysis, the number of Cyanobacteria, Acitinobacteria, and *Streptococcus* was positively connected with weight increment; however, the abundance of these bacteria was lower in new protein diets than in whole fishmeal diet. Furthermore, the *occludin* was identified as a biomarker for growth performance by the Mantel test, and the lower *occludin* in diets containing novel proteins compared with the complete fishmeal diet may be responsible for the reduced growth performance. These joint analysis may provide new insight into the decreased growth performance in fish. Although lower growth performance was seen throughout the present investigation, the non-significant changes in intestinal structure and villus height/width demonstrated the growth potential of novel protein diets in the long-term alternate feeding.

4.2 The oxidative stress of juvenile largemouth bass under an alternate feeding strategy

Antioxidant enzymes are essential parts of the antioxidant defense system and help to reduce oxidative damage. MDA, an indication of oxidative stress injury in animals, is a lipid peroxidation product (21). In addition, it is well known that the *nrf-2*/*keap-1* pathway protects cells from oxidative stress, and the activation of *nrf2* might up-regulate downstream antioxidant genes that serve as a key cellular defense against the cytotoxic consequences of oxidative stress (22). The present research discovered a decrease in the level of *keap-1*, thus the GSH-Px activity in algae protein *C. vulgaris* diet increased. This was similar to the findings of Mohsen et al. (3), Hassnaa et al. (23), and Li et al. (19) when feeding *Oreochromis niloticus* and *M. salmoides* with a meal containing *C. vulgaris* observing the increased antioxidant capacity. This may be attributed to antioxidant bioactive components, which are pigments and vitamin C contained in this diet (24, 25), thus these factors promoted the colonization of oxidative stress tolerant bacteria ultimately leading to an increase in antioxidant capacity of *C. vulgaris* diet. According to several studies (26, 27), cottonseed protein concentrate, in which free-gossypol content was about 243.94mg/kg is detrimental to the intestinal health in grass carp and Nile tilapia. These fish were believed to tolerate anti-nutritional factors better than carnivorous fish. Our study observed decreased CAT activity in this plant protein diet, and this is largely owing to the poor resistance of carnivorous fish species to free-gossypol content in cottonseed protein concentrate diet (19), which caused intestinal inflammation when its level beyond 13.98mg/kg in a carnivorous fish, *Sillago sihama Forsskál* (28). Surprisingly, *gpx* and *sod* was dramatically reduced in algae diet *C. vulgaris* and cottonseed protein concentrate diets compared to whole fishmeal diet, although their GSH-Px as well as T-SOD enzyme activity was unaffected. In addition, the *keap*/*nrf-2* ratio did not differ between the diets containing novel proteins and the whole fishmeal diet. These results indicated that the antioxidant system may inhibit the expression of these genes through feedback control (29).

4.3 The intestinal physical, immunological, and chemical barriers of juvenile largemouth bass under an alternate feeding strategy

The intestinal tight junction genes (*claudin-1*, *zo-1*, *occludin*, and *claudin-4*) and serum IL-1β, TNF-α and LPS can affect intestinal permeability in largemouth bass (17, 30), thus can reflect the intestinal cell-cell integrity. In the current study, the expression of tight-junction genes was suppressed in the cottonseed protein concentrate, algae protein *C. vulgaris*, and insect protein *T. molitor* diets, despite no considerable change in plasma LPS concentration. While the level of *claudin-4* was up-regulated in the bacterial protein *C. autoethanogenum* diet. Additionally, this diet had the lowest LPS level in plasma and intestine compared to other protein diets. However, these results contradicted prior research that claimed the improved physical barrier was detected in yellow worm meal, bacterial protein *C. autoethanogenum*, and algal protein Chlorella diets (17, 19). Possible explanation is that the Gram-negative bacterium, i.e. *Pseudomonas*, which could disrupt the intestinal physical barrier and cause diseases in freshwater fish (31), was most prevalent in whole fishmeal. Therefore, the favorable effects that *T. molitor*, bacterial protein *C. autoethanogenum*, and algal protein *C. vulgaris* diets had were removed during the second fishmeal stage.

Immunity in fish is reliant on the immunological response, which is intimately connected to innate and adaptive immune components such as immunoglobulin (32). In addition, MUC2, LYS, and digestive enzyme can prevent intestine from being invaded by exogenous pathogens (15, 17). These parameters form a critical component of the intestinal immunochemical barrier and play a vital role in maintaining intestinal homeostasis. Our earlier investigation confirmed that a 56-day administration of novel protein diets might increase chemical and immunological intestinal barriers in largemouth bass (17), however the present study did not find any overall changes. Only the content of LYS and sIgA-like rose on the algae protein *C. vulgaris* diet compared with whole fishmeal diet. Similar findings were made by Maliwat et al. (33), Mohsen et al. (3), and Chen et al. (34), who discovered that feeding Chlorella to *Macrobrachium rosenbergii*, *O. niloticus*, and *Oncorhynchus mykiss* increased their immunity. These may be

attributed to water-soluble polysaccharides (35), D-Lactic acid (36), and water-soluble alpha-glucans (37) contained in algae protein *C. vulgaris* diet. For other diets containing novel proteins, generally, it seems that the enhanced physiological functions from diets containing novel proteins were overshadowed by the second 29-day fishmeal stage. Also, it is interesting that there was a high correlation between lipase activity and the composition of the microbiota, although there were no significant differences between diets containing novel proteins and whole fishmeal. This is verified in the finding by Yang et al. (38), who also found that supplemental *Aspergillus* lipase can change the microbiota composition in rats. This may be due to the intestine's enhanced hydrolysis of undigested macronutrients such as proteins and lipids into nutrients useable by the intestinal flora.

4.4 The gut microbiota barriers and SCFAs production of juvenile largemouth bass under alternate feeding strategy

The intestinal microbiota may generate toxic compounds that result in barrier abnormality and disease processes, but it could also generate favorable metabolites like SCFAs, which have anti-inflammatory, anti-oxidant, and enteric-epithelial-repair functions that could impact animal wellness and disease progression (39). Thus, the structure and composition of the intestinal microbiota are regarded as crucial for intestinal health, since they improve host physiology and immunity in the intestine and affect body's immunological response (40). Usually, the dominant bacteria play important role in the health status, and the most abundant bacteria in this research were *Lactococcus*, Firmicutes, *Pseudomonas*, and Protebacteria. Protebacteria, *Pseudomonas*, and *Streptococcus*, which have been recognized as detrimental to aquatic organisms, were shown to be less prevalent in the bacterial protein *C. autoethanogenum* diet. In contrast, a rise in beneficial bacteria, namely *Lactococcus* and Firmicutes, was seen in this diet. Consequently, a significant quantity of acetic acid, butyric acid, and propionic acid were generated. In agreement with the current findings, earlier research has revealed that feeding a diet containing *C. autoethanogenum* increased Firmicutes and decreased Protebacteria in largemouth bass (17, 19, 41). These were also confirmed by the bugbase functional predictions, which indicated that this diet included the greatest concentration of anaerobic bacteria and the lowest concentration of aerobic bacteria. This may be due to the fact that *C. autoethanogenum* contains a significant amount of carbohydrates, lipids, and vitamins that may regulate gut microbiota and enhance intestinal health (6, 42). However, no significant differences in microbiota were identified across other diets containing novel proteins and whole fishmeal diet when using this alternate feeding strategy. A possible explanation for these results may be that the other three protein diets were not as well adapted to the alternate feeding strategy as the bacterial protein *C. autoethanogenum* diet.

SCFAs were recognized to supply energy to intestinal mucosal cells, boost cell metabolism and development, as well as decrease the expansion of pathogens and prevent intestinal dysfunction. The recent research discovered very favorable associations between SCFAs and Shannon/Simpson. These indicators were used to assess the intestinal health (9, 43, 44) in earlier investigations. In addition, our findings were confirmed by a study conducted on mice, which indicated that increasing α-diversity linked to higher levels of short-chain fatty acids (45). Our discovery provides new insight into the elevated intestinal SCFAs in fish, albeit with further study for underlying mechanism. According to the Mantel test, *claudin-4* is important for SCFAs contents, and lower expression was found in yellow meal worm diet, algae protein Chlorella diet, and cottonseed protein concentrate, resulting in lower SCFAs contents in these diets. This finding broadly supported our previous work, which found that tight junctions may affect the production of SCFAs (17).

Pearson's correlation analysis revealed favorable associations between SCFAs/*claudin-4* and probiotics, suggesting that these bacteria may aid in the production of SCFAs and the maintenance of intestinal barrier integrity. In contrast, the association between Proteobacteria/*Pseudomonas*/Aerobic bacteria and SCFAs yielded contradictory results. These pathogens were previously discovered to cause death or intestinal inflammation in numerous fish, including juvenile hybrid grouper (46) and largemouth bass (17, 30). Nonetheless, significant relationships were also discovered between SCFAs and possible pathogens (*Plesiomonas*/*Aeromonas*). The reason for this is not clear, but it may have something to do with the synergy between microbes, as Ding et al. (47) found the probiotic-treated diet could improve the health status of juvenile *Megalobrama amblycephala* while simultaneously increasing the abundance of both intestinal beneficial bacteria and harmful bacteria.

Despite the fact that physiological and microbial indicators were not significantly altered in the cottonseed protein concentrate diets, yellow worm meal diet, and algae protein *C. vulgaris* diet, compared to the whole fishmeal diet, we sought to assess their function holistically. Therefore, Z-score value of each diet from different physiological biomarkers were computed and summarized. Based on the amount of area occupied by each diet, the order is as follows: bacterial protein *C. autoethanogenum* diet (CAP/FM) > algae protein *C. vulgaris* diet > (ChM/FM) > whole fishmeal diet (FM/FM) > cottonseed protein concentrate diet (CSM/FM) > insect protein *T. molitor* diet (TM/FM). This result is inconsistent with our previous research (17), which indicated that the ranking functions in the algae protein *C. vulgaris* diet (ChM) were the highest, followed by the bacterial protein *C. autoethanogenum* diet (CAP), the insect protein yellow worm meal diet (TM), the fishmeal diet (FM), and the cottonseed protein concentrate diet (CSM). This discrepancy could be attributed to the different feeding strategies and adaptations of juvenile largemouth bass to different novel protein diets. It can thus be suggested that, compared with the whole fishmeal diet, the bacterial protein *C. autoethanogenum* diet and the algae protein *C. vulgaris* diet have better intestinal health under this alternate

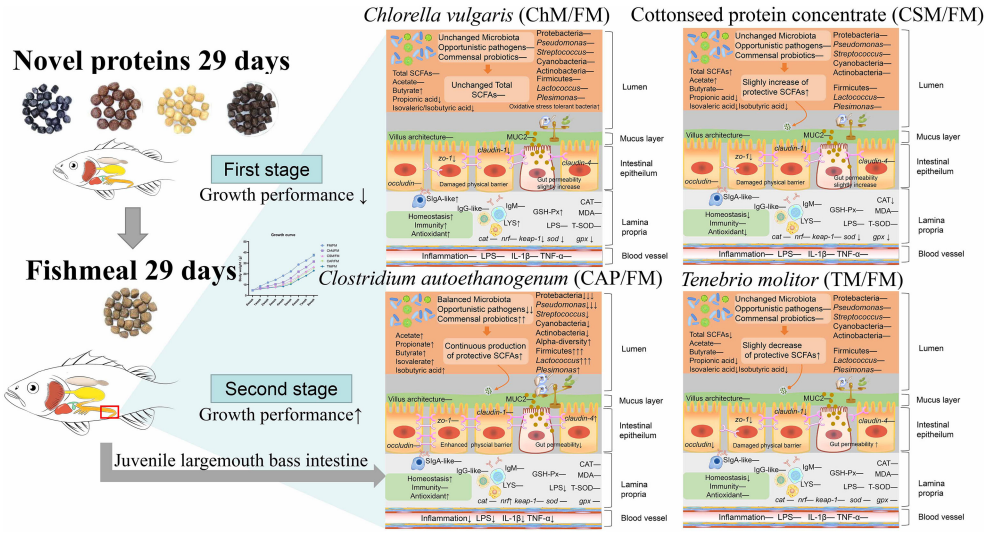

FIGURE 9
A schematic model showing the mechanism of the effects of novel protein diets on the intestinal health of juvenile largemouth bass under an alternate feeding strategy.

feeding strategy, which is an effective strategy to feed largemouth bass. However, This alternate feeding technique may not be acceptable for the insect protein *T. molitor* diet or the cottonseed protein concentrate diet.

5 Conclusion

This study set out to evaluate the functions of novel protein diets and a whole fishmeal diet under alternate feeding strategy. From the schematic model of this study (Figure 9), the growth performance of all diets containing novel proteins was elevated. In addition, the *C. autoethanogenum* diet and the *C. vulgaris* diet improved intestinal health. However, our comprehensive evaluation of the *T. molitor* diet and the cottonseed protein concentrate diet using the Z-score value revealed that intestinal health performance was inferior to that of the whole fishmeal diet. This work contributes to the existing application of novel protein diets by providing an alternate feeding strategy. Nonetheless, the alternate feeding strategy should be introduced with caution when fed novel proteins, since not all diets performed better than a fishmeal diet when employing this strategy. Future investigation and experimentation into the *C. autoethanogenum* diet as well as the *C. vulgaris* diet, when introduced with alternate feeding, is strongly recommended.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number PRJNA932585.

Ethics statement

The animal study was reviewed and approved by Chinese order no. 676 of the state Council revised 1 March, 2017. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

LL: conceptualization, data curation, formal analysis, investigation, methodology, writing - original draft. YW: sampling, resources, and software. YH: funding. CW: supervision, project administration, visualization writing, editing. All people who made significant contributions to the work presented in the article, including those who offered editing and writing assistance but are not authors, are acknowledged in the paper's Acknowledgments section and have given their written permission to be included. All authors contributed to the article and approved the submitted version.

Funding

This work was financially supported by the National Key Research and Development Program of China (2019YFD0900200) and Fundamental Research Funds for the Central Universities (grant number: 2662020SCPY005).

Acknowledgments

Thanks to reviewers for their constructive comments to help improve the paper. Dr. Xuemei Xiong, Dr. Jie Wang, Dr. Gang Yang, and Ms. Wenyu Feng were thanked for their assistance in improving the quality of our work. Prof. Kenichi Tsuda and his team members were also thanked for their gracious assistance in supplying lab equipment and space for experiments during our lab's summer 2022 power cut.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1110696/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 28 April 2023
DOI 10.3389/fimmu.2023.1140012

OPEN ACCESS

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RECEIVED 08 January 2023
ACCEPTED 07 April 2023
PUBLISHED 28 April 2023

CITATION
Luo Q, Qian R, Qiu Z, Yamamoto FY, Du Y, Lin X, Zhao J and Xu Q (2023) Dietary α-ketoglutarate alleviates glycinin and β-conglycinin induced damage in the intestine of mirror carp (*Cyprinus carpio*). *Front. Immunol.* 14:1140012. doi: 10.3389/fimmu.2023.1140012

Dietary α-ketoglutarate alleviates glycinin and β-conglycinin induced damage in the intestine of mirror carp (*Cyprinus carpio*)

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This study investigated the glycinin and β-conglycinin induced intestinal damage and α-ketoglutarate alleviating the damage of glycinin and β-conglycinin in intestine. Carp were randomly divided into six dietary groups: containing fish meal (FM) as the protein source, soybean meal (SM), glycinin (FMG), β-conglycinin (FMc), glycinin+1.0% α-ketoglutarate (AKG) (FMGA), β-conglycinin +1.0% AKG (FMcA). The intestines were collected on 7th, and the hepatopancreas and intestines were collected on 56th. Fish treated with SM and FMc displayed reduced weight gain, specific growth rate, and protein efficiency. On 56th day, Fish fed on SM, FMG and FMc presented lower superoxide dismutase (SOD) activities. FMGA and FMcA had higher SOD activity than those fed on the FMG and FMc, respectively. In intestine, fish fed on the SM diets collected on 7th presented upregulated the expression of transforming growth factor beta (TGFβ1), AMP-activated protein kinase beta (AMPKβ), AMPKγ, and acetyl-CoA carboxylase (ACC). Fish fed FMG presented upregulated expression of tumor necrosis factor alpha (TNF-α), caspase9, and AMPKγ, while downregulated the expression of claudin7 and AMPKα. FMc group presented upregulated expression of TGFβ1, caspase3, caspase8, and ACC. Fish fed FMGA showed upregulated expression of TGFβ1, claudin3c, claudin7, while downregulating the expression of TNF-α and AMPKγ when compared to fish fed FMG diet. FMcA upregulated the expression of TGFβ1, claudin3c than fed on the FMc. In intestine, the villus height and mucosal thickness of the proximal intestine (PI) and the distal intestine (DI) were decreased and crypt depth of the PI and mid intestine (MI) were increased in SM, FMG and FMc. In addition, fish fed on SM, FMG and FMc presented lower citrate synthase (CS), isocitrate dehydrogenase (ICD), α-ketoglutarate dehydrogenase complex (α-KGDHC) Na^+/K^+-ATPase activity in DI. FMGA had higher CS, ICD, α-KGDHC, and Na^+/K^+-ATPase activity in PI and MI than those fed on the FMG. FMcA had higher Na^+/K^+-ATPase activity in MI. In conclusion, dietary soybean meal destroys the intestine's health, the adverse

effects are related to the presence of β-conglycinin and glycinin, especially glycinin. AKG may regulate intestinal energy *via* tricarboxylic acid cycle, thereby alleviating the damage intestinal morphology caused by the dietary soybean antigen proteins.

KEYWORDS

α-ketoglutarate, glycinin, β-conglycinin, intestine immunity, *Cyprinus carpio*

1 Introduction

Given the increased demand and limited capture of forage fish, fishmeal prices have reached all-time high, with limited supply (1). As an alternative to fishmeal, soybean meal is a plant-based ingredient with high levels of protein, balanced amino acid profile, and other nutrients required by aquatic animals (2). However, soybeanmeal cannot completely replace fishmeal because of the antinutritional factors such as allergens, trypsin inhibitor and saponins (3). High inclusion of soybean meal in fish aquafeeds decreased growth performance in Japanese seabass (*Lateolabrax japonicus*) (4), orange-spotted grouper, and (*Epinephelus coioides*) (5). These adverse effects are often related to the presence of anti-nutritional factors, especially β-conglycinin and glycinin (6). These two antigenic proteins are considered the major cause of reduced growth and increased intestinal inflammation in aquatic animals (7). Previous studies have showed that 4% to 8% β-conglycinin and/or glycinin can damage the structural integrity of the intestine, reduce the immune function and compromise the growth performance of juvenile golden crucian carp (*Carassius auratus*) (8, 9) and grass carp (*Ctenopharyngodon idella*) (10, 11).

In this context, supplementation of functional additives is an important strategy to maintain the intestine health. α-ketoglutaric acid (AKG) is an intermediate for the tricarboxylic acid (TCA) cycle is involved in pleiotropic metabolic and regulatory pathways in the cell, including energy production (12). AKG has the function of improve intestinal immunity and inhibit apoptosis in response to inflammatory stimuli (13), which can ultimately compensate the energy consumption in the intestinal health. In addition, AKG has alleviative effect on oxidative stress as a source of energy and an antioxidant in mammalian cells (14). Similar results were also observed on juvenile red drum (*Sciaenops ocellatus*) (15). Thus, it is hypothesized that AKG could protect fish against glycinin and β-conglycinin injury.

Nevertheless, the alleviating effects of α-ketoglutarate on the damage of glycinin and β-conglycinin in intestinal mucosa of Mirror carp have not been elucidated. Our research group showed that the intestinal inflammation and tight junction protein showed a trend of first serious and then recovery when addition β-conglycinin and Glycinin was 4% to 16%, respectively (16). Therefore, the objective of this study was to investigate the effects of high-lever glycinin and β-conglycinin on growth performance, antioxidant capacity, intestinal health and energy of mirror carp (*Cyprinus carpio*), and evaluated whether AKG supplementation had potential alleviating effects on the glycine and β-conglycine induced injuries in the intestinal mucosa.

2 Materials and methods

2.1 Experimental diets

Glycinin (purity, 85%) and β-conglycinin (purity, 88%) were provided by the China Agricultural University. α-ketoglutarate (purity, 98%) was purchased from Sigma-Aldrich. For this study, six experimental diets were formulated to be isonitrogenous (33%) and isolipidic (6%). The ingredients and nutrient composition of all diets are shown in Table 1. For the control group (FM), fish meal was used as the main protein source, and the other five diets were formulated with either 56.0% soybean meal (SM), 22.8% glycinin (FMG), 22.0% β-conglycinin (FMc), 22.8% glycinin + 1.0% α-ketoglutarate (FMGA), or 22.0% β-conglycinin + 1.0% α-ketoglutarate (FMcA). Dietary ingredients were ground into fines to pass through a 60 mesh sieve. Homogenized ingredients were thoroughly mixed with fish oil, soybean oil, phospholipid, and water, and mixtures were extruded (twin screw extruded F-26, South China University of Technology, Guangzhou, China), using a 1.5 mm die-plate. The diets were dried in a ventilated oven at 40 °C, until reached a constant dry matter, and stored at -20 °C until feeding.

2.2 Feeding management

The feeding trial was carried out at the College of Life Science, Huzhou University. Prior commencement of the feeding trial, fish were acclimated in the recirculating system for two weeks. A total of 450 carps of similar sizes (mean initial body weight 2.08 ± 0.06 g) were randomly distributed into six treatments, 3 replicate tanks per treatment, with 25 fish per tank (500 L water). Fish were fed to apparent satiation thrice daily (08:00, 13:00, and 18:00) for 8 weeks; and uneaten feed was collected after 45 min, dried and weighed to record feed intake (17). During the experimental period, the carp were cultured in a recirculating aquaculture system with continuous aeration, with 1/3 of the water being changed every day. The water

TABLE 1 Formulation of the experimental diets fed to Mirror carp (*Cyprinus carpio*), and their analyzed nutrient composition.

Ingredients	Groups					
	FM	SM	FMG	FMc	FMGA	FMcA
Fish meal	38.00	5.00	5.00	5.00	5.00	5.00
Soybean meal	0.00	56.00	0.00	0.00	0.00	0.00
Glycinin	0.00	0.00	22.80	0.00	22.80	0.00
β-conglycinin	0.00	0.00	0.00	22.00	0.00	22.00
α-ketoglutarate	0.00	0.00	0.00	0.00	1.00	1.00
Wheat middling	49.90	28.72	57.10	57.80	56.10	56.80
Cellulose	3.60	0.00	3.65	3.69	3.65	3.69
Fish oil	0.00	3.20	3.20	3.20	3.20	3.20
Soybean oil	3.10	0.30	1.40	1.40	1.40	1.40
Soybean lecithin oil	1.00	1.00	1.00	1.00	1.00	1.00
Vitamin premix[a]	0.50	0.50	0.50	0.50	0.50	0.50
Mineral premix[b]	0.20	0.20	0.20	0.20	0.20	0.20
Choline chloride	0.30	0.30	0.30	0.30	0.30	0.30
$Ca(H_2PO_4)_2$	1.20	2.00	2.00	2.00	2.00	2.00
Carboxymethyl cellulose	2.00	2.00	2.00	2.00	2.00	2.00
Lysine	0.10	0.23	0.00	0.00	0.00	0.00
Methionine	0.10	0.51	0.65	0.65	0.65	0.65
L-Threonine	0.00	0.04	0.20	0.26	0.20	0.26
Proximate analysis (%)						
Moisture	5.70	5.90	5.87	6.30	5.88	5.25
Crude protein	33.36	34.08	33.62	33.42	33.59	33.75
Crude lipid	6.50	6.35	6.48	6.42	6.40	6.34
Ash	11.52	7.41	4.54	4.73	4.66	4.61

[a]The premix provided the following per kg of diets: A 8 000IU, VC 500mg, VD 3 000IU, VE 60mg, VK 35mg, VB_1 15mg, VB_2 30mg, VB_6 15mg, VB_{12} 0.5mg;
[b]The premix provided the following per kg of diets:$FeSO_4 \cdot H_2O$ 960 mg, $CuSO_4 \cdot 5H_2O$ 12 mg, $MnSO_4 \cdot H_2O$ 33 mg, $ZnSO_4 \cdot H_2O$ 70 mg, Na_2SeO_3 1.2 mg, $Ca(IO_3)_2$ 1.4mg, $CoCl_2 \cdot 6H_2O$ 2.4 mg, Zeolite meal 920 mg.

temperature ranged from 24.6 to 30.4°C, dissolved oxygen was no less than 6.0 mg l^{-1}, and the ammonia nitrogen concentrations was less than 0.3 mg L^{-1}.

2.3 Growth performance

At the beginning and end of the trial, fish in each tank were group weighed and counted to calculate growth performance, including Weight gain (WG %), Specific growth rate (SGR), Survival rate (SR %), PE (Protein efficiency %), Feed conversion ratio (FCR) (18). The production performance were calculated as follows:

$$Weight\ gain\ (WG,\ \%) = (FBW - IBW)/IBW \times 100;$$

$$Specific\ growth\ rate\ (SGR,\ \%/d) = [ln(FBW) - ln(IBW)] \times 100\ /d;$$

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

$$Protein\ efficiency\ (PE,\ \%) = (FBW - IBW)/(F \times P) \times 100;$$

$$Feed\ conversion\ ratio\ (FCR) = F/(FBW - IBW);$$

Abbreviations: Number of initial fish, N_0; number of final fish, N_t; initial body weight, IBW; final body weight, FBW; Feeding days, d; total diet intake, F; feed crude protein content, P.

2.4 Sample collection and analysis

Intestine samples collected on the 7th day were dissected quickly on ice. 3 fish was pooled as one sample, and six samples per treatment. The intestine samples were stored at -80°C to analyze the relative gene expression during a short-term exposure to the experimental feed.

On the 56th day, after weight and length were measured, three fish from each tank (nine per treatment). The fish were then dissected to obtain the hepatopancreas and intestine quickly on ice. The hepatopancreas stored at -80°C for antioxidant capacity analysis. The intestinal tract was immediately divided into proximal intestine (PI), mid intestine (MI), and distal intestine (DI), stored at -80°C for key enzymes in tricarboxylic acid cycle and Na^+/K^+-ATPase analysis. An additional segment of PI, MI, and DI was fixed using Bouin solution for histology.

2.5 Antioxidant status

Glutathione (GSH, Cat. No. A006-2-1), malondialdehyde (MDA, Cat. No. A003-1) content; the activities of total superoxide dismutase (SOD, Cat. No. A001-1), and catalase (CAT, Cat. No.A007-1-1) were determined using the commercial kits purchased from Nanjing Jiancheng Bioengineering Institute.

2.6 Gene expression analysis

The total RNA was extracted from the proximal intestine, mid intestine, and distal intestine by Rapid Extraction Kit extracted total RNA (RN28; Aidlab Bio Inc., Beijing, China). RNA concentration and purity were measured by the spectrophotometry analysis (A260:A280 nm ratio) within the ratio specified by the kit (1.8-2.2). Subsequently, cDNA was synthesized by a MonScript™ RTIII All-in-One Mix (MR05001M; Monad Bio Inc., Wuhang, China). The MonAmp™ SYBR® Green qPCR Mix (MQ10101S; Monad Bio Inc., Wuhang, China) used mRNA level analysis by quantitative real-time PCR on a CFX-96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., USA). The reactions were done in a volume of 20 µL containing 0.4 µL of 10 µM each of reverse and forward primers, 1 µL of diluted cDNA, 10 µL of MonAmp™ SYBR® Green qPCR Mix, and 8.2 µL of Nuclease-Free Water. PCR conditions were as follows: 95 °C for 5 min followed by 40 cycles of 95 °C for 10 s, 60°C for 10 s and 72 °C for 30 s. The online Primer3 (http://primer3.ut.ee) software was used to design the primers. Target gene mRNA relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and corrected for the expression of the normalizing gene β-actin, according to Luo et al. (19). The primers used for quantitative fluorescence analysis are shown in Table 2.

2.7 Histology of the intestine

The 1 cm segments were cut from the PI, MI, and DI. The 1 cm intestinal segments were processed, embedded, and stained according by Hangzhou Haoke Biotechnology Co., Ltd. Randomly selected fields were observed and pictures were taken by a camera coupled with a light microscope (40×). The images were measured and calculated for villus height, crypt depth, mucosal thickness according to the method previously described by Lin et al. (20) using the software Adobe Photoshop 2021. The average value was calculated from the measurement results from each sample index.

2.8 Key enzymes in tricarboxylic acid cycle and Na^+/K^+-ATPase in the intestine

Citrate synthase (CS, Cat. No. MM-204201), Isocitrate dehydrogenase (ICD, Cat. No. MM-3280701), α-ketoglutarate dehydrogenase complex (α-KGDHC, Cat. No. MM-9176301), and Na^+/K^+-ATPase (Cat. No. MM-166201), were determined by the enzyme-linked immunosorbent assays (ELISA), following the manufacturer's procedures (Jiangsu Meimian Industrial Co., Ltd., China)

2.9 Statistical data analysis

All statistical analyses were performed using SPSS, version 26.0 software. The level of significance was set to $P< 0.05$. Data were subjected to the one-way analysis of variance (ANOVA) followed by Tukey's test was performed for comparison of means, and data is presented as means ± standard error (SE).

3 Results

3.1 Growth performance

The effect of soybean antigen proteins and α-ketoglutarate on growth performance of mirror carp is shown in Figure 1. Compared with the FM group, there were no significant differences in SR. The WG, SGR, and PE were significantly reduced in SM and FMc group. In addition, the FCR was significantly increased in SM. Feeding the fish with the FMG diets did not affect significantly WG, SGR, SR, PE, and FCR.

Feeding the fish with the FMGA diets did not affect significantly WG, SGR, SR, PE, and FCR, when compared to the fish fed FMG diets. Feeding the fish with the FMcA diets did not affect significantly SR, PE, and FCR, but WG and SGR were significantly decreased, when compared to the fish fed FMc diets.

3.2 Antioxidant capacity in the hepatopancreas

The effect of soybean antigen proteins and α-ketoglutarate on antioxidative ability in the hepatopancreas was given in Figure 2. Compared with the FM group, there was no significant change in MDA content, however, SOD activity were significantly decreased in SM, FMG and FMc group; the CAT activity in FMG and FMc group and the GSH content in FMc group were decreased significantly.

Compared with the FMG group, there were no significant change in GSH content, MDA content, and CAT activity. However, the SOD activity of FMGA group increased significantly. Compared with the FMc group, there was no significant change in MDA content, CAT activity. However, the GSH content, SOD activity of FMcA group increased significantly.

TABLE 2 Primer sequences of target genes from mirror carp (*Cyprinus carpio*), to measure the relative expression of the genes through quantitative real-time PCR.

Target genes	Primer sequence (5′-3′)	Gene ID
TNF-α[a]	AAGTCTCAGAACAATCAGGAA TGCCTTGGAAGTGACATT	XM_019088899.2
TGFβ1[b]	ACACGGTCACTTTGGTGTCA CAATGTGGGTTGCAGAACAG	XM_019072371.2
IL-1β[c]	AACTTCACACTTGAGGAT GACAGAACAATAACAACAAC	XM_019080073.2
claudin3c	GCACCAACTGTATCGAGGATG GGTTGTAGAAGTCCCGAATGG	XM_042710912.1
claudin7	CTTCTATAACCCCTTCACACCAG ACATGCCTCCACCCATTATG	XM_042732468.1
occludin	ATCGGTTCAGTACAATCAGG GACAATGAAGCCCATAACAA	KF975606.1
caspase3	CTCTACGGCACCAGGTTACTACTC GCCATCATTTCACAAAGGGACT	XM_019110173.2
caspase8	AAGCTCCTCATTGAAGAACCG ATCGTCCTGAACCACAACCTC	XM_042759183.1
caspase9	GCAAGCCCAAACTGTTCTTCAT CGTCCATCTGGTCATCTATCCC	XM_042750058.1
AMPKα[d]	GATGCCCTCTGGATGCTCTC GATGTCGTATGGTTTGCTCTGG	XM_042777568.1
AMPKβ[e]	AAACCTGAGGAACGCTTCAA CATCACATGGTTGGGTTCTG	XM_042711618
AMPKγ[f]	TGGGACAACAAACTGCAGAG GCAAGGGAATGAAGGAAACA	XM_042716989.1
ACC[g]	GTCACTGGCGTATGAGGATATT TCCACCTGTATGGTTCTTTGG	XM_042724983.1
TOR[h]	ATCTACGGCAAGACGAGAGG GTTGGTGGAGAGTGGGATCA	XM_042761449.1
4E-BP[i]	GGTGCTGATCAACGAGTCAA CAGGACGTTCTTGCTTGTCA	XM_042737175.1

[a]TNF-α, tumor necrosis factor alfa;
[b]TGF-β1, transforming growth factor beta;
[c]IL-1β, interleukin-1 beta;
[d]AMPKα, AMP-activated protein kinase alfa;
[e]AMPKβ, AMP-activated protein kinase beta;
[f]AMPKγ, AMP-activated protein kinase gamma;
[g]ACC, acetyl-CoA carboxylase;
[h]TOR, target of rapamycin;
[i]4E-BP, eIF4E-binding protein.

3.3 Relative expression levels of inflammatory cytokines genes in the intestine

The effect of soybean antigen proteins and α-ketoglutarate on the mRNA levels of inflammatory cytokines genes in the intestines are shown in Figure 3. Compared with the FM group, the mRNA expression levels of TNF-α and IL-1β were not significantly different, but the mRNA expression levels of TGFβ1 was significantly increased in SM and FMc group; the mRNA expression levels of TGFβ1, IL-1β were not significantly different, but the mRNA expression levels of TNF-α was significantly increased in FMG group.

Compared with the FMG and FMc group respectively, the mRNA level of IL-1β was not significantly different in FMGA and FMcA group, but the mRNA expression levels of TGFβ1 were significantly increased in FMG and FMcA group, respectively.

3.4 Relative expression levels of junction protein in the intestine

The effect of soybean antigen proteins and α-ketoglutarate on the mRNA levels of junction protein in the intestines are shown in Figure 4. Compared with the FM group, the mRNA expression levels of claudin3c, claudin7, and occludin were not significantly different in SM and FMc group; the mRNA expression levels of claudin3c and occludin were not significantly different, but the mRNA expression levels of claudin7 was significantly decreased in FMG group.

Compared with the FMG group, the mRNA level of occludin was not significantly different, but the mRNA expression levels of claudin3c and claudin7 were significantly increased in FMGA group. Compared with the FMc group, the mRNA level of occludin and claudin7 were not significantly different, but the mRNA expression levels of claudin3c was significantly increased in FMcA group.

3.5 Relative expression levels of apoptosis factors in the intestine

The effect of soybean antigen proteins and α-ketoglutarate on the mRNA levels of apoptosis factors in the intestines was given in Figure 5. Compared with the FM group, the mRNA expression levels of caspase3, caspase8, and caspase9 were not significantly different in SM group. In FMG group, the mRNA expression levels of caspase3 and caspase8 were not significantly different, but the mRNA expression levels of caspase9 was significantly increased; the mRNA expression levels of caspase9 was not significantly different, but the mRNA expression levels of caspase3 and caspase8 were significantly increased in FMc group.

Compared with the FMG and FMc group, the mRNA expression levels of caspase3, caspase8, and caspase9 were not significantly different in FMGA and FMcA group.

3.6 Relative expression levels of AMPK/ACC and TOR signaling pathway in the intestine

The effect of soybean antigen proteins and α-ketoglutarate on the mRNA levels of AMPK/ACC and TOR signaling pathway in the intestines was given in Figure 6. Compared with the FM group, the mRNA expression levels of AMPKα, target of rapamycin (TOR), and eIF4E-binding protein (4E-BP) were not significantly different, but the mRNA level of AMPKβ, AMPKγ, and acetyl-CoA carboxylase (ACC) were significantly increased in SM group; the mRNA expression levels of AMPKβ, ACC, TOR, and 4E-BP were

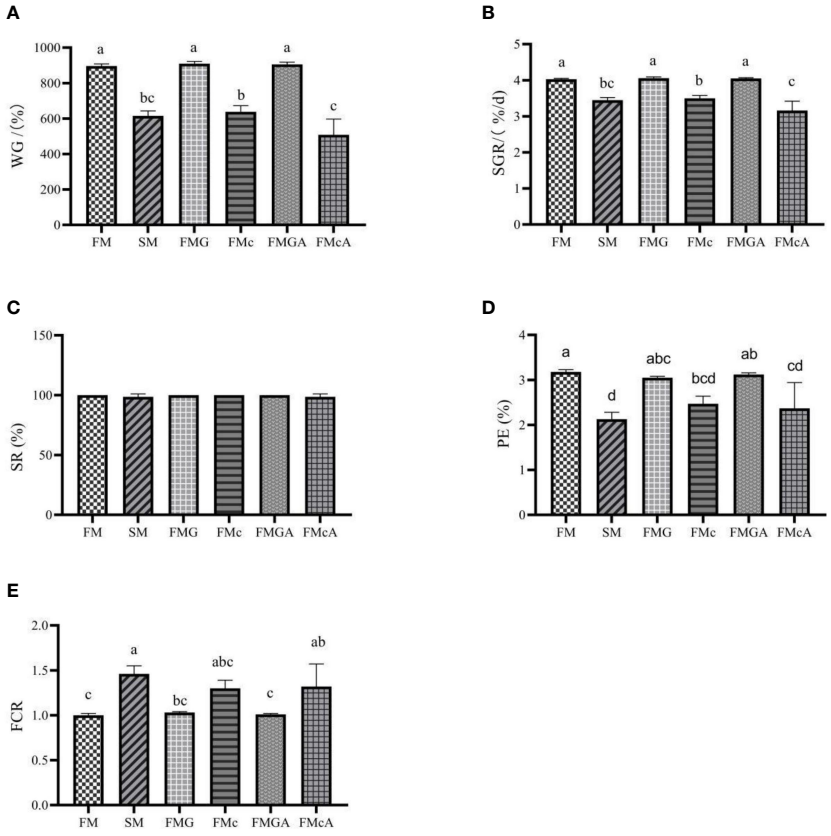

FIGURE 1
Growth performance of mirror carp at different treatments fed with dies containing fishmeal (FM), soybean meal (SM), glycinin (FMG), B-conglycinin (FMc), AKG+glycinin (FMGA), AKG+B-conglycinin (FMcA) after eight weeks. **(A)** WG, weight gain (%); **(B)** SGR, specific growth rate (%/day); **(C)** SR, survival rate (%); **(D)** PE, protein effiency (%); **(E)** FCR, feed conversion ratio; Data are presented as mean + SE, (n = 3). [a, b, c] Mean values with different letters were significantly different (P<0.05).

not significantly different, but the mRNA level of AMPKγ were significantly increased and the mRNA level of AMPKα were significantly decreased in FMG group; the mRNA expression levels of AMPKα, AMPKβ, AMPKγ, TOR and 4E-BP were not significantly different, but the mRNA level of ACC was significantly increased in FMc group.

Compared with the FMG group, the mRNA expression levels of AMPKα, AMPKβ, ACC, TOR, and 4E-BP were not significantly different, but the mRNA level of AMPKγ was significantly decreased in FMGA group. Compared with the FMc group, the mRNA expression levels of AMPKα, AMPKβ, AMPKγ, ACC, TOR and 4E-BP were not significantly different.

3.7 Morphology of the intestine

The effect of soybean antigen proteins and α-ketoglutarate on morphology of the intestines was given in Figure 7. The fish fed on the SM diet, there were no significant change in crypt depth in DI; but the crypt depth were significantly increased in PI and MI. The villus height and mucosal thickness were significantly decreased in PI, MI, and DI. The fish fed on the SM diet, there were no significant change in villus height (MI) and crypt depth (DI); but the villus height (PI and DI) and mucosal thickness (PI, MI, and DI) were significantly decreased and crypt depth (PI and MI) were significantly increased. The fish fed on the FMc diet, the villus height and mucosal thickness were significantly decreased and crypt depth were significantly increased in PI, MI, and DI.

Compared with the FMG group, there were no significant change in villus height, crypt depth (MI and DI), mucosal thickness (PI and MI), but crypt depth (PI) was significantly decreased and villus height (PI), mucosal thickness (DI) was significantly increased in FMGA group. Compared with the FMc group, there were no significant change in villus height (PI, MI and DI), crypt depth (MI), mucosal thickness (PI and MI), but crypt depth (PI and DI) was significantly decreased and mucosal thickness (DI) was significantly increased.

3.8 Key enzymes in tricarboxylic acid cycle and Na^+/K^+-ATPase in the intestine

The effect of soybean antigen proteins and α-ketoglutarate on key enzymes in tricarboxylic acid cycle and Na^+/K^+-ATPase in the intestines was given in Figure 8. Compared with the FM group, there were no significant change in CS, ICD, and α-KGDHC (PI and MI) activity, but Na^+/K^+-ATPase (PI) was significantly increased and α-KGDHC, ICD, CS (DI), and Na^+/K^+-ATPase

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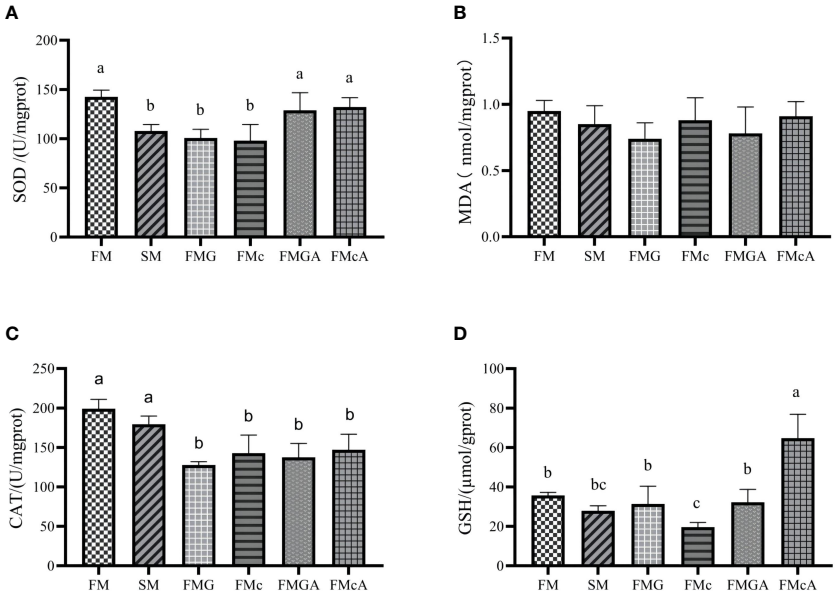

FIGURE 2
Antioxidative ability in the hepatopancreas of mirror carp at different treatment fed with diets containing fishmeal (FM), soybean meal (SM), glycinin (FMG), B-conglycinin (FMc), AKG+B-conglycinin (FMcA) after eight weeks. **(A)** T-SOD, total superoxide dismute (U/mg prot); **(B)** MDA, malondialdehyde (mmol/mg prot). **(C)** CAT, catalase (U/mg prot); **(D)** GSH, glutathione (umol/gprot); Data are presented as mean +SE, (n = 6) [a, b, c] Mean values with different letters were significantly different (P<0.05).

(MI and DI) activity were significantly decreased in SM group; there were no significant change in CS, Na^+/K^+-ATPase (PI), ICD (MI), and α-KGDHC (PI and MI) activity, but CS, Na^+/K^+-ATPase (MI and DI), ICD (PI and DI), α-KGDHC (DI) were significantly decreased in FMG group; there were no significant change in ICD, α-KGDHC (PI and MI), and Na^+/K^+-ATPase (PI) activity, but CS (PI, MI and DI), α-KGDHC, ICD (DI), Na^+/K^+-ATPase (MI and DI) were significantly decreased in FMc group.

Compared with the FMG group, there were no significant change in CS, ICD, α-KGDHC and Na^+/K^+-ATPase (DI) activity, but CS, α-KGDHC, ICD and Na^+/K^+-ATPase (PI and MI)activity were significantly increased in FMGA group. Compared with the FMc group, there were no significant change in CS (PI and MI), ICD, α-KGDHC (PI, MI, and DI), and Na^+/K^+-ATPase (PI and DI) activity, but Na^+/K^+-ATPase (MI) activity were significantly increased and CS (DI) activity was significantly decreased in FMcA group.

4 Discussion

In the present study, carp fed diets with soybean meal presented lower WG, SGR, and PE than in the control, which is corroborated by the findings of other studies investigating dietary soybean meal

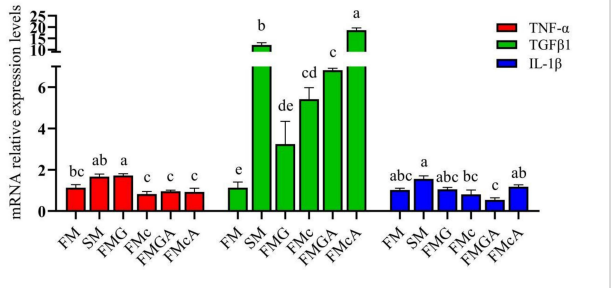

FIGURE 3
Relative expression levels of inflammatory cytokins in the intestine of mirror carp at different treatments fed with diets containing fishmeal (FM), soybeans meal (SM), glycinin (FMG), B-conglycinin (FMc), AKG+B-conglycinin (FMcA) after eight weeks. TNF-a, tumor necrosis factor alfa; TGF-B1, transforming growth factor beta; IL-1B, interleukin-1 beta. Data are presented as means + SE, (n = 3). [a, b, c] Mean values with different letters were significantly different (P<0.05).

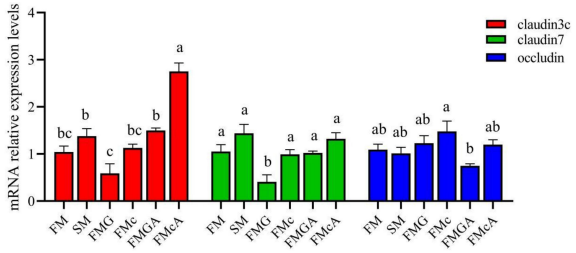

FIGURE 4
Relative expression levels of junction protein in the intestine of mirror carp at different treatment fed with the diets containing fishmeal (FM), soybean meal (SM), glycinin (FMG), B-conglycinin (FMcA)< AKG+B-conglycinin (FMGA), AKG+B-conglycinin (FMcA) after eight weeks. Data are presented as means + SE, (n = 3). [a, b, c] Mean values with different letters were significantly different (P<0.05).

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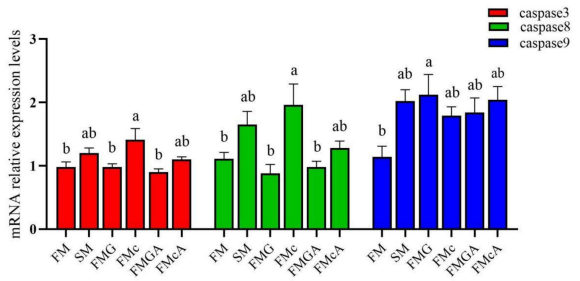

FIGURE 5
Relative expression levels of apoptosis factors in the intestine of mirror carp at different treatments fed with diets containing fishmeal (FM), soybean meal (SM), glycinin (FMG), B-conglycinin (FMc), AKG+ glycinin (FMGA), AKG+B-conglycinin (FMcA) after eight weeks. Data are presented as means + SE, (n = 3). [a, b, c] Mean values with different letters were significantly different (P<0.05).

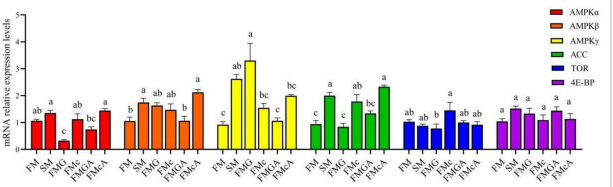

FIGURE 6
Relative expression levels of AMPK/ACC and TOR signaling pathways in the intestine of mirror carp at different treatments fed with diets containing fishmeal (FM), soybean meal (SM), glycinin (FMG), B-conglycinin (FMc), AKG+ glycinin (FMGA), AKG+B-conglycinin (FMcA) after eight weeks. AMPKa, AMP-activated protein kinase gamma; ACC, acetyl-CoA carboxylase; TOR, target of rapamycin; 4E-BP, eIF4E-binding protein. Data are presented as means + SE, (n = 3). [a, b, c] Mean values with different letters were significantly different (P<0.05).

(21, 22). Dietary soybean meal increased the FCR, implying that the growth inhibition can be partly attributed to low feed efficiency by adding soybean meal (23). Glycinin and β-conglycinin are the main anti-nutritional factors in soybean meal, which can also adversely affect the growth performance of aquatic animals (3). In this study, dietary β-conglycinin reduced growth performance. This finding is consistent with studies in golden crucian carp (8). β-conglycinin cannot be digested and broken down into small molecules, and it is known to cause allergic reaction in intestinal tract, destruction of intestinal integrity, dysfunction of digestion and absorption of nutrients, inflammation, and oxidative damage, and finally suppressed fish growth performance (24). It was surprising to observe that growth performance was not influenced by the dietary Glycinin. These striking differences in growth performance between these two anti-nutrients may happen due to their functional properties. Glycinin has a higher content of sulfur-containing amino acids than β-conglycinin (25). The composition of essential amino acids is more balanced, so it can better meet the body 's intake of essential amino acids. After adding the same dose of soybean antigen proteins, β-conglycinin could reduce the growth performance of carp (26), while glycinin did not affect the growth performance of turbot (27). Moreover, in this study fish were fed to apparent satiation (~9% of their body weight/day), and this high feed intake may have met the requirement of the growth performance of carps, which can be observed on the growth performance. Dietary AKG supplementation can promote growth performance by enhancing the activities of the antioxidant defense system (28). The supplementation of 1.0% AKG diet has also presented positive effects on growth performance of juvenile hybrid sturgeon (*Acipenser schrenckii* ♀ × *A. baerii* ♂). However, in this study, 1.0% AKG diet has not positive on the WG and SGR of carp, similar results were also observed in juvenile red drum (15). This lack of production performance may have been caused by the physiological differences among these fish species, breeding environments, growth phases, and dietary nutrient levels.

It is widely accepted that inflammation and oxidative stress usually occur simultaneously (29).Various stress factors may cause an imbalance between pro-oxidant and antioxidant systems, which will lead to oxidative stress. MDA, SOD and CAT were used as oxidative stress biomarkers (30). The concentration find in their tissues can reflect their antioxidant capacity (31) The oxidative stress induced by soybean antigen proteins, as evidenced by the depressed levels of SOD and CAT, indicates that glycinin and β-conglycinin can cause SBM-induced oxidative stress in carp. These deleterious effects may be caused by the strong immunogenicity induced by soybean antigen proteins (32). Our results on the elevated activity of SOD suggest that AKG may suppress oxidative damage by glycinin and β-conglycinin. Besides these set of enzymes, GSH is one of the main non-enzymatic antioxidants that protect cells against oxidative damage (33), and it is protects many kinds of cells in body (34). AKG can be converted into glutamine by glutamate dehydrogenase and glutamine synthetase, which can be a marker of antioxidative function (14). It is evident that AKG can improve antioxidative capacity by increasing available glutamine and aiding antioxidative systems (14). Fish fed AKG supplemented feed presented an improvement of antioxidant enzyme levels, including GSH and SOD, indicating its protective efficacy (35). It has also been reported that AKG can positively alleviate the oxidative stress damage in cells, ultimately contributing to cell redox homeostasis (14), and also serve as an energy donor and antioxidant agent *via* the TCA cycle (36).The prooxidant effect of AKG seems to be due to the altered intensity of the TCA cycle in which AKG is preferentially metabolized (37). These data demonstrate that 1.0% AKG administration can alleviate the oxidative stress caused by the dietary soybean antigen proteins *via* the TCA cycle.

After feeding, soybean antigenic protein enters the intestine, destroying the structural stability of the intestine, activating the immune system and causing intestinal inflammation (38). TNF-α plays an essential role in mediating the inflammatory, regulating immune function and other pathophysiological reactions (39). IL-1β is a pleiotropic inflammatory cytokine and plays an important role in the inflammatory process (40). Both TNF-α and IL-1β are important pro-inflammatory factors (41). Intestinal inflammation is related to elevated pro-inflammatory cytokines (42). Enteritis is aggravated by up-regulating pro-inflammatory cytokines (43). In the present study, the expression of pro-inflammatory factor (TNF-

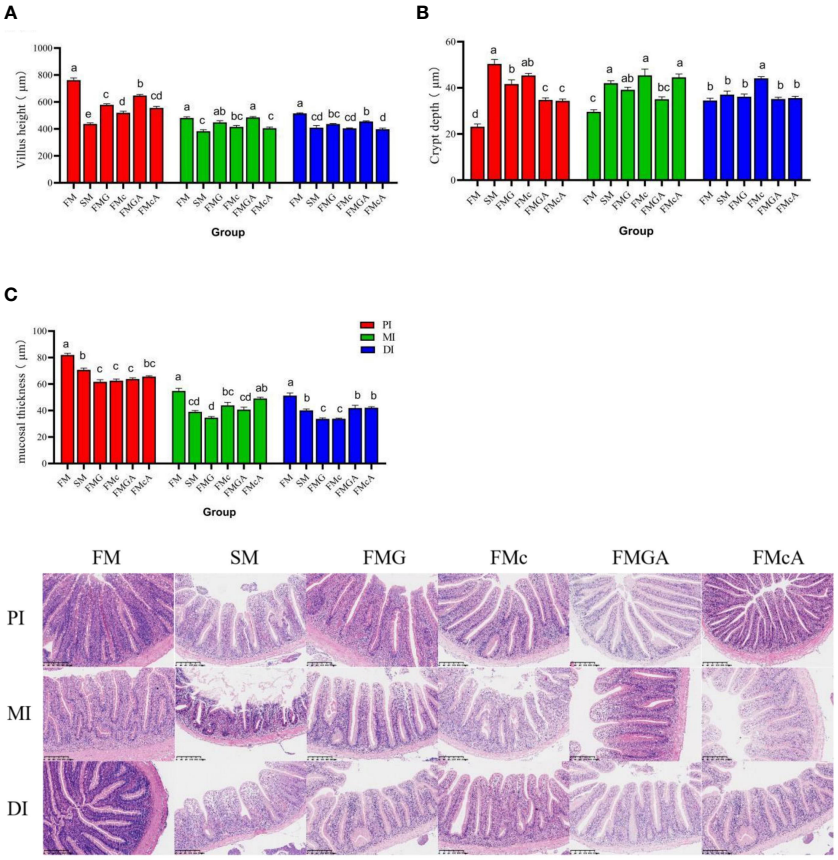

FIGURE 7
Morphology of the intestines of mirror carp at different treatments fed with diets containing fishmeal (FM), soybean meal (SM)m glycinin (FMG), B-conglycinin (FMc), AKG+ glycinin (FMGA), AKG+B-conglycinin (FMcA) after eight weeks. PI, proximal intestine; MI, mid intestine; DI, distal instestine. **(A)** villus height (um); **(B)** crypt depth (um); **(C)** mucosal thickness (um). Data are presented as means + SE, (n = 3). [a, b, c] Mean values with different letters were significantly different (P<0.05).

α) was upregulated by dietary FMG group, which revealed the occurrence of glycinin-induced enteritis. Similarly, the phenomenon of glycinin-induced enteritis has also been observed in juvenile *Rhynchocypris lagowskii* (44). In addition, the mRNA level of TNF-α was decreased by dietary FMGA group. AKG can improve intestinal immunity and promotes intestinal health (45). Previously, Zhang et al. (34) found that glutamine suppressed TNF-α concentration. It is further confirmed by our study that AKG as a precursor for the biosynthesis of glutamine, increasing intestinal immunity. TGF-β is considered to be important anti-inflammatory cytokines that can inhibit inflammation (46). TGF-β has also played an important role in gut wound repair by promoting epithelial reconstitution through up-regulation of epidermal growth factor (47). Compared with the FMG/FMc group, the mRNA level of TGF-β1 was increased by dietary AKG. These data demonstrate that 1.0% AKG administration can alleviate the intestinal inflammation caused by the dietary soybean antigen proteins.

The intestinal epithelial barrier is the first line of defense against pathogenic microorganisms and toxins in the intestines (48). The connection between intestinal epithelial cells mainly relies on the TJ (49), and mainly composed of occludin, claudins, and others (50), which forms a highly selective barrier and protect animal health. In our study, we found that the mRNA levels of claudin7 was suppressed by glycinin. Claudins are the major barrier-forming proteins of tight junction structure (51). Decreased gene expression of TJ usually represents the weakened intestinal barrier function (52). Similarly, Zhao et al. (53) reported that changes in TJ expression induced by soybean antigen protein lead to intestinal epithelial barrier dysfunction. It demonstrates that glycinin increase intestinal paracellular permeability, decrease tight junction proteins' expressions, suggesting that glycinin induce intestinal tight junction barrier dysfunction. The result in this study is supported by the findings in carp (54). This might be due to the fact that glycinin, as a foreign antigen, caused immune injury and inflammation, and then inflammatory factors destroyed intestinal epithelial tissue proteins (32). A lot of studies have proved that the impaired intestinal barrier is directly related to the intestinal inflammation (55, 56) and attenuate gut injury through an anti-inflammatory role (57). In the study, the down-regulation of claudin3c, claudin7 observed after dietary soybean antigen protein was abolished by AKG. These observations were partly attributed to AKG supplementation improving glutamine synthesis activity and concentrations of glutamine and glutamic acid in the intestines (58). Dietary alanyl-glutamine in soy protein diets elevated the expression of TJ proteins (59). A number of studies have were reported that dietary glutamine enhance gut

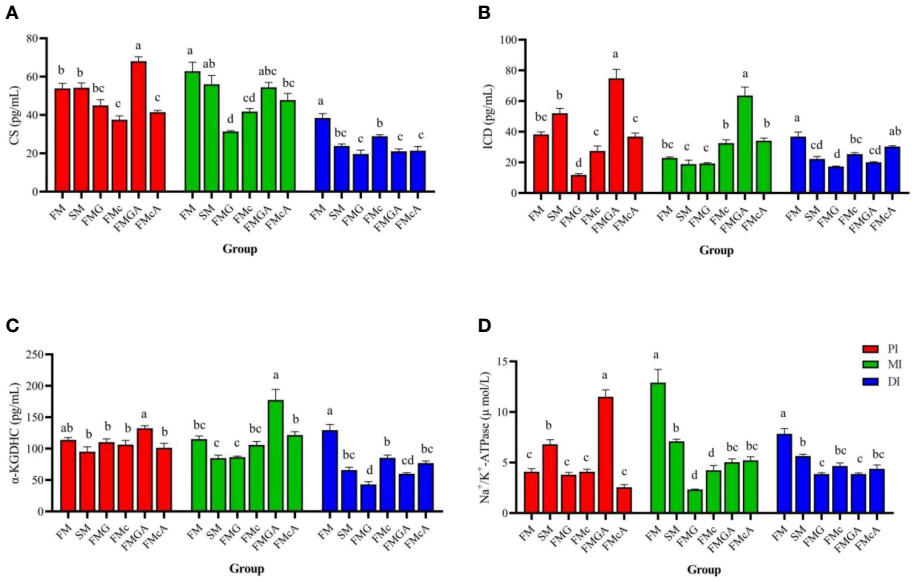

FIGURE 8
Key enzymes in the tricarboxylic acid cycle and Na^+/K^+-ATPase in the intestine fed with diets containing fishmeal (FM), soybean meal (SM), glycinin (FMG). B-conglycinin (FMc), AKG+ glycinin (FMGA) AKG+B-conglycinin (FMcA) after eight weeks. PI, proximal intestine; MI, mid intestine; DI, distal intestine. **(A)** CS, Citrate synthase; **(B)** ICD, Isocitarte dehydrogenase; (pg/mL) **(C)** a-KGDHC, a-ketaglutarte dehydrogenase complex (pg/mL); **(D)** Na+/K+-ATPase (umol/L). Data are presented as means +SE, (n = 3). $^{a, b, c}$ Mean values with different letters were significantly different (P<0.05).

development and regulate intestinal barrier function in multiple animal models (60). In this study, for the first time, demonstrate that 1.0% AKG administration can alleviate the damage of the epithelial barrier caused by the dietary soybean antigen proteins.

Apoptosis is a physiological programmed cell death through the activation of cell intrinsic suicide machinery (61). Apoptosis is a cascade amplifying reaction regulated by a cysteine family of proteases (62). It is well accepted that caspase family proteins is the central regulator of apoptosis (63). We found that the expressions of caspase3 and caspase8 were significantly elevated with FMc group. The expressions of caspase9 was significantly elevated with FMG group, consistent with the results of Peng et al. (64), who showed that soybean antigen protein increased the levels of caspase3 activity and induced piglet intestinal cell apoptosis. Additionally, glycinin or β-conglycinin accelerates enterocyte proliferation, apoptosis and migration for piglets (65). Hence this suggesting that soybean antigen protein causes intestinal damage. However, AKG administration could not alleviate the intestinal apoptosis caused by the dietary soybean antigen proteins in one week. Maybe the longer experimental period might have showed some positive on intestinal apoptosis.

AMPK, a sensor of the availability of intracellular energy, is activated at low energy levels and regulates cellular processes accordingly (66). AMPK is a heterotrimeric Ser/Thr kinase composed of an α, β and γ subunit (67). AMPK is activated by phosphorylation of Thr172 within the activation segment of the KD of the α subunit (68). The β subunits contains a region termed the carbohydrate binding module (68) and allows AMPK to interact with glycogen particles (69). AMPK preserves the level of ATP *via* stimulation of energy producing pathways and suppression of energy-consuming metabolisms (70). Under lowered intracellular ATP levels, AMP or ADP can directly bind to the γ regulatory subunits of AMPK, leading to a conformational change that promotes facilitates the phosphorylation of AMPKα and the activation of AMPK (71). In the current study, the mRNA expression of AMPKγ was significantly elevated and AMPKa was decreased with glycinin. This might be attributed to the fact that soybean antigen protein destroyed the structural stability of the intestine through pro-inflammatory and TJ proteins (54), which required more energy for maintenance, thus increased AMPKγ gene expression. Maintaining the intestinal barrier is energy-consuming, and impairing mitochondria function is associated with intestinal inflammation (72). In addition, AMPK phosphorylation are reported to be involved in promote apoptosis (73). A similar phenomenon was also observed in the study that the expressions of TNF-α, caspase8, and AMPKγ were significantly elevated and claudin7 was significantly decreased in FMG. It indicates that glycinin-induced injure intestine was partially related to the activate of AMPK signaling. In addition, the mRNA level of AMPKγ was decreased in FMGA group. AKG as a source of energy (14), the oxidation of AKG through the tricarboxylic acid cycle can provide large amounts of ATP. AKG stimulates AMPK phosphorylation and oxidation of energy substrates in the intestinal mucosa, thereby enhancing ATP supply and supporting cell function (20). This suggested that AKG increased the intestinal ATP supply partly by down-regulating AMPKγ mRNA expression. It indicates that AKG increases intestinal ATP supply by down-regulating AMPKγ gene expression, but the change of AMP/ATP is not enough to affect AMPK phosphorylation.

AMPK stimulates fatty acid oxidation as a way to increase energy levels. ACC is one of the first proteins identified as a target of AMPK (74). The mRNA level of ACC was increased with the

soybean meal and β-conglycinin in this study. ACC is a critical enzyme of the lipogenic pathway (75); simultaneously, it is regulated by AMPK (76). This may indicate that dietary soybean meal and β-conglycinin could increase lipogenesis. In addition, the mTOR, it is also one of the target proteins of the downstream signaling pathway after AMPK (77). It is a limiting step in animal protein synthesis (78). And 4E-BP is one of the mTOR-regulated target sites. In this study, the mRNA level of TOR and 4E-BP were not significantly different with the SM, FMG and FMc for one week. However, studies have showed that β-conglycinin and/or glycinin could decrease the P-TOR protein expression for seven weeks in grass carp (24, 42). The mainly reason is unknown presently, maybe the longer experimental period might have showed some effect of the mTOR signaling pathway.

The fish growth highly depends on the healthy development of the intestine, which can be reflected by the villus height and mucosal thickness (9). Soybean allergy usually induces intestinal inflammatory diseases, characterized by the atrophy and proliferation of crypt villi, which accelerate the apoptosis and migration of intestinal cells (29). In this study, the VH and MT of the proximal intestine, mid intestine, and the distal intestine (except for the FMG group in MI) were decreased and crypt depth of the PI, MI, and DI (except for the SM, FMG group in DI) were increased in SM, FMG and FMc, indicates varying degrees of damage and inflammation. Similar results were also observed in turbot (8) and grass carp (79). Stimulation of antigen-active macromolecules can prompt an immune response that causes atrophy of the intestinal villi, thus damaging the intestinal morphology (9). In addition, dietary AKG has been reported to improve mucosal morphology and function of the intestine (57). In this study, the VH (PI) and MT (DI) were increased and crypt depth (PI) was decreased in FMGA group. The MT (DI) was increased and crypt depth (PI and DI) was decreased in FMcA, which was consistent with studies in piglets (80). In summary, soybean meal, glycinin and β-conglycinin damage the intestinal morphology and AKG improving intestinal morphology

Soybean antigen protein destroys the structural stability of the intestine and causes digestive and absorption disorders (54). Maintaining the intestinal barrier is energy-consuming, and impairing mitochondria function is associated with gut inflammation (72). Under stress, the body's energy metabolism disorders, ATP content decreased significantly, cell dysfunction or death, leading to damage to intestinal structure and function (81). Citrate synthase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase are the three rate-limiting enzymes of TCA cycle, which regulate the energy metabolism and biosynthesis of organisms. Citrate synthase can determine the rate of acetyl-CoA into TCA cycle, which is an important indicator of energy metabolism (82). Isocitrate dehydrogenase exists in mitochondria and cytoplasm and catalyzes isocitrate to produce α-ketoglutarate (83). The α-ketoglutarate dehydrogenase system exists in the mitochondrial matrix and catalyzes the oxidative decarboxylation of α-ketoglutarate to produce succinyl coenzyme A and reduced coenzyme I (NADH) (84). Na^+/K^+-ATPase mainly exists in the basement membrane of intestinal absorptive cells (85). It produces energy by decomposing ATP to maintain the reverse concentration gradient of sodium and potassium. Its activity can indirectly reflect the absorptive capacity of intestinal mucosa (86). In this study, soybean meal, glycinin and β-conglycinin significantly decreased α-KGDHC, ICD, CS and Na^+/K^+-ATPase in DI, indicates that tricarboxylic acid cycle is inhibited and energy production is reduced. The AKG is an important intermediate product in the tricarboxylic acid cycle of organisms, and is also a biosynthetic precursor of glutamic acid, glutamine and arginine in organisms. In addition, glutamine and arginine play an important role in promoting the repair of damaged intestine (87). The AMPKγ, VH (PI), MT (DI), α-KGDHC, ICD, CS, and Na^+/K^+-ATPase (PI and MI) were increased in FMGA. The Na^+/K^+-ATPase (MI) and MT (DI) were increased in FMcA. These data demonstrate that AKG in enterocytes may beneficially regulate the intracellular endogenous amino acid concentrations *via* TCA cycle (88) and then influence various signaling pathways (89), thereby alleviating the damage intestinal morphology caused by the dietary soybean antigen proteins. In addition, study has shown that AKG can be used as a substrate for intestinal energy metabolism (90). AKG could produce plenty of ATP in the TCA cycle and provide energy for intestinal cell processes (14). Dietary supplementation of AKG can improve the energy metabolism of intestine after soybean antigen protein stimulation.

5 Conclusions

In conclusion, dietary soybean meal can compromise the intestine health, and the adverse effects are related to the presence of β-conglycinin and glycinin, especially glycinin. 1.0% AKG may regulate intestinal energy *via* TCA cycle, thereby alleviating the damage intestinal morphology caused by the dietary soybean antigen proteins. Moreover, it is recommended the dietary supplementation of AKG when high levels of soybean meal are used in carp aquafeeds.

Data availability statement

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

Ethics statement

This study was conducted following the China's Guidance of the Care and Use of Laboratory Animals. The experimental protocol was approved by the Committee on the Ethics of Animal Experiments of Huzhou University.

Author contributions

QL: project administration, writing the original draft preparation, and formal analysis; ZQ and RQ: formal analysis and investigation; FY: writing the manuscript and critical revision; YD and XL: investigation; QX: experiment design, management and

manuscript revision. All authors contributed to the article and approved the submitted version.

Funding

This work was financially supported by the Natural Science Foundation of China, NSFC (31972800), and the China Agriculture Research System of MOF and MARA.

Acknowledgments

The authors would like to express their sincere thanks to the personnel of these teams for their kind assistance. The author also thank International Science Editing (http://www.internationalscienceediting.com) for editing this manuscript.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 17 May 2023
DOI 10.3389/fimmu.2023.1140678

OPEN ACCESS

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RECEIVED 09 January 2023
ACCEPTED 11 April 2023
PUBLISHED 17 May 2023

CITATION
Zhao J, Yang X, Qiu Z, Zhang R, Xu H and Wang T (2023) Effects of tributyrin and alanyl-glutamine dipeptide on intestinal health of largemouth bass (*Micropterus salmoides*) fed with high soybean meal diet.
Front. Immunol. 14:1140678.
doi: 10.3389/fimmu.2023.1140678

Effects of tributyrin and alanyl-glutamine dipeptide on intestinal health of largemouth bass (*Micropterus salmoides*) fed with high soybean meal diet

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To investigate the effects of dietary tributyrin (TB) and alanyl-glutamine (AGn) on the intestinal health of largemouth bass (*Micropterus salmoides*) fed with high-level soybean meal (SM) diet, six isonitrogenous (41.36%) and isolipidic (10.25%) diets were formulated and fed to largemouth bass (initial body weight 25.5 ± 0.5g) for 8 weeks. The two control diets contained 34.8% peanut meal (PM) and 41.3% SM, while the other four experimental diets supplemented TB at 0.1% (TB0.1), 0.2% (TB0.2) and AGn at 1% (AGn1), 2% (AGn2) in SM, respectively. The results showed that there were no significant differences in weight gain, survival rate, and hepatosomatic index among all groups (P>0.05), while feed coefficient rate in AGn1, AGn2 and TB0.2 groups was significantly lower than that in SM group (P< 0.05). Compared with the PM group, the intestinal inflammation of largemouth bass in SM group were obvious, accompanied by the damage of intestinal structure, the decrease of digestive enzyme activity, and the up-regulation of proinflammatory cytokines. Compared with the SM group, the activities of intestinal trypsin, lipase and foregut amylase in TB and AGn groups increased significantly (P<0.05), and the gene expression levels of acetyl-CoA carboxylase (ACC), caspase-3, caspase-8, caspase-9, tumor necrosis factor alpha (TNF-α), and interleukin-1 beta (IL-1β) were down-regulated, while the gene expression levels of target of rapamycin (TOR) and eIF4E-binding protein (4E-BP) were up-regulated in all experimental groups (P<0.05). It can be concluded that supplementation of 1%-2% AGn and 0.1%-0.2% TB can alleviate enteritis caused by high-level soybean meal, and the recommend level is 2% AGn and 0.2% TB.

KEYWORDS

tributyrin, alanyl-glutamine, intestinal morphology, inflammation, soybean meal, largemouth bass

1 Introduction

Fish meal is widely used in aquatic animal feed. However, due to the limitation of natural resources and the climate change, the shortage of fish meal supply is increasing. In order to improve the sustainability and profitability of aquaculture, plant protein was paid attention in recent years. Soybean meal (SM) has become one of the main protein sources in aquatic feed because of its high protein content, relatively balanced amino acid composition and reasonable price. However, SM contains some anti-nutritional factors including trypsin inhibitors, antigen proteins, lectins, tannins, saponins and alkaloids (1–3). Previous studies on Atlantic salmon (*Salmo salar*) (4, 5), zebrafish (*Danio rerio*) (6), rice field eel (*Monopterus albus*) (7), Japanese seabass (*Lateolabrax maculatus*) (8), largemouth bass (9) and Mirror carp (*Cyprinus carpio*) (10) have shown that, excessive use of SM in feed can cause intestinal inflammation and injury, intestinal digestion and absorption dysfunction, and ultimately reduce growth performance (11, 12), especially in carnivorous fish.

Intestine is an important organ of fish, involved in digestion, absorption and immunity (13). Intestinal mucosal barrier dysfunction is a prerequisite for intestinal diseases (14). In this case, it is necessary to adopt nutritional strategies such as supplementing some functional feed additives to alleviate intestinal inflammation and disease in animals. Both glutamine and butyric acid play important roles in providing energy to the intestine and improving intestinal immunity (15, 16). Glutamine is a major fuel source for rapidly dividing cells such as intestinal epithelial cells, macrophages, and lymphocytes in the intestine, suggesting that glutamine can maintain intestinal integrity and activate the immune system (15). However, due to the low solubility and instability of glutamine in water, and its easy decomposition into toxic pyroglutamate and ammonia at high temperature, these characteristics limit its wide application in animal feed. Glutamine dipeptide is a dipeptide formed from glutamine and other amino acids (mainly alanine or glycine) that acts physiologically similar to glutamine. Due to its good stability, high water solubility and high utilization efficiency *in vivo*, glutamine dipeptide has been applied to practices related to growth and intestinal immunity in terrestrial (15, 17) and aquatic animals (18–21). For example, glutamine dipeptide can promote the growth of carp (22, 23), increase the weight gain and specific growth rate of hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂), improve intestinal structure, and decrease the expression of intestinal proinflammatory genes (TNF-α, IL-1β) (24). Butyric acid is a short-chain fatty acid which plays an important role in regulating growth performance, gastrointestinal function, immunity, intestinal pH, and gastrointestinal microecological balance (25). As the main respiratory fuel source of the colonic bacteria, butyrate helps to enhance epithelial cell proliferation and differentiation and improve intestinal absorption function. In addition, many researches have proved that butyrate has potential immunomodulatory and anti-inflammatory properties in the intestine (26). However, butyric acid is very volatile, it is mostly added in the form of sodium butyrate and tributyrin (TB) in feeds. However, sodium butyrate is easy to absorb moisture and agglomerate, and has a special odor, which is not conducive to its wide application. As the precursor of butyric acid, TB can be decomposed by pancreatic lipase in animal intestine to produce 3 molecules of butyric acid and 1 molecule of glycerol, which are then transported by blood to various tissues and organs of the body. The results showed that TB as a feed additive can improve the growth performance of piglets (27), snakehead (*Channa argus*) (28) and yellow catfish (*Pelteobagrus fulvidraco*) (29), and improve the intestinal structures of carps (30), yellow drums (*Nibea albiflora*) (31), and improve digestive enzyme activity and immune performance of juvenile black sea breams (*Acanthopagrus schlegelii*) cultured with high plant protein diet (32).

Largemouth bass, a carnivorous fish native to North America, has been cultivated in many countries, including China, because of its fast growth, delicious meat, and no intermuscular bones. According to statistics, the production of largemouth bass in China had exceeded 700,000 tons in 2021 (China Fishery statistics Year-book, 2022). The commercial feed of largemouth bass typically contains 35%-50% fish meal, which greatly increases feed costs (10). In order to reduce costs, SM is often adopted to replace fish meal. However, replacing fish meal with a high proportion of SM often caused serious negative effects on growth performance, intestinal health and immunity in largemouth bass (33–36). Therefore, there is an urgent need to explore dietary strategies to reduce the negative effects of high percentage of SM on the growth and intestinal health of largemouth bass. In addition, there are few studies on TB and alanyl-glutamine (AGn) related to largemouth bass; in particular, the effect and mechanism on growth and gut health of feeding high soybean meal diet were not clarified (9, 33). Based on the above information, glutamine dipeptide and TB that could provide energy for intestinal epithelial cells and improve intestinal immunity were selected as feed additives in this study. The purpose of this study was to verify the alleviating effects of AGn and TB on intestinal health of largemouth bass cultured with a high proportion of SM by detecting growth performance, intestinal morphology and digestibility, genes related to protein synthesis and inflammation.

2 Materials and methods

2.1 Experimental diets

A total of 6 diets with isonitrogenous and isolipidic were prepared (37–39). In order to maintain the same animal/plant protein ratio, peanut meal, a plant protein with relatively few anti-nutritional factors and little damage to fish intestinal tract, was selected (40) and the peanut meal group contained 34.80% peanut meal supplemented with methionine, lysine and threonine, which were used as the positive control group (PM); the high SM group with 41.30% SM was supplemented with methionine as the negative control group (SM). With reference to the results of previous studies on TB (41–44) and Ala-Gln (18, 23, 45, 46),

0.10%, 0.20% of TB (group TB0.1 and TB0.2) and 1.00%, 2.00% of Ala-Gln (group AGn1 and AGn2), respectively, were added to SM feed as experimental groups. The feed formula and nutrient content of each experimental group was shown in Table 1. Dietary ingredients were ground into fine powder and sieved through a 60-mesh sieve, the ingredients were thoroughly mixed and water was added to form a dough. Then, pellets with 2 mm particle size were produced by using a twin screw extruder. The diets were dried in a ventilated oven at 40 °C and stored at -20 °C (3).

2.2 Feeding and management

Largemouth bass for the experiment was purchased from a farm in Huzhou, Zhejiang of China, and were domesticated in the circulating water system for 2 weeks. At the beginning of the feeding trial, healthy fish with an average body weight of 25.5 ± 0.5 g were randomly distributed to 18 tanks (300 L water), with three replicate tanks for each treatment group and 20 fish per tank. Fish was fed twice daily to apparent satiation at 7 a.m. and 6 p.m. for 8 weeks. During the experiment, one-third of the water was changed every day to maintain water quality. Water temperature, pH, dissolved oxygen, ammonia nitrogen and nitrite were monitored daily and recorded as 27°C to 30°C, above 6.5 mg/L, and below 1.0 mg/L respectively.

2.3 Growth performance

At the end of feeding trial, all fish were counted and weighed. Growth performance was determined by calculating the survival rate (SR), weight gain (WG), specific growth rate (SGR), feed

TABLE 1 Feed formula and nutrient levels of diets (air-dry basis).

Ingredients	Composition of diet %					
	PM	SM	TB1	TB2	AGn1	AGn2
Fish meal	35.00	35.00	35.00	35.00	35.00	35.00
Soybean meal	0.00	41.30	41.30	41.30	41.30	41.30
Peanut meal	34.80	0.00	0.00	0.00	0.00	0.00
Pregelatinized starch	5.00	5.00	5.00	5.00	5.00	5.00
Soybean oil	0.00	2.00	2.00	2.00	2.00	2.00
Soy lecithin	2.00	2.00	2.00	2.00	2.00	2.00
Fish oil	1.26	2.00	2.00	2.00	2.00	2.00
$Ca(H_2PO_4)_2$	2.05	2.00	2.00	2.00	2.00	2.00
Met	1.12	1.20	1.20	1.20	1.20	1.20
Lys	0.13	0.00	0.00	0.00	0.00	0.00
Thr	0.10	0.00	0.00	0.00	0.00	0.00
Vitamin premix[a]	0.50	0.50	0.50	0.50	0.50	0.50
Mineral premix[b]	0.20	0.20	0.20	0.20	0.20	0.20
Choline chloride	0.50	0.50	0.50	0.50	0.50	0.50
$MgSO_4$	0.30	0.30	0.30	0.30	0.30	0.30
Microcrystalline cellulose	11.04	2.00	1.90	1.80	1.00	0.00
Carboxymethyl cellulose	3.00	3.00	3.00	3.00	3.00	3.00
Yeast hydrolysate	3.00	3.00	3.00	3.00	3.00	3.00
Tributyrin	0.00	0.00	0.10	0.20	0.00	0.00
Alanyl-glutamine	0.00	0.00	0.00	0.00	1.00	2.00
Total	100.00	100.00	100.00	100.00	100.00	100.00
Nutrient levels						
Crude protein	40.84	40.36	40.73	40.74	40.63	40.43
Crude lipid	9.28	9.88	9.53	9.46	9.58	9.47

[a]Vitamin premix provided the following per kg of diets: VA 2000 IU, VC 175.00 mg, VD_3 1000 IU, VE 100.00 mg, VK_3 4.80 mg, VB_1 14.70 mg, VB_2 28.00 mg, VB_6 19.60 mg, VB_{12} 0.07 mg, nicotinamide 78.40 mg, D-biotin 0.07 mg, inositol 122.50 mg, folic acid 0.98 mg, D-calcium pantothenate 22.50 mg; 2. [b]Mineral premix provided the following per kg of diets: $MnSO_4{\cdot}H_2O$ 179.00 mg, $CoCl_2$ 1.50 mg, $FeSO_4{\cdot}7H_2O$ 66.00 mg, $CuSO_4{\cdot}5H_2O$ 8.06 mg, $ZnSO_4{\cdot}H_2O$ 150.80 mg, Na_2SeO_3 0.35 mg, KI 6.05 mg.

coefficient rate (FCR), hepatosomatic index (HSI) and viscerosomatic index (VSI), as follows:

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

$$WG(\%) = 100 \times (\text{final body weight} - \text{initial body weight})/\text{initial body weight};$$

$$SGR(\%/\text{day}) = 100 \times (\text{Ln final body weight} - \text{Ln initial body weight})/\text{Feeding days};$$

$$FCR = \text{feed intake}/\text{weight gain};$$

$$HIS(\%) = 100 \times (\text{final liver weight}/\text{final body weight});$$

$$VSI(\%) = 100 \times (\text{final visceral weight}/\text{final weight}).$$

2.4 Sample collection

Then, the fish were anesthetized with tricaine methanesulfonate (MS-222), five fish from each tank were randomly selected and stored at -20°C for whole body proximate composition analysis. Three fish from each tank were individually measured body weight, body length, visceral weight, liver weight, to calculate hepatosomatic index (HSI) and viscerosomatic index (VSI). After the dissection, the intestinal tissues were cut into three parts (fore-, mid- and hindgut) and part was stored at −80°C for the digestive enzyme activity analysis, and part was washed with 75% normal saline, foregut, midgut and hindgut were quickly gathered from three fish per tank, then transferred to 4% polyformaldehyde (PFA) fixative for histology structure analysis. Then the whole intestinal tissues of another six fish from each tank were quickly removed and frozen in liquid nitrogen and stored at -80°C for gene expression analysis.

2.5 Proximate composition analysis

The moisture, crude protein, crude lipid and crude ash contents were analyzed according to the method of the Association of Official Analytical Chemists (47). The moisture content was determined by drying samples in an oven at 105 °C. The crude protein content was determined using the combustion method by the Dumas nitrogen determination apparatus. The crude lipid content was measured using the ether method in the Soxhlet extraction system. The crude ash was determined, and heated sample was carbonized to smokeless and transferred to a muffle furnace at 550°C for 6 h.

2.6 Intestinal histology analysis

Intestinal samples were fixed in the 4% polyformaldehyde (PFA) fixative for 24 h, and then dehydrated in a series of alcohol solutions and embedded in paraffin. The sliced sections (5 μm) of each sample were stained with hematoxylin-eosin (H&E) (Hangzhou Haoke Biotechnology Co., Ltd., Hangzhou, China). The tissue sections were examined using light microscopy with Image-Pro Plus 6 soft-ware (Media Cybernetics, Maryland, USA). It was observed and randomly selected 3 fields of view to take pictures. Villus height (VH), villus width (VW) and muscular thickness (MT) were measured.

2.7 Enzyme activity analysis

The frozen guts were thawed at 4 °C, then homogenized with 9 times saline and then centrifuged at 4000 rpm for 10 min (4 °C). The supernatants were collected to measure enzyme activities within 24 h. The trypsin, lipase, α-amylase activities of the gut were tested using the kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions.

2.8 Gene expression analysis

Total RNA was extracted from the whole intestinal tissue using RNAiso Plus (Takara Biotech, Dalian, China). RNA concentration and purity were measured by the spectrophotometry analysis (A260:A280 nm ratio) within the ratio specified by the kit (1.8-2.2). The RNA was reverse transcribed into cDNA with a reverse transcription kit (TaKaRa, Japan) and stored at -20°C for qRT-PCR analysis. The online Primer3 was used to design the primers used for qRT-PCR of the required genes. The primer sequences are shown in Table 2. Reverse transcription is carried out according to the instructions of TaKaRa RR047A, and real-time fluorescent quantitative PCR is carried out according to the instructions of the TaKaRa RR420A kit. The transcript levels of genes encoding AMP-activated protein kinase α (AMPKα), the target of rapamycin (TOR), 4E-binding proteins (4E-BPs), acetyl-CoA carboxylase (ACC), caspase-3, caspase-8, caspase-9, tumor necrosis factor (TNF), Interleukin-1β (IL-1β) and transforming growth factor-β (TGFβ1 and TGFβ2) were determined. The relative expression levels of target genes were analyzed using the $2^{-\triangle\triangle Ct}$ comparison Ct value method.

2.9 Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) using SPSS 25.0 statistical software (IBM Corp., Armonk, NY, USA). Tukey's test was used for multiple comparisons if the difference between groups was significant ($P<0.05$). Differences were considered significant at $P< 0.05$. All data are expressed as means ± SE.

3 Results

3.1 Growth performance and body composition of fish

As shown in Table 3, there were no significant differences in SR, WG, SGR and HSI among all groups ($P>0.05$). And there were no

TABLE 2 Primers sequences for qRT-PCR of largemouth bass.

Primer name	Primer sequence	Primer type	Primer ID
AMPK-α	CAGAAACACGAAGGCAGGGT	forward	119901030
	GAGACTGCGGATCTTCTGTCTG	reverse	
TOR	GAGGAGGCAGAGAAAGGCTTC	forward	119905919
	TCCCTCCATGCTGCTGATG	reverse	
4E-BP	GCAGGTCAGACCTCCAGGAG	forward	119914828
	CGGTGTAGTGCTGAACAGAGTCC	reverse	
ACC	ATCCCTCTTTGCCACTGTTG	forward	119896220
	GAGGTGATGTTGCTCGCATA	reverse	
caspase-3	GCTTCATTCGTCTGTGTTC	forward	119898808
	CGAAAAAGTGATGTGAGGTA	reverse	
caspase-8	GAGACAGACAGCAGACAACCA	forward	119902447
	TTCCATTTCAGAAAACACATC	reverse	
caspase-9	CTGGAATGCCTTCAGGAGACGGG	forward	119915381
	GGGAGGGGCAAGACAACAGGGTG	reverse	
TNF-α	CTTCGTCTACAGCCAGGCATCG	forward	119897144
	TTTGGCACACCGACCTCACC	reverse	
IL-1β	TGATGAGGGACTGGACCTGG	forward	119914255
	ACCAGGCTGTCCATGATCATG	reverse	
TGFβ1	AGCGCATTGAGGCCATTAG	forward	119882881
	GATGTCTGGTGGGCTCTCG	reverse	
TGFβ2	TGTCGTCCGTGTTCTCCTCAC	forward	119896887
	GTAAATGGCGACAATCTCACG	reverse	
β-actin	GATGGTGGGTATGGGCCAG	forward	119885147
	GAGCCTCTGTGAGCAGGACAG	reverse	

significant differences in FCR between SM group and PM group, while FCR in AGn1, AGn2 and TB0.2 groups was significantly lower than that in SM group (P<0.05). Meanwhile, the VSI of AGn2 group was significantly lower than that of PM and SM groups (P<0.05). As shown in Table 4, there were no significant differences in crude protein, crude lipid, crude ash and moisture among all groups (P>0.05).

3.2 Digestive enzymes activity in the intestine

As shown in Figure 1, compared with the PM group, the activities of lipase and amylase in the foregut, midgut and hindgut, trypsin in the foregut of largemouth bass decreased significantly in SM group (P<0.05). Compared with SM group, lipase and trypsin in the whole intestine increased significantly by the addition of high or low dose of the two additives (P< 0.05 or P<0.01), and the activities of high dose groups were greater than those of low dose groups (P<0.05). In terms of amylase, compared with SM group, both additives significantly increased the activity of amylase in the foregut (P<0.05), but decreased the activity of amylase in the midgut and hindgut (P<0.05), and there were no significant differences in high dose and low dose groups except activities in TB of the midgut (P>0.05).

3.3 Intestinal morphology

The histological features of the foregut, midgut, and hindgut of largemouth bass were presented in Figures 2–4, and the villus height (VH), crypt depth (CD), and mucosal thickness (MT) were shown in Table 5. There were cleavage and necrosis at the top of villus in the foregut of SM group. Compared with the PM group, VH and MT decreased significantly in the foregut, midgut, and hindgut of SM group (P<0.05), while CD increased significantly in the foregut (P<0.05) rather than in the midgut and hindgut. Compared with SM group, VH and MT in the foregut, midgut, and hindgut in the AGn and TB groups increased significantly (P<0.05). In the foregut, in terms of VH and MT, they were obviously higher in AGn groups

TABLE 3 Growth performance of largemouth bass fed with different diets.

Groups	SR (%)	WG (%)	SGR (%/d)	FCR	HSI (%)	VSI (%)
PM	76.67 ± 10.14	185.51 ± 9.80	1.87 ± 0.06	1.17 ± 0.12^{ab}	1.71 ± 0.17	7.56 ± 0.18^{a}
SM	80.00 ± 15.28	179.85 ± 7.95	1.84 ± 0.05	1.30 ± 0.13^{a}	1.85 ± 0.06	7.89 ± 0.21^{a}
AGn1	93.33 ± 4.41	189.05 ± 16.49	1.89 ± 0.10	0.99 ± 0.03^{b}	1.54 ± 0.07	7.77 ± 0.54^{ab}
AGn2	100.00 ± 0.00	212.70 ± 11.58	2.00 ± 0.04	0.88 ± 0.08^{b}	1.05 ± 0.26	6.46 ± 0.49^{b}
TB0.1	80.00 ± 17.56	164.22 ± 14.43	1.73 ± 0.10	1.61 ± 0.32^{a}	1.87 ± 0.08	8.24 ± 0.15^{a}
TB0.2	86.67 ± 10.93	185.28 ± 14.73	1.87 ± 0.09	0.90 ± 0.06^{b}	1.55 ± 0.07	7.31 ± 0.52^{ab}

Survival rate (SR), weight gain (WG), specific growth rate (SGR), feed coefficient rate (FCR), hepatosomatic index (HSI), viscerosomatic index (VSI). The value is expressed as mean ± SE. There were significant differences in the data within the parameters of each column with different lowercase letters (P< 0.05).

than those in TB groups (P<0.05), and in AGn1 group they are higher than those in AGn2 group (P<0.05), but there were no significant differences between TB0.1 and TB0.2 (P>0.05). In the midgut, VH and MT in AGn2 group were significantly higher (P<0.05) and AGn1 were significantly lower (P<0.05) than those in TB0.1 and TB0.2 groups, and there was no significant difference between TB0.1 and TB0.2 (P>0.05). In the hindgut, there was no significant difference in VH and MT among the two additives and different concentration (P>0.05). Compared with PM group, CD in SM group increased significantly in the foregut, but no significant changes in the midgut and hindgut. Compared with SM group, CD in the foregut, midgut, and hindgut reduced significantly in different concentrations of the two additives (P<0.05) except AGn1 in hindgut. In the foregut, CD in TB2 group was significantly lower than that in AGn1, AGn2 and TB1, but no obvious differences were found between AGn1 and AGn2 groups. In the midgut, CD in AGn groups were obviously lower than those in TB groups, but no obvious differences between the two doses of AGn or TB. In the hindgut, CD in the high dose groups were obviously lower than those in low dose groups of the two additives, but no obvious differences between the two low dose groups.

3.4 Genes expression of intestinal immunity

The expression of metabolism and immune related genes in the intestine of largemouth bass was shown in Figures 5, 6. Specifically, mRNA expression levels of TOR and 4E-BP in SM group were significantly lower than those in PM group (P<0.05). (Figures 5A, B). However, the expressions of ACC, caspase, TNF-α, and IL-1β showed opposite trend (P< 0.05) (Figure 5C, Figures 7A–C, Figures 6A, B). Compared with SM group, higher expressions of TOR and 4E-BP were observed in the experimental groups (AGn and TB0.2), while no significant differences were found in TB0.1 group (P > 0.05) (Figures 5A, B). The mRNA expression levels of ACC, caspase, TNF-α, and IL-1β in the experimental groups were significantly lower than those in SM groups (P< 0.05) (Figure 5C, Figure 7, Figures 6A, B). The mRNA expressions of AMPK-α, TGFβ1, and TGF-β2 were not significant different among all experimental groups (P > 0.05) (Figure 5D, Figures 6C, D).

4 Discussion

Supplementing butyrate and glutamine has a positive effect on the growth performance of fish fed with plant-based diet (15, 24). It has been reported that TB could enhance the WG of grass carp (*Ctenopharyngodon idellus*) (43, 48), hybrid grouper (49) and blunt snout bream (*Megalobrama amblycephala*) (44), but had no significant effects on SR, FCR, HSI, VSI and body composition of grass carp (43, 48). Dietary glutamine or alanyl-glutamine increased the WG of hybrid grouper (24), Jian carp (50), Pacific white shrimp (*Litopenaeus vannamei*) (21), yellow catfish (20) and largemouth bass (9, 33). However, in this study, two additives had no significant effects on the growth performance and body composition of largemouth bass fed with high soybean meal. These inconsistent research results may be related to the variety and status of

TABLE 4 Composition of largemouth bass fed with different diets.

Groups	Crude protein %	Crude lipid %	Crude ash %	Moisture %
PM	17.24 ± 0.03	4.48 ± 0.08	3.86 ± 0.03	70.59 ± 0.83
SM	17.01 ± 0.05	4.62 ± 0.05	3.89 ± 0.04	70.81 ± 0.28
AGn1	17.00 ± 0.09	4.51 ± 0.05	3.89 ± 0.04	70.46 ± 0.66
AGn2	17.09 ± 0.11	4.55 ± 0.06	3.95 ± 0.02	70.50 ± 0.60
TB1	16.96 ± 0.08	4.65 ± 0.06	3.88 ± 0.03	71.05 ± 0.46
TB2	16.95 ± 0.06	4.64 ± 0.06	3.87 ± 0.04	70.65 ± 0.68

The value is expressed as mean ± SE. There were significant differences in the data within the parameters of each column with different lowercase letters (P<0.05).

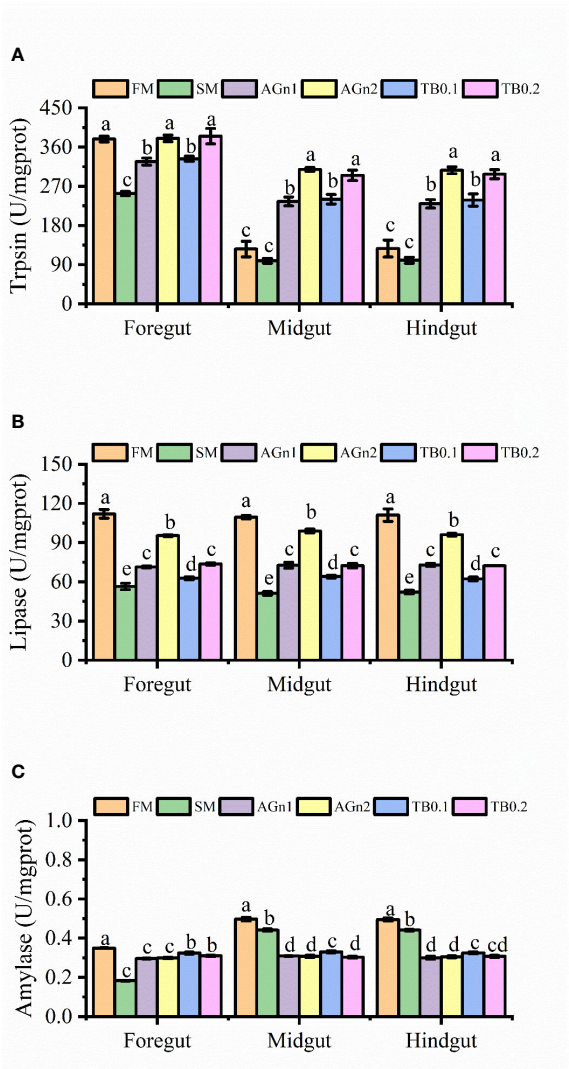

FIGURE 1
The results of digestive enzyme activities **(A)**, trypsin; **(B)**, Lipase; **(C)**, amylase) in fore-, mid- and hindgut of largemouth bass fed with different diets. Data are shown as mean ± SE. Value with different superscripts are significantly different ($P<0.05$).

experimental animals (51). Interestingly, the FCR of largemouth bass decreased after TB or AGn supplementation and the VSI also decreased after AGn addition, which indicated that both additives improved the feed utilization of fish in our study. Similar results were also found in studies of Ala-glutamine on hybrid grouper (24), *Penaeus vannamei* (21), grass carp (52), and on studies of TB in hybrid grouper (49), blunt snout bream (44), grass carp (43). So, the improved FCR may be due to more energy provided by the two additives for intestinal tract, further improving the health of intestine.

The morphology and structure of animal intestine can affect the absorption and utilization of nutrients as well as the growth and development of animals, among which the thickness of intestinal mucosa, villus height and crypt depth are usually used as indicators of intestinal digestion and absorption capacity (29, 53). Studies have shown that feeding high SM to aquatic animals causes damage to intestinal epithelial cells (6, 54, 55). Due to a variety of anti-nutritional factors contained in plant components, dietary plant proteins usually disrupt the digestibility of fish, resulting in reduced intestinal absorption (30, 33, 41, 51). In this study, the gut of largemouth bass, especially the foregut, was damaged in the fish fed soybean meal, and the supplementation of two additives, especially Ala-glutamine, showed obviously improvement on the intestinal barrier. Similar to the results of this study, intestinal damage occurred in black sea bream cultured by high SM diet, which was manifested as reduced intestinal villi height, disruption and atrophy (32). However, intestinal structure was improved when butyrate glycerides and TB were added to the feed (30, 56). The intestinal villus height of grass carp (43), yellow drum (31), yellow catfish (29) and Mirror carp (26) increased significantly after adding appropriate amount of TB in the diet. Dietary supplementation of glutamine or dipeptide products had positive effects on the mucosal thickness and crypt depth of hybrid grouper (24), turbot (*Scophthalmus maximus*) (57) and grass carp (52). The improvement of intestinal structure and morphology may be due to the fact that TB and AGn are the main energy sources of intestinal epithelial cells (15, 58), which can provide energy for intestinal epithelial cells and stimulate their proliferation and differentiation, especially when the intestinal tract is damaged (34, 59, 60). In addition, the present study showed that AGn was superior to TB in improving the intestinal morphology of largemouth bass, especially in the fore- and midgut, and 2% AGn was the most effective. In another study, by adding 0.6%-0.9% AGn in the high-soybean meal diet, the intestinal villus height of largemouth bass was improved, but growth performance was not improved (33). So, the optimal dose of Ala-glutamine in largemouth bass to improve both intestinal structure and growth needs to be further studied.

The activity of digestive enzymes in fish digestive tract is one of the important indexes reflecting the ability of aquaculture animals to digest nutrients in feed (29). In this experiment, a high proportion of SM decreased the activities of lipase and trypsin in the fore-, mid- and hindgut of largemouth bass, and the supplementation of AGn and TB effectively increased the intestinal activities of the two enzymes. This is consistent with the results of studies which added triglyceride to the diet of black sea bream (32), carp (30), yellow catfish (29) and Pacific white shrimp (61), as well as those which supplementation of glutamine or dipeptide to the diet of grass carp (52), Jian carp (50) to improve intestinal trypsin and lipase. The addition of butyrate products increases the activity of digestive enzymes in fish, which may be related to organic acids, such as butyrate, which can reduce the pH of chyme and thus greatly stimulate the secretion of digestive enzymes (62). As the main energy substance of gut (63), glutamine promotes the proliferation of intestinal mucosal cells and the repair of damaged intestinal epithelial cells; in addition, it may also promote the development of pancreatic tissue, an important organ secreted by digestive enzymes (18). The high SM diet decreased the intestinal amylase activity of largemouth bass, but the amylase activity was only increased in the foregut and decreased in the midgut and hindgut when the dietary AGn or TB was supplemented. This is consistent with the study that amylase

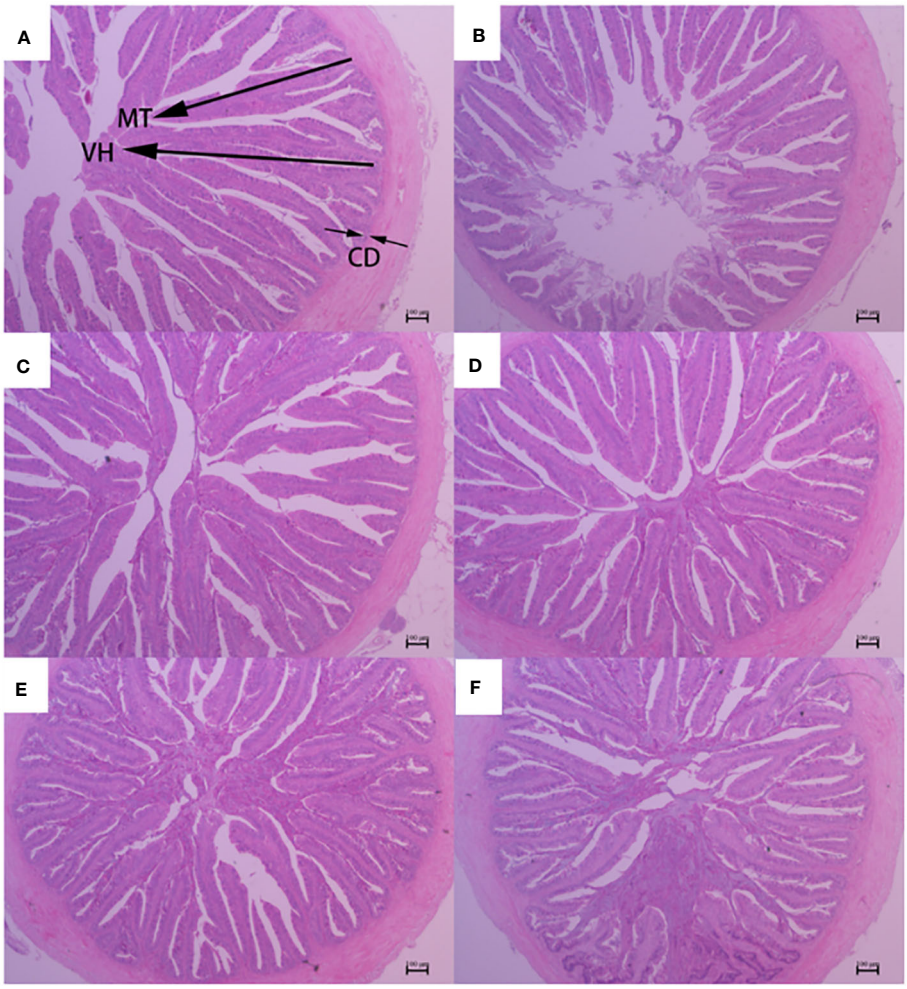

FIGURE 2
Foregut morphology of largemouth bass fed diets containing different levels of AGn and TB for 8 weeks. **(A)**; PM group, **(B)**; SM group, **(C)**; AGn1 group, **(D)**; AGn2 group, **(E)**; TB0.1 group, **(F)**; TB0.2 group. VH, villus height, CD, crypt depth, MT, mucosal thickness. (HE staining, original magnification ×40). The arrows indicate the positions of MT (mucosal thickness), VH (villus height) and CD (crypt depth).

activity was enhanced by supplementing TB in the diets of young black bream (41) and snakehead (28), and glutamine dipeptide in the diets of grass carp (52). However, this is contrary to the result of grass carp supplemented TB (43). These differences may be due to the low intestinal amylase activity of fishes, especially carnivorous fishes (64).

In addition to the capacity of digestion and absorption, the intestine is the largest immune organ of fish. Previous studies have shown that the use of SM has caused some intestinal health problems in fish, such as impaired intestinal development, inflammation and oxidative damage, but there are few studies on whether TB and AGn can solve these problems in fish. Antinutritional factors in SM are highly immunogenic (2), and are easy to induce immune response, leading to fish intestinal inflammation (65, 66). Both IL-1β and TNF-α are proinflammatory cytokines, while TGF-β1 and TGF-β2 are anti-inflammatory cytokines (67). During cellular oxidative stress, both TNF-α and IL-1β secretes inflammatory cytokines, causing neutrophil aggregation and activating redox sensitive transcription factors, leading to increased inflammatory response and tissue damage (29, 31, 34). In this experiment, expression levels of TNF-α and IL-1β were upregulated in the SM group, but significantly downregulated after TB/AGn supplementation. This indicated that the high proportion of soybean meal did cause intestinal damage, and TB/AGn could effectively inhibit the inflammatory process caused by high level SM in the intestine of largemouth bass. It is generally believed that the inflammation is caused by the anti-nutritional factors contained in soybean meal (30, 51). Studies in this aspect have been confirmed in the studies of various fish species such as yellow drum (*Nibea albiflora*) (31), common carp (30), black sea bream (56), turbot (68), and hybrid grouper (24). Many studies have shown that dietary supplementation of TB or AGn has a protective effect on intestinal inflammation in both terrestrial (17) and aquatic animals (24, 30). Studies have shown that adding a moderate amount of TB to a high plant protein diet can down-regulate the intestinal proinflammatory factors TNF-α and IL-1β in yellow drum (31) and common carp (30). The suppression of the

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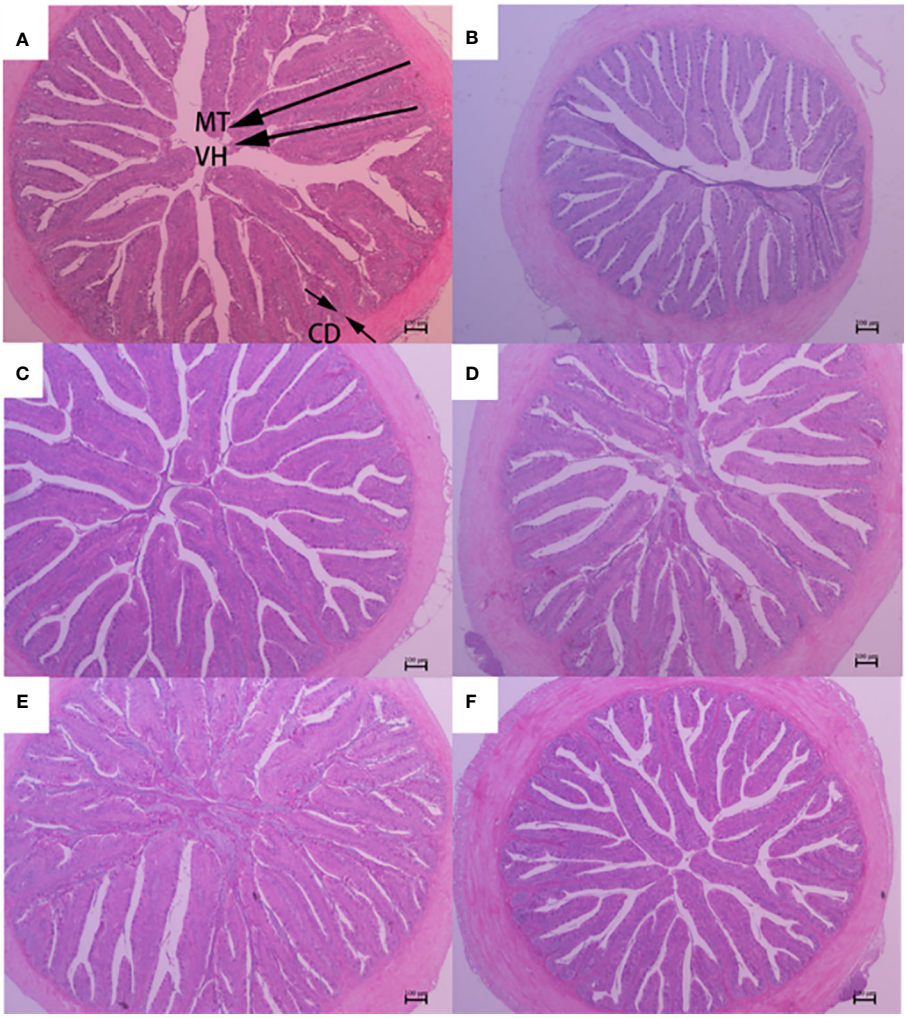

FIGURE 3
Midgut morphology of largemouth bass fed diets containing different levels of AGn and TB for 8 weeks. **(A)**; PM group, **(B)**; SM group, **(C)**; AGn1 group, **(D)**; AGn2 group, **(E)**; TB0.1 group, **(F)**; TB0.2 group. VH, villus height, CD, crypt depth, MT, mucosal thickness. (HE staining, original magnification ×40). The arrows indicate the positions of MT (mucosal thickness), VH (villus height) and CD (crypt depth).

proinflammatory response by TB or Ala-Gln supplementation in soybean meal-based diets may be related to the improvement of the gut physical barrier, as a developed gut physical barrier which protects the gut from pathogenic microorganisms (33, 49). However, no significant differences between TGF-β1 and TGF-β2 anti-inflammatory cytokines was observed in our study, which may be because the TGF was not sensitive to such responses. Similar results were also found in the studies of yellow drum (31).

Apoptosis plays an important role in maintaining homeostasis in the organism. Intestinal inflammation is usually accompanied by an increase in apoptosis (69). Cysteine protease (caspase) is a key enzyme, that causes cell apoptosis and its apoptosis regulatory pathways, include mitochondrial pathway, death receptor pathway and endoplasmic reticulum pathway. Once this signal transduction pathway is activated, caspase will be activated, followed by apoptosis protease cascade reaction. Previous studies have shown that glutamine attenuated the apoptosis induced by 2,4,6-trinitrobenzene sulfonic acid in the rat colon (70), and intestinal caspase-3 and -9 activities of hybrid grouper were significantly lower in 2% Ala-Gln group (33). This is consistent with the current study, implying that glutamine and Ala-Gln both have a potential apoptosis prevention effect by reducing caspase activities. In the case of tributyrin, studies have shown that adding 0.08% TB to a high plant protein diet can downregulate the apoptosis genes(caspase-2 and caspase-8)in hybrid grouper (49). Similar results were also observed in the current study, these apoptosis-related factors caspase-3, -8 and -9 were significantly upregulated in SM group, but downregulated in the experimental groups. Intestinal inflammation is usually accompanied by an increase in apoptosis (69), so the intestinal damage may be alleviated by the decreased expression of genes related to apoptosis after TB/AGn supplementation. This shows that compared with peanut meal, SM can significantly accelerate the apoptosis of intestinal cells of largemouth bass, and TB and AGn supplementation can alleviate the damage of SM to intestinal cells of largemouth bass by inhibiting mitochondrial apoptosis pathway.

Studies in aquatic animals have shown that similarly to butyric acid, glutamine and its dipeptide can downregulate the mRNA

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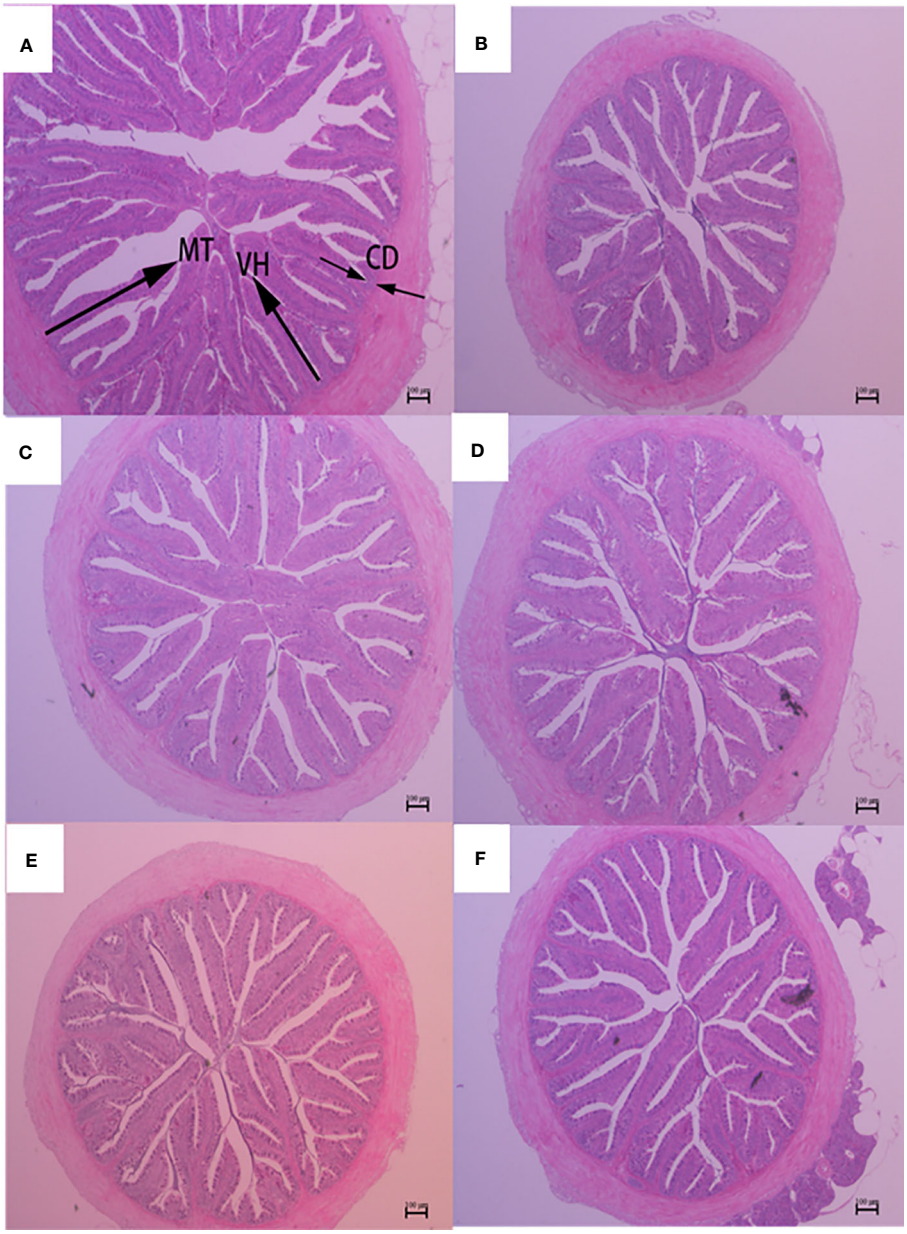

FIGURE 4
Hindgut morphology of largemouth bass fed diets containing different levels of AGn and TB for 8 weeks. **(A)**; PM group, **(B)**; SM group, **(C)**; AGn1 group, **(D)**; AGn2 group, **(E)**; TB0.1 group, **(F)**; TB0.2 group. VH, villus height, CD, crypt depth, MT, mucosal thickness. (HE staining, original magnification ×40). The arrows indicate the positions of MT (mucosal thickness), VH (villus height) and CD (crypt depth).

expression of TNF-α and IL-1β in the intestine of hybrid grouper (24) and turbot (68), and effectively inhibit the fish enteritis induced by soybean meal. The nuclear factor NF-κB is an important regulator of gene expressions involved in inflammation. It induces inflammatory cytokines and recruits immune cells *via* translocation to the nucleus (71). Activation of NF-κB may lead to inflammation and hyperpermeability of intestinal epithelial cells. These results demonstrate that dietary glutamine probably enhances the intestinal immune defense to alleviate enteritis by inhibiting the MyD88/NF-κB pathway (24). In addition, medical studies have shown that the therapeutic effects of sodium butyrate and AGn on respiratory tract inflammation are exerted by affecting signaling pathways including AMPK, TOR, NF-κB and STAT3. This revealed that glutamine and its dipeptide and butyrate products may have a similar mechanism of action in the treatment of certain inflammations (17). However, the exact mechanism and the involved signal pathway underlying the anti-inflammatory role of these two supplements in fish with soybean meal induced enteritis are not clear and warrant further investigation. Adenylate activated protein kinase (AMPK), whose activity is regulated by many factors such as body energy status, is known as the "cell energy regulator" (72, 73). AMPK is activated by sensing changes in AMP/ATP and ADP/ATP ratios in cells, and promotes catabolic processes of ATP production by inhibiting the anabolic processes of ATP

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TABLE 5 Intestine morphology indexes of largemouth bass fed with different diets.

Tissues	Groups	Villus height (μm)	Crypt depth (μm)	Mucosal thickness (μm)
Foregut	PM	1015.58 ± 1.11^{c}	30.66 ± 0.96^{b}	1049.49 ± 0.92^{b}
	SM	871.04 ± 18.98^{e}	41.60 ± 2.32^{a}	915.59 ± 16.71^{d}
	AGn1	1037.50 ± 2.28^{a}	28.20 ± 0.80^{b}	1068.34 ± 2.60^{a}
	AGn2	1020.00 ± 1.62^{b}	26.28 ± 1.03^{b}	1048.83 ± 0.91^{b}
	TB0.1	984.83 ± 4.73^{d}	25.82 ± 0.64^{b}	1013.56 ± 4.51^{c}
	TB0.2	953.03 ± 8.54^{d}	21.01 ± 0.95^{c}	977.75 ± 8.96^{c}
Midgut	PM	855.50 ± 21.58^{b}	47.06 ± 1.37^{a}	900.77 ± 21.57^{b}
	SM	557.82 ± 31.01^{d}	42.69 ± 0.87^{a}	598.25 ± 31.37^{d}
	AGn1	765.88 ± 5.49^{c}	30.37 ± 1.32^{c}	798.91 ± 6.36^{c}
	AGn2	1001.75 ± 24.60^{a}	25.11 ± 1.90^{c}	1052.34 ± 25.43^{a}
	TB0.1	857.00 ± 6.54^{b}	38.16 ± 0.76^{b}	898.38 ± 6.21^{b}
	TB0.2	855.89 ± 6.37^{b}	36.71 ± 1.06^{b}	896.37 ± 5.96^{b}
Hindgut	PM	547.99 ± 1.66^{b}	51.52 ± 2.93^{a}	578.56 ± 0.97^{b}
	SM	509.46 ± 4.66^{c}	53.25 ± 3.13^{a}	559.31 ± 2.50^{c}
	AGn1	654.64 ± 13.42^{a}	47.90 ± 2.54^{a}	705.28 ± 15.93^{a}
	AGn2	734.28 ± 23.55^{a}	27.47 ± 1.05^{c}	788.92 ± 26.38^{a}
	TB0.1	730.90 ± 22.97^{a}	34.47 ± 1.29^{b}	768.20 ± 23.79^{a}
	TB0.2	673.45 ± 16.02^{a}	25.11 ± 1.90^{c}	729.37 ± 18.94^{a}

The value is expressed as mean ± SE. There were significant differences in the data within the parameters of each column with different lowercase letters (P<0.05).

consumption, thus restoring energy balance (74). Acetyl coenzyme A carboxylase (ACC) is the rate limiting enzyme for fatty acid synthesis, which is the main target protein of AMPK regulating fatty acid oxidation (75, 76). When cells are under stress or energy consumption increases and the body energy level is low, the intracellular AMPK content increases, which reduces ACC activity, thereby reducing fatty acid synthesis and improving fatty acid decomposition to meet the demand for energy (77, 78). In this study, the addition of TB and Ala-Gln to soybean meal diets did not affect AMPK expression. The possible reason was that ATP levels did not significantly change during the experiment, and the changes in AMP/ATP and ADP/ATP were not sufficient to affect AMPK phosphorylation. Compared with SM group, the transcription and expression of ACC in intestine of largemouth bass decreased significantly in PM and experimental groups, which was consistent with the research results of broilers (79) and piglets (80). These results indicated that the intestinal damage of largemouth bass in SM group was more serious than that of PM group, and supplementation of TB and Ala-Gln could significantly reduce the intestinal damage caused by soybean meal. As the target protein of rapamycin, TOR is also one of the target proteins regulated by AMPK. Studies have shown that the regulation of nutrient metabolism in animals is all involved in TORC1, a complex formed by TOR (81, 82). TORC1 regulates the overall translation level and regulates protein synthesis and metabolism through its downstream phosphorylation of S6K1 and 4E-BP1 (82). It has been proved that the addition of glutamine increases protein synthesis by increasing phosphorylated TOR and its main downstream effective substrates S6K1 and 4E-BP1 (74, 83, 84). In this study, the PM, AGn and TB0.2 groups all upregulated the expression of TOR and 4E-BP, indicating that they could help the intestine of largemouth bass to deposit protein.

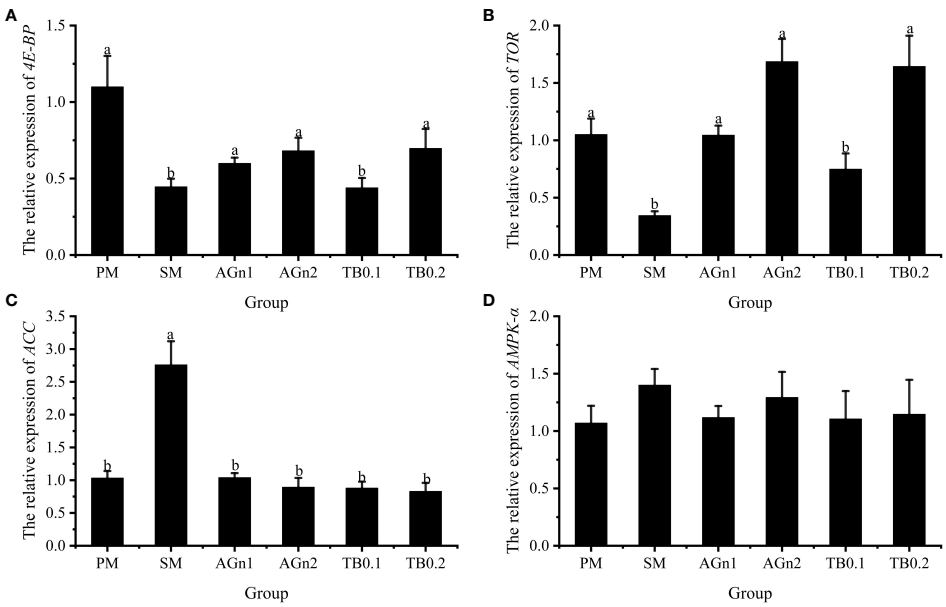

FIGURE 5
Effects of different treatments on the mRNA levels of 4E-BP **(A)**, TOR **(B)**, ACC **(C)** and AMPK-α **(D)** in intestines of largemouth bass. Data are shown as mean ± SE. Value with different superscripts are significantly different (P< 0.05).

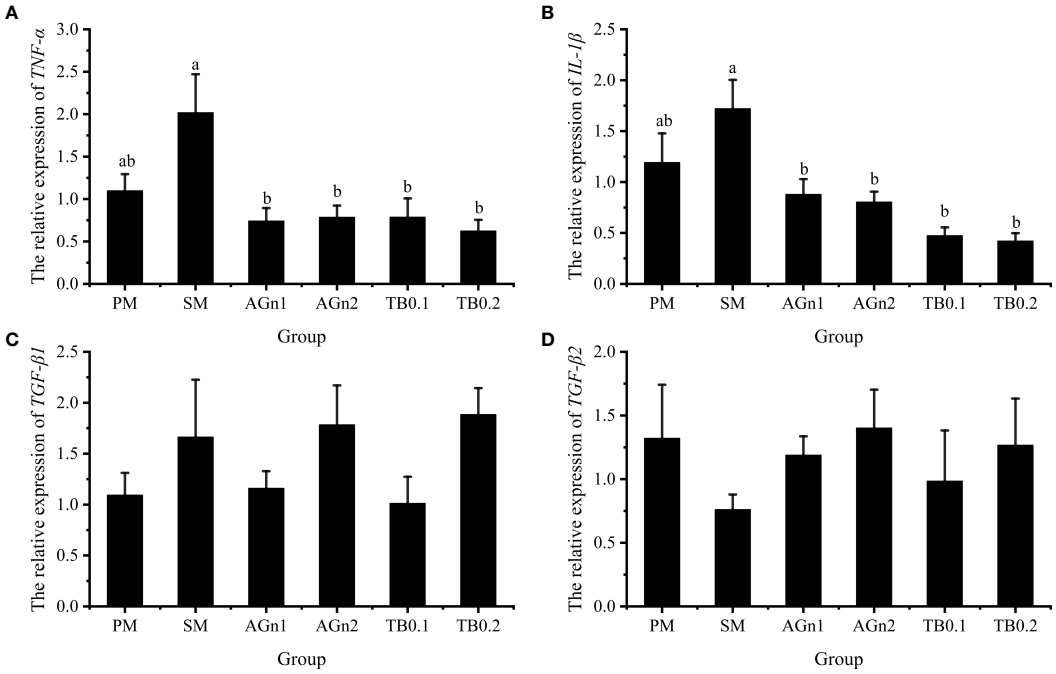

FIGURE 6
Effects of different treatments on the mRNA levels of TNF-α **(A)**, IL-1β **(B)**, TGFβ1 **(C)** and TGFβ2 **(D)** in intestines of largemouth bass. Data are shown as mean ± SE. Value with different superscripts are significantly different (P<0.05).

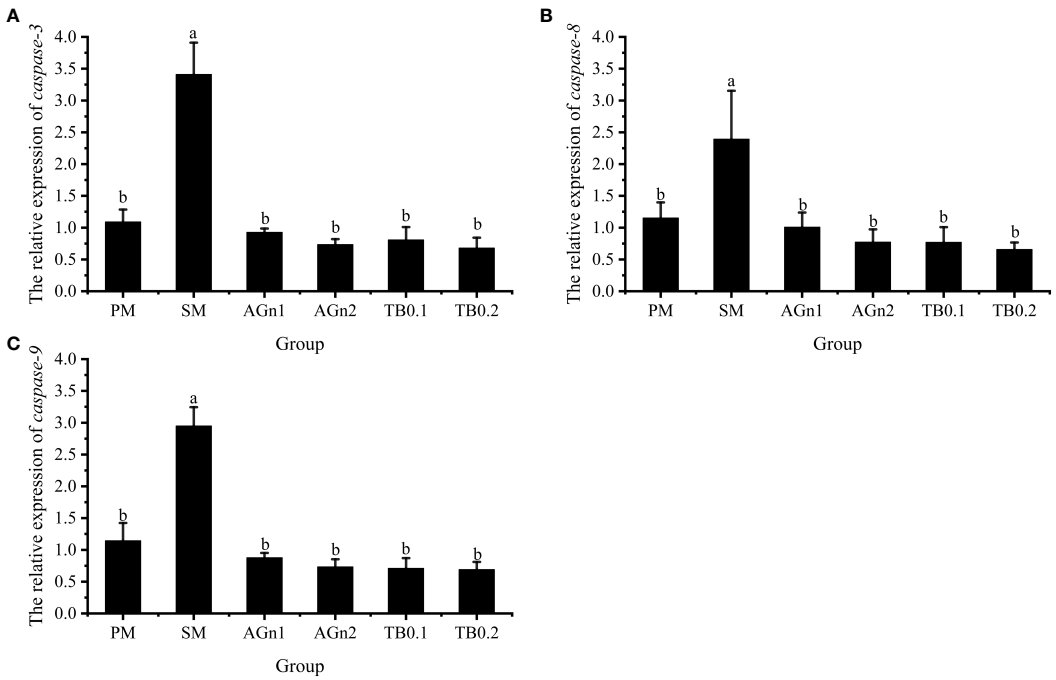

FIGURE 7
Effects of different treatments on the mRNA levels of caspase3 **(A)**, caspase8 **(B)** and caspase9 **(C)** in intestines of largemouth bass. Data are shown as mean ± SE. Value with different superscripts are significantly different (P<0.05).

5 Conclusion

To sum up, high proportion of soybean meal induces intestinal inflammation, damages intestinal structure and reduces the activities of digestive enzymes of largemmouth bass. Fortunately, the intestinal inflammation was alleviated by the addition of TB and AGn in the feed. The recommended level is 2% AGn and 0.2% TB. This study helps to promote the application of TB and AGn as functional feed additives in aquatic feed in the aspects of theories and practices.

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 Animal Experiment Ethics Committee of Huzhou University. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

JZ project administration, writing the original draft preparation, and formal analysis. XY and ZQ, formal analysis and investigation. RZ, experiment design and management. HX, investigation. TW, writing the manuscript and critical revision. All authors contributed to the article and approved the submitted version.

Funding

This work was financially supported by the Natural Science Foundation of China, NSFC (31972800), and the China Agriculture Research System of MOF and MARA.

Acknowledgments

The authors would like to express their sincere thanks to the personnel of these teams for their kind assistance. The authors also thank the kind people who gave us help and advice in the process of experiment and article writing and publishing.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 25 May 2023
DOI 10.3389/fimmu.2023.1158390

OPEN ACCESS

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RECEIVED 03 February 2023
ACCEPTED 09 May 2023
PUBLISHED 25 May 2023

CITATION
Rawling M, Schiavone M, Apper E, Merrifield DL, Castex M, Leclercq E and Foey A (2023) Yeast cell wall extracts from *Saccharomyces cerevisiae* varying in structure and composition differentially shape the innate immunity and mucosal tissue responses of the intestine of zebrafish (*Danio rerio*). *Front. Immunol.* 14:1158390. doi: 10.3389/fimmu.2023.1158390

Yeast cell wall extracts from *Saccharomyces cerevisiae* varying in structure and composition differentially shape the innate immunity and mucosal tissue responses of the intestine of zebrafish (*Danio rerio*)

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With the rising awareness of antimicrobial resistance, the development and use of functional feed additives (FFAs) as an alternative prophylactic approach to improve animal health and performance is increasing. Although the FFAs from yeasts are widely used in animal and human pharma applications already, the success of future candidates resides in linking their structural functional properties to their efficacy *in vivo*. Herein, this study aimed to characterise the biochemical and molecular properties of four proprietary yeast cell wall extracts from *S. cerevisiae* in relation to their potential effect on the intestinal immune responses when given orally. Dietary supplementation of the YCW fractions identified that the α-mannan content was a potent driver of mucus cell and intraepithelial lymphocyte hyperplasia within the intestinal mucosal tissue. Furthermore, the differences in α-mannan and β-1,3-glucans chain lengths of each YCW fraction affected their capacity to be recognised by different PRRs. As a result, this affected the downstream signalling and shaping of the innate cytokine milieu to elicit the preferential mobilisation of effector T-helper cell subsets namely Th17, Th1, Tr1 and FoxP3^{+}-Tregs. Together these findings demonstrate the importance of characterising the molecular and biochemical properties of YCW fractions when assessing and concluding their immune potential. Additionally, this study offers novel perspectives in the development specific YCW fractions derived from *S. cerievisae* for use in precision animal feeds.

KEYWORDS

α-mannans, yeast cell wall polysaccharides, Bioactivities, zebrafish, β-1,3-glucans

1 Introduction

The development of nutritional solutions to improve the health and welfare of aquatic and terrestrial farmed species is at the forefront of contemporary research interests. Indeed, the desire to "prevent rather than cure", and to minimize the use of antibiotics, are driving the development of functional feed additives (FFAs) technologies such as prebiotics, probiotics and postbiotics to improve animal health and performance. Further success in the implementation of FFA technologies in precision animal feeds reside in the finer characterisation of their specific functional properties *in vivo*. In particular, understanding the interaction between mucosa-associated lymphoid tissues (MALT) and the FFA is critical.

The intestinal environment is largely exposed to self, commensal and potentially pathogenic non-self antigens that are constantly screened and processed by the diverse immune cells of the gut-associated lymphoid tissue (GALT), suggesting a central role of GALT in intestinal immune response during homeostasis (1–3). Accordingly, the gastrointestinal system contains substantial amounts of organised lymphoid tissues with large populations of scattered innate and adaptive effector immune cells. Indeed, in humans nearly 70% of the immune system is housed in the gastrointestinal tract (GIT) where it interacts with gastrointestinal function in a dynamic manner (4); for instance, directing the immune response towards the luminal contents allowing for either tolerance or degradation of luminal antigens. The immune mechanisms implicated in this action are very complex and are governed by both innate and adaptive immunity. Innate immunity is an immediate response that is indispensable before a specific adaptive immunity is mobilised, that requires a longer period to be efficacious (5). In teleost fish immune system, most of the innate immune functions are similar to mammals but there are some differences in the structure of GALT (6–8). Although there is no clear evidence that teleost fish have Peyer's patches and mesenteric lymph nodes, the presence of innate immune cells, TCR$\gamma\delta^+$ T-cells and B1-B cells in and under the gut epithelium across the entire intestinal tract (9–13) make teleost fish a valid comparative model to higher vertebrates.

Zebrafish (*Danio rerio*) is a good comparative model to investigate the mode of action of functional feed compounds on mucosal immune responses due the high presence (~70%) of human orthologous genes including genes associated with diseases (14). Indeed, counterparts of mammalian pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs), as well as downstream signalling components, have been demonstrated to play important roles in zebrafish host defence (15). Furthermore, upon comparison of the zebrafish GIT with that of the stickleback, mouse and human, Lickwar and colleagues (6) reported a core set of genes that were highly expressed in all vertebrate intestinal epithelial cells (IECs) and displayed functional conservation across a broad range of IEC biology involved in lipid transport, metabolism, response to microbes and inflammation. Moreover, from a cellular level zebrafish have an abundance of innate immune cells that interplay with γδT-cells in the recognition and processing of antigens that cross the luminal barrier of the intestine (16).

Within the last decades, FFAs derived from *Saccharomyces cerevisiae* yeast have become among the most widely used functional ingredients in animal and human feed. *S. cerevisae* yeast cell wall (YCW) holds an important position among multifarious prebiotics and have been implicated to suppress the adhesion of potentially pathogenic bacteria *via* GIT agglutination (17, 18) and exert beneficial effects on growth performance, intestinal structure and immunity as well as gut microflora in farmed and companion animals (19–21). *S. cerevisae* is composed of 90% of polysaccharides typically consisting of 30 to 40% mannans and 50 to 60% of highly branched β-1,3/1,6-glucans. Mannans, which constitute the outermost layer of the cell wall, have a complex structure that includes α-1,6-1,3-1,2-mannosyl linkages and are covalently attached to the cell wall proteins *via* either asparagine (N-linked mannans) or serine/threonine (O-linked mannan) residue to form mannoproteins (22). β-glucans, which are found beneath the mannans layer, consist of long β-1,3-glucose polymer with β-1,6-glucan side branches (23). Mannans and β-glucans are documented to be recognised by different PRRs such as C-type lectins including Dectin 1, 2 and 3, DC-SIGN and Mannose-binding receptor; and Toll-like receptors (24).

YCWs are highly diverse in their biochemical composition, molecular structure and cell wall architecture which can vary with the yeast species and strain, the growth conditions of the yeast and the YCW production process (25). Such diversity in the biophysical properties of the YCW can be expected to elicit distinct host-immune responses but this has, to date, largely been under-evaluated. Therefore, contemporary research on the use of yeast-based technologies requires more effort to link the structural characteristics of YCW with their subsequent functional effects *in vivo*.

The current investigation aimed at characterising the biochemical and molecular properties of four proprietary *S. cerevisiae* YCW fractions of discrete origins in relation to their potential effect on the intestinal immune responses when administered orally. Our hypothesis was that the immune properties of a given *S. cerevisiae* YCW varies with its structural and molecular characteristics which, to the authors' knowledge, has not been previously addressed using a purposely designed comparative study. This information will facilitate the development of specific YCW fractions eliciting targeted immune functionalities for use in precision animal nutrition.

2 Materials and methods

2.1 Experimental system and fish

Experimentation was carried out at the Aquaculture and Fish Nutrition Research Aquarium, University of Plymouth (Plymouth, UK) within an indoor freshwater recirculated aquaculture system (RAS) equipped with mechanical and biological filtration, aeration, photo-and-thermo control. The RAS system consisted of 15 rectangular fibreglass tanks (15 litre/tank) each set with a water flow rate of 15 litre/hr. Wild type zebrafish stocks were originally sourced

from the European zebrafish resource centre (EZRC, Germany); F2 generations were bred from the original stock and used in the experiments. For this trial, 375 fish were randomly distributed into the experimental system (25 fish/tank, initial mean body-weight (BW_i) = 0.80 ± 0.02 g) at the beginning of the trial. During the trial, fish were kept under a constant 12:12 hr light:dark photoperiod and water quality parameters were maintained within a suitable range for zebrafish as follows: water temperature = 25 ± 0.5 °C, pH = 6.8 to 7.5, dissolved oxygen = 7.5 to 8.0 mg/l, total ammonia = 0.04 to 0.08 mg/l, nitrite = 0.02 to 0.06 mg/l and nitrate = 54 to 58 mg/l. Approval was given by the University of Plymouth's animal ethical review board under number: ETHICS-32-2019.

2.2 Experimental diets

A basal diet was formulated (36% crude protein and 8% crude lipid) using feed formulation software (Feedsoft®) to meet the known nutritional requirements of cyprinids (26). The test diets were produced by supplementing, prior to cold press, the mash basal diet with one of four YCW fractions (YCW1, 2, 3 or 4; Lallemand SAS, Blagnac, France) at 2.0 kg/T of feed each (Table 1). Each YCW fraction was provided in the form of a pure, dry (moisture ≤ 8%), fine, light tan powder. The diets were produced by mechanically stirring the ingredients into a homogenous mixture using a Hobart food mixer (Hobart Food Equipment, Australia, model no: HL1400 — 10STDA mixer). Warm water was added to reach a consistency suitable for cold press to form 1 mm pellets (PTM Extruder system, model P6, Italy). Each diet was then dried, ground and sieved to isolate pellets of ϕ600-800 µm for the trial. The nutritional profile of the diets was determined according to AOAC protocols (27).

2.3 Experimental design and feeding

The trial lasted 5 weeks during which zebrafish were fed one of five diets in triplicate tanks: 1) Control (basal diet), 2) YCW1, 3) YCW2, 4) YCW3, and 5) YCW4. Fish were hand-fed at 4.0% biomass per day distributed in three equal meals (0900, 1300 and 1600 h). Biomass per tank was estimated daily based on predicted growth rate and adjusted weekly by bulk-weighing each tank following a 24 h starvation period.

2.4 Sampling schedule

At the end of the trial, 9 fish per tank (27 fish/treatment) were randomly netted and euthanized following UK Home Office schedule 1 procedures prior to sampling under a microdissection microscope. Among these, 3 fish per tank (9 fish/treatment) were

TABLE 1 Formulation (g/kg) and proximate composition (%) of the experimental diets.

	Control diet	YCW diets
Formulation (g/kg)		
Soybean meal dehulled[1]	150.0	150.0
Soy Protein Concentrate (SPC60)	154.0	154.0
Sunflower meal	220.0	220.0
Wheat meal	166.4	166.4
Fabameal	220.0	220.0
Rapeseed oil	59.2	59.2
Vitamin and mineral premix[2]	10.0	10.0
Lysine HCL[3]	9.3	9.3
DL-methionine[3]	8.3	8.3
Gelatin[3]	10.0	10.0
Yeast cell wall fraction[4]		2.0

Proximate composition	Control	YCW1	YCW2	YCW3	YCW4
Dry matter (DM; %)	94.8 ± 0.9	94.4 ± 0.5	94.7 ± 0.4	94.7 ± 0.1	94.5 ± 0.3
Crude protein (% DM)	36.4 ± 0.6	36.4 ± 0.5	36.2 ± 0.1	36.3 ± 0.4	36.5 ± 0.1
Crude lipid (% DM)	6.4 ± 0.4	6.5 ± 0.2	6.6 ± 0.2	6.8 ± 0.3	6.7 ± 0.2
Ash (% DM)	4.3 ± 0.2	4.2 ± 0.1	4.2 ± 0.1	3.6 ± 0.5	3.9 ± 0.4

[1]HP-110, Hamlet Protein A/S (Horsens, Denmark): crude protein 57.5%; ash 6.8%; moisture 6.5%; lipid 2.5%).
[2]Premier Nutrition (Rugeley, UK): Calcium 12.1%, magnesium 1.6%, phosphorous 0.5%, vit A 1.0µg/kg, vit D3 0.1 µg/kg, vit E (as alpha tocopherol acetate) 7,000 mg/kg, copper (as cupric sulphate) 250.0 mg/kg, ash 78.7%.
[3]Sigma-Aldrich (Poole, UK).
[4]Lallemand SAS (Blagnac, France).

dissected for histology as follows. Posterior intestinal (PI) samples were excised and digesta was removed using phosphate buffer saline (pH 7.2, Sigma Aldrich, UK), fixed in 10% neutral buffered formalin (pH 7.0; Sigma Aldrich, UK) kept at 4°C for 24 h followed by long-term storage in 70% ethanol at room temperature until processing.

The remaining 6 fish per tank were sampled for gene expression analysis as follows. PI samples were excised and stored in 500 µl of RNA later solution (Applied Biosystems, UK) and kept at 4°C for 24 h then at -80°C until processing. For each biological replicate, PI from 3 fish per tank were pooled together giving 2 samples per tank (6 samples/treatment).

2.5 Biochemical composition of YCW

The biochemical composition of the YCW samples was determined by the sulfuric acid method according to the protocol of (28), followed by analysis of the released monosaccharides (glucose and mannose) to determine mannans and β-glucans content as the sum of β-1,3 and β-1,6-glucans, respectively. Sugars monosaccharides (mannose and glucose) were analysed by high performance liquid chromatography with an evaporative light scattering detector (Varian-385-LC ELSD). A Rezex^{TM} RCM-Monosaccharide Ca+2 column (300 x 7.8 mm; Phenomenex) was used to separate monosaccharides at 80°C by an isocratic elution for 20 min at 0.6 mL/min with ultrapure water.

2.6 Scanning electronic microscopy (SEM) and Atomic Force Microscopy (AFM) imaging of YCW

YCW samples were imaged by SEM on a SEM Quanta 250 FEG FEI at 10 kV. The surface of the YCW was analysed by a Nanowizard III Atomic Force Microscope (AFM; Bruker) after immobilisation of yeast cells by mechanical trapping into polydimethylsiloxane (PDMS) stamps (29). Single-molecule force spectroscopy experiments were carried out using MLCT probes (Bruker) with a 0.002 $N.m^{-1}$ spring constant, that were functionalised with concanavalin A (ConA; Sigma-Aldrich) and a mouse monoclonal anti-β-1,3-glucan (Biosupplies) as described by Schiavone and colleagues (30). Force mapping were obtained by recording 1024 force-distance curves on each cell (at least 8 cells were analysed per YCW). The binding was calculated as the percentage of retract curves presenting adhesion events on the total of force-distance curves analysed (n=8192). Force curves with adhesion force <20 pN or a distance tip-sample (rupture length) of zero were considered as non-adhesive curves. All the curves were analysed with JPK Data Processing software (Bruker-JPK Instruments). The distance required to break the interaction between ConA or anti-β-1,3-glucan at the apex of the AFM tip and the α-mannan or β-1,3-glucan respectively at the yeast cell surface was measured and used to determine the contour length of the corresponding polysaccharides.

To characterize the stretching of polysaccharides at the surface of the cell, elongation forces on the force-distance curves were analysed with the worm-like chain (WLC) model introduced in Bustamante and colleagues (31), which describes the polymer as a curved filament. The contour length from this model represents the length of the polysaccharide stretched or unfolded. All adhesion and contour length values were considered for the histograms, which were generated using OriginPro version 2020 (OriginLab, Northampton, MA) and fitted with a Gaussian curve to obtain the most probable value of the adhesion force and the length of α-mannan or β-1,3-glucan unfolded for each YCW.

2.7 Intestinal morphometry by light microscopy

Formalin-fixed PI samples were dehydrated in a gradient ethanol series (Leica TP1020), embedded in paraffin wax for longitudinal sectioning at 3 µm thickness (Leica RM2235 microtome). Multiple consecutive sections for each sample were stained with haematoxylin and eosin (H&E) to assess muscularis thickness (MT), mucosal fold height (VL), lamina propria width (LPW), and intraepithelial leukocyte abundance (IELs) per 100 enterocytes after Rawling and colleagues (32). Alcian blue-period acid Schiff (AB-PAS) stain was used to assess goblet cell density (GCD) and goblet cell mucin chemotype after Rawling and colleagues (32). Quantitative measurements of each image were taken using Image 'J' 1.47v software (National Institutes of Health, USA).

2.8 Transcriptomic analysis

RNA extraction and cDNA synthesis were performed according to Rawling and colleagues (28). Briefly, 20 mg PI sample was transferred into a microcentrifuge tube containing 1 ml of TRI reagent and homogenised using ceramic beads for 40 sec on the FastPrep-24 5^{G} machine following the manufacturer's instructions (MP Biomedical, EU). A 200 µl volume of chloroform was added, mixed and centrifuged (12,000 x *g*; 15 min; 4°C). The supernatant was removed, and 500 µl of isopropanol was added and centrifuged (14,000 x *g*; 15 min; 4°C), to precipitate the RNA. RNA was cleaned using 70% molecular grade ethanol. Total RNA was dissolved in diethylpyrocarbonate (DEPC) treated water. Any contaminating genomic DNA was removed using the DNase max kit following manufacturer's instructions (Qiagen, UK). The concentration and quality of RNA in each sample was determined by measuring 260/280 nm and 260/230 absorbance ratios (NanoDrop Technologies, Wilmigton, USA). The integrity of RNA was confirmed by running samples on a 1% agarose gel and RNA samples were stored at -80°C. A total amount of 1 µg of RNA was used for cDNA synthesis, employing iScript cDNA synthesis kit (Bio-Rad, UK). The reaction was placed at 25°C for 5 min, then 46°C for 20 min and inactivated at 95°C for 1 min. The iScript cDNA synthesis kit contains a combination of oligo dTs and random hexamers to work with a wide variety of targets.

The real-time PCR assay was performed according to Rawling and colleagues (32). Briefly, PCR reactions were set on a 384 well

plate in duplicate per sample. Each reaction was mixed with 2 µl of diluted (1/10) cDNA and 5.5 µl 2 x concentrated iQ™ SYBR Green Supermix (Bio-Rad), 0.3 µM forward primer and 0.3 µM reverse primer. The primer used and their sequences are presented in Table 1. Florescence monitoring occurred at the end of each cycle and an additional dissociation curve analysis was performed showing a single peak per sample. *Elf1-α* and *metap1* were used as reference genes in each sample to standardise the results by eliminating variation in mRNA and cDNA quantity and quality (33). The stability of *elf1-α and metap1* as reference genes were confirmed by an expression stability value 'M' generated by the geNorm™ software. Modification of gene expression was presented with respect to the controls being sampled at the same time as the treatment. PCR efficiencies for primer sets were determined using 10-fold serial dilutions of cDNA and presented using the equation E (PCR efficiency) = 10(-1/slope; Table 2 and Supplementary Table 3). The expression of target genes was displayed as fold change (FC (Log2) and were calculated based on Ct deviation (δCt) of the unknown sample versus a control sample and expressed in comparison to the reference genes *elf1-α* and *metap1*.

2.9 Statistical analysis

All statistical analyses were carried out using R version 3.4.1 (34). Rt-qPCR data were analysed using the permutation after Ohmel (35). Redundancy analysis of gene expression profiles for cytokines was performed using vegan package in R (36). All other data were assessed by one-way ANOVA tests with Tukey HSD *post-hoc* analysis where differences occurred. Data are presented as mean ± standard deviation (SD). Gene expression data showing comparisons with the control group are presented as means ± standard error of the mean (SEM; fold change (Log2)). AFM data represent cumulative results of all experiments performed and are shown as means ± S.D. The significative differences between YCW were determined by one-way analysis of variance (ANOVA) test with Tukey HSD *post-hoc* analysis. For all analysis, significance was accepted at $P < 0.05$.

3 Results

3.1 YCWs imaging, composition and functional characterisation

To assess the biochemical and nanomechanical properties of each yeast cell wall fraction several assays were performed. Scanning electronic microscopy imaging revealed well round-shaped YCWs with a preserved cellular integrity, i.e. without apparent breakage (Figure 1A). YCW fraction 4 presented a rougher cell wall surface due to the removal of the outer layer of the cell wall.

The biochemical composition in bioactive compounds, i.e. mannans and β-glucans, was significantly different between each YCWs (Figure 1B). YCW1 had the highest content of mannans (24.5%) and YCW group 4 the lowest (13%). Inversely, YCW1 had the lowest and YCW4 the highest β-glucans content (25.8% and 36.7%, respectively).

Yeast cell surface was mapped using Concanavalin A (ConA) and anti-β-1,3-glucan functionalised AFM tips. The occurrence of binding events between α-mannan and ConA-functionalised AFM tips, as well as between β-1,3-glucan and anti-β-1,3-glucan tip at the surface of YCW1 was significantly different from YCW3 and 4 (Figure 1C; $p < 0.05$). Over the total number of force curves analysed, 26.1%, 30.4%, 35.8% and 7.1% of interactions with ConA were detected respectively for YCW1, 2, 3 and 4. While the proportion of total mannans and occurrence of binding with ConA were consistent for YCW1 (24.5% and 26.1%, respectively), the surface of YCW2 and 3 interacted more frequently with ConA compared to YCW1 despite their significantly lower total mannan content ($p < 0.05$; Figure 1B). Finally, YCW4 had both the lowest levels of mannans and binding with ConA but these showed contrasted levels (13% and 7.1%, respectively). The mechanical properties of the cell wall α-mannans at the cell surface of the yeast were measured by rupture distance that corresponds to the distance at which the binding of the polysaccharide with the AFM-tip ConA was broken. The results suggest that the elongation forces on the cell surfaces of all YCW fractions were better described by the WLC model, which resulted in contour lengths as large as 600 nm in YCW fraction 2 and 3 and up to 400 nm in YCW fractions 1 and 4. As a result it could be proposed that in all YCW fractions the entire mannoproteins and not solely the α-mannan chains were stretched out from the cells wall and there were detectable differences in the length of these polysaccharide structures (Figure 1D). The mannans contour length averaged 258.1 ± 5.8 nm and 290.4 ± 10.2 nm for YCW2 and 3, respectively, which was approximately double to that of YCW1 and 4 (Figure 1D).

Mapping of β-1,3-glucans at the surface of YCW using a monoclonal mouse anti-β-1,3-glucan measured 9.3%, 7.3%, 5.9% and 12.4% of interactions for YCW1, 2, 3 and 4, respectively (Figure 1C); overall showing a lower ratio of interactions to composition compared to that of mannans - ConA. This observation was expected as the AFM technique allows to map only β-1,3-glucans accessible at surface of the cell, while the biochemical analysis quantifies the amount of total β-glucans without distinguishing β-1,3-glucan from β-1,6-glucan. Similar to the detection method used for α-mannans, β-1,3-glucans stretching was described using the WLC model, indicating a semi-flexible conformation of this polysaccharide, in accordance with the triple helix structure of β-1,3-glucans. The contour length of β-1,3-glucans chains from YCW 1 and 3 were distributed over a narrow range of 10 to 400 nm compared to YCW2 and 4 for which values of up to approximately 600 nm were measured. The average β-1,3-glucan contour lengths were 127.0 ± 17.1 nm and 145.2 ± 7.1 nm for YCW1 and 3 which were lower than the values measured for YCWs 2 and 4 (Figure 1E).

3.2 Intestinal morphometry reveals strengthening of intestinal barrier

The PI of zebrafish fed the different YCWs for 5 weeks were closely examined as the intestine is a primary site of endocytic and pinocytotic activity for antigen sensing and uptake (Romboult et al.,

TABLE 2 Primer pair sequences, gene name abbreviations, annealing temperature (Aneal Tm in °C), amplicon size (bp) and primer efficiency (Eff) for genes used for real-time PCR.

Gene	Functional annotation	Accession number	Primer sequence (5′-3′)	Efficiency
Reference genes				
elf1α	elongation factor 1 alpha	L23807.1	F-AGATGCCGCCATTGTTGAGA R-CTCTTGGTCGCTTTGCTGTG	2.1
metap1	methionyl aminopeptidase 1	NM_001025165.2	F-GACGAGGGAGCCAAGAGATT R-TCTGTGAAGCCTGGTATCCG	2.1
MAMP recognition				
tlr2	toll-like receptor 2	NM_212812.1	F-GTCCCATCGGTTCAGTCTCTT R-GTTTCAGGGTGGGAGACATCT	2.1
tlr4bb	toll-like receptor 4b, duplicate b	NM_212813.2	F-TCAACCAGAGCTGACACATCT R-CAGAAAGGTTCATGGGCAACTT	2.2
marco	macrophage receptor with collagenous structure	KJ955494.1	F-CTGGGAGGAAGGGAGATTCAG R-ACCAGTCCTGCCATCTTGAC	1.9
Signal transduction				
myd88	myeloid differentiation factor 88	NM_212814.2	F-GACTGACACCTGAGACCTTTGA R-TCGGTGTGTTCCAACTGTTTG	1.9
traf6	Tumor necrosis factor (TNF) receptor-associated factor 6	NM_212814.2	F-GAGAAGGAGAGGGAGTCGTTTC R-TGCTGGTCAGGAGGCATACT	1.9
tollip1	toll interacting protein (tollip), transcript variant 1	NM_207061.2	F-CCTGTGGTTCTGATGCCTACA R-GGCACCACACCCTGATTATACA	1.8
Transcription factors				
nfκB	Nuclear factor kappa B	NM_001001840.3	F-TGCTGACACTCACCCATCTG R-GACCACCACTCAACTGATAGC	1.8
stat4	signal transducer and activator of transcription 4	NM_001004510.1	F-AGTCCACTGGCTGTCTGTCT R-CAGCGGACCCTCATTTCCTT	2.1
rorc	related orphan receptor c	EF107094.1	F-CTGAAGAGATCCGTGTCTACCA R-TGGCAAACTCCACCACATACT	2.1
stat5a	signal transducer and activator of transcription 5a	NM_194387.2	F-ACAAGTAGTGCCAGAGTTTGC R-GCTGGAGATGATGCTACATGGT	1.9
foxp3a	forkhead box P3a	NM_001329567.1	F-TGCGTGTTGAAGGAAGGAAAG R-GGTGCCATCCAGTCCATATCA	2.2
Cytokines				
tnfα	tumor necrosis factor alpha	NM_212859.2	F-CCATAAGACCCAGGGCAATCA R-GGCAGCCTTGGAAGTGAAATTG	1.9
ifnγ (ifng)	interferon gamma 1	NM_212864.1	F-CCCATCTTCCTGCGAATCCT R-GCTTCATCCACGCTGTCATTC	2.1
il17a	interleukin 17a/f1	NM_001020787.1	F-ACATAACGAGAGCCTGTATCCT R-CCTCAACGCCGTCTATCAGA	2.2
il10	interleukin 10	NM_001020785.2	F-CCCTATGGATGTCACGTCATG R-TCCCGCTTGAGTTCCTGAAA	2.2
tgfβ	transforming growth factor, beta 3	NM_194386.2	F-AGGACAACACTGAGACTGAGTA R-GCAGTAGGGCAGGTCATTGT	2.0

2011[28]). The sampled intestine revealed no signs of enteritis or necrosis-like pathologies; there was no effect of feeding the YCW fractions on muscularis thickness and mucosal fold length, but there was a significant effect of YCW group 2 on lamina propria width which was lower compared to all other treatments (Supplementary Figure 1 and Table 1).

Compared to the control group, goblet cell density (GCD; Figure 2A) was significantly higher in YCW groups 1, 2 and 3 (+32.4%, +37.4% and +32.8%, respectively). Further, there were significant elevations in the prevalence acidomucin goblet cells (Figures 2B, C) in YCW 1, 2 and 3 fed groups (+13.7 pp, +15.5 pp, +18.4 pp, respectively) compared to the control (Figure 2A).

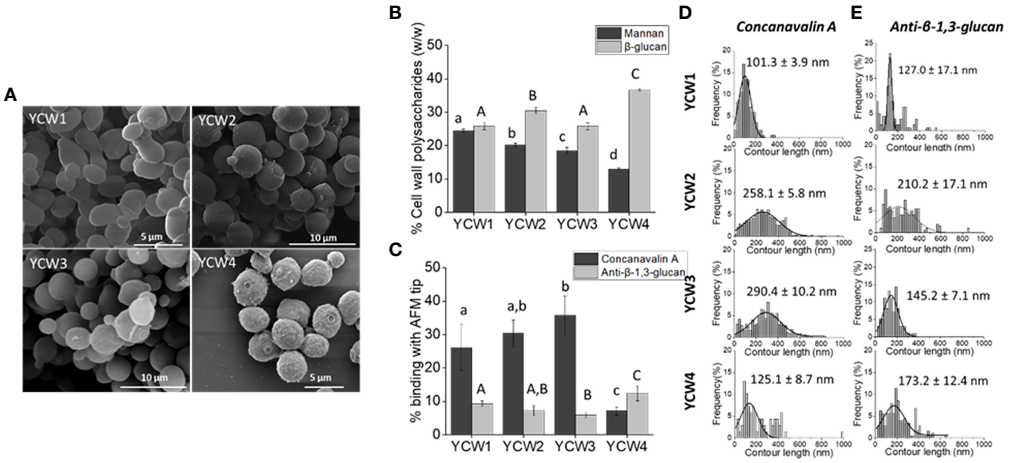

FIGURE 1
YCW fractions are characterised by differences in cells surface structures and composition. **(A)** Scanning electron microscopy images of each YCW fraction. **(B)** YCW biochemical composition of bioactive compounds, as the total percentage of α-mannans and total β-glucans for β-1,3 and β-1,6-glucans present in each YCW fraction. **(C)** Occurrence of binding with Concanavalin A and anti-β-1,3-glucan tip at the surface of each YCW fraction. Frequency distribution plots of **(D)** mannan-chains and **(E)** β-1,3-glucans contour length mapped at the surface of each YCW fraction using an AFM tip functionalised with Concanavalin A and anti-β-1,3-glucan, respectively; shown with mean values. All contour length values are plotted. Results are expressed as mean ± standard deviation value. Different letters indicate significative differences (One-way ANOVA, $p < 0.05$) between YCWs within each separate parameter as distinguished using small or large letters.

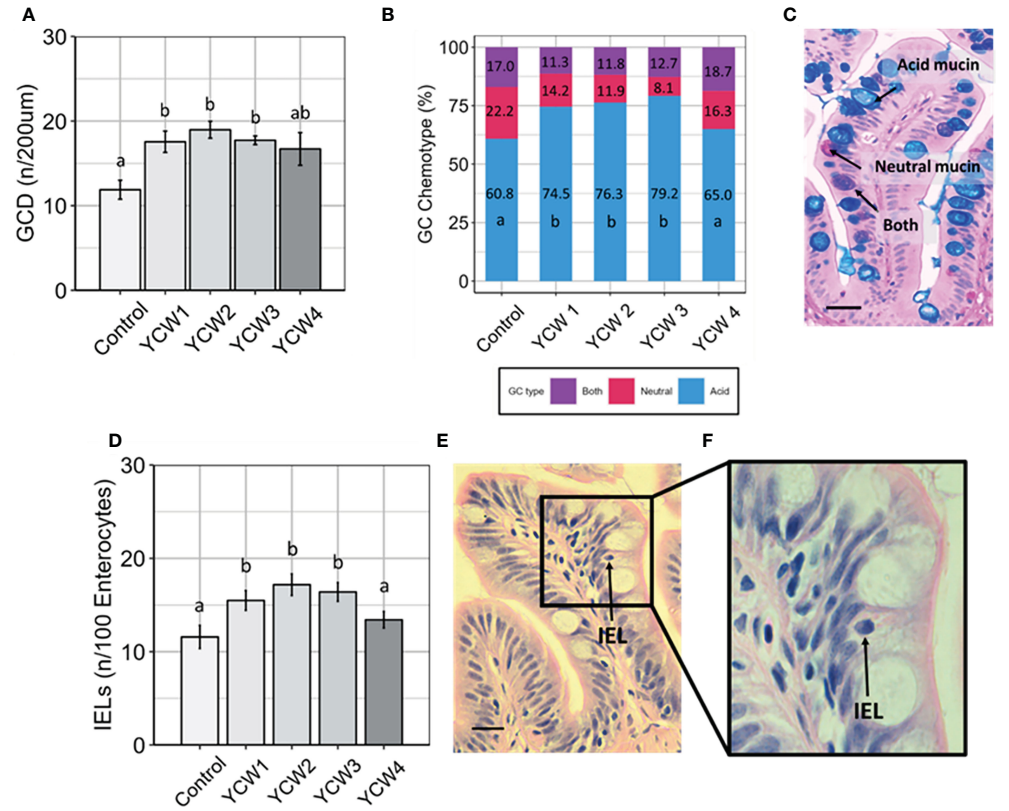

FIGURE 2
Fortification of the intestinal barrier by elevation of goblet cell density, acidomucin secreting cells and intra-epithelial leucocytes (IELs) when fed different yeast cell fractions. **(A)** Goblet cell density (GCD, n/200 µm of intestinal villi; mean ± SD with 9 fish/group assessed; **(B)** Relative abundance of goblet cell (GC) chemotypes as identified using Ab-PAS differential staining; **(C)** Example of Ab-PAS stained villi showing acidic (blue), neutral (pink), both (purple) mucins (scale bar= 10 µm). **(D)** Abundance of IELs (n/100 enterocytes; mean ± SD with 9 fish/group assessed; **(E, F)** Example of H&E stained mucosal fold showing an intraepithelial leukocyte (black arrow; Figure E, scale bar 10 = µm; **(F)** Different letters indicate significant differences between treatments ($p < 0.05$).

Similar to the results observed with GCD, IEL abundance (Figures 2D–F) was significantly elevated in YCW groups 1, 2 and 3 compared to control (+25.2%; +29.2% and +32.6% respectively; Figure 2D) which was not observed in YCW group 4.

3.3 Different YCW fractions differentially modulate of innate immune PRRs and signal transduction

Innate immune cells express different classes of innate immune PRRs, including scavenger receptors (SRs) and the microbe sensing toll-like receptors (TLRs). Indeed, members from both families have been implicated for the detection of microbial associated molecular patterns (MAMPs) derived from the yeasts such as *Saccharomyces cerevisiae*. Compared to the control, there was a significant elevation in the expression of *tlr2* in YCW groups 2, 3 and 4 (+84.1%, +58.6% and +64.6% respectively, $p < 0.05$) while the expression of *tlr4bb* was significantly elevated in YCW groups 1 (+74.3%; $p = 0.006$) and 4 (+47.4%; $p = 0.01$) and that of *marco* in YCW groups 1, 3 and 4 (+91.1%; +91.2% and +90.9%; $p = 0.002$). Although not significant, the expression of *tlr4bb* and *marco* was down regulated by 9.1% and 8.3%, respectively in the YCW 2 group compared to the control (Figures 3B, C).

Regards to signal transduction markers (Figure 3B) and compared to the control; *myd88* expression was significantly upregulated in all four YCW groups (from +74.5% in YCW1 to +93.0% in YCW2 group) while *traf6* was upregulated in the YCW groups 3 and 4 (+71.4%, $p = 0.01$; +77.8%, $p = 0.002$, respectively) and *tollip1* in YCW groups 1, 3 and 4 (+52.5%, $p = 0.04$; +68.2%, $p = 0.04$ and +62.8%, $p = 0.006$, respectively). Notably and although not significant, the expression level for *tollip1* was down regulated by 30.7% in the YCW 2 group compared to the control (Figure 3F).

3.4 Transcriptional gene expression markers show distinct profiles for different YCW fractions

Transcriptional factors for induction of innate immune responses that can prime antigen specific responses are important markers to identify and link cell mediated innate responses with adaptive T-cell responses. In this context the expression level of *stat4*, an important transcription factor for Th1 differentiation

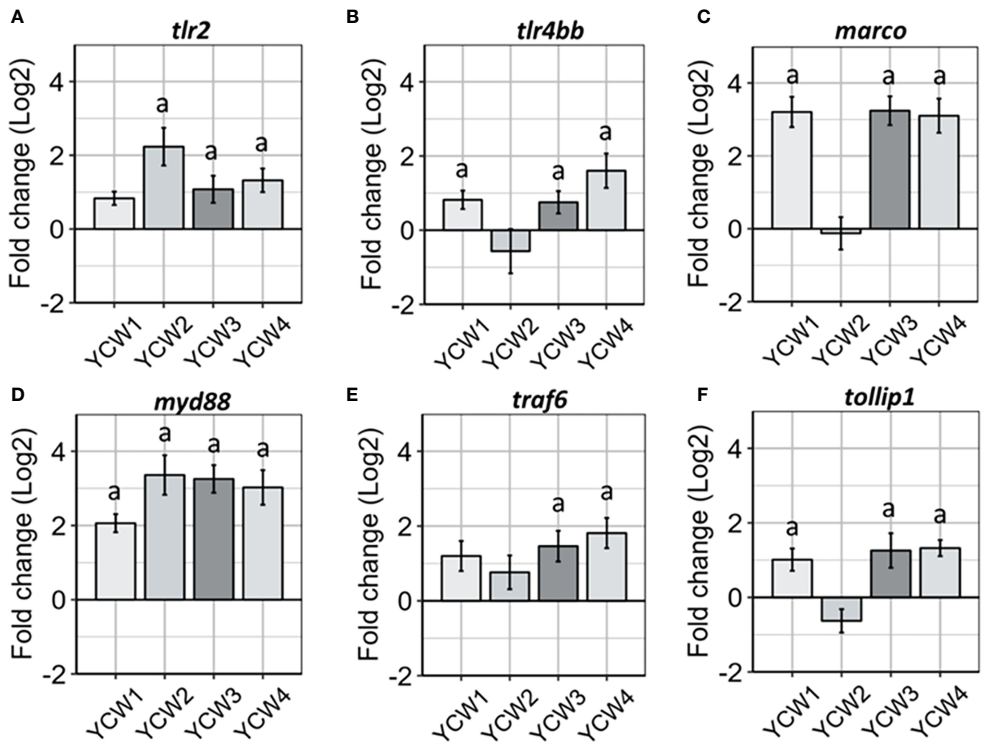

FIGURE 3
Oral administration of different YCW fractions differentially modulate the gene expression of host PRRs and innate immune signal transduction markers. Total RNA was isolated from the posterior intestine of zebrafish and gene expression of **(A–C)** PRR (*tlr2, tlr4bb* and *marco*) and **(D–F)** signal transduction markers (*myd88, traf6* and *tollip 1*) were evaluated by RT-qPCR. Data expressed as fold-change (Log2) relative to the control and shown as mean ± SEM (n = 6 per group); Presence of a letter highlight significant differences to the control and different letters highlight significant differences between treatments ($p < 0.05$).

through the IL-12 signalling cascade (Figure 4A), was up-regulated across all YCW groups compared to the control (from +67.6% in YCW 1 group to +76.3% in YCW 4 group; $p < 0.01$). In contrast and compared to the control group, the expression of *rorc* gene was significantly elevated in the YCW 1 group (+63.1%, $p = 0.02$) and to a further extent in YCW groups 3 (89.7%; $p = 0.002$) and 4 (+91.9%; $p = 0.002$), but not in YCW group 2 (Figure 4B).

Compared to the control (Figure 4C), the expression of *stat5a* was significantly elevated in all YCW groups and the highest in the YCW group 2 (+85.1%, $p = 0.002$). In contrast, the expression of *foxp3a* was differentially modulated in the different YCW groups (Figure 4D). While YCW group 1 had no apparent effect on *foxp3a* expression level, it was significantly upregulated in YCW groups 3 (47.3%; $p = 0.03$), 2 (+71.4%; $p = 0.004$) and 4 (+86.7%, $p = 0.002$) compared to the control. Notably, the expression of *foxp3a* was significantly down-regulated in the YCW 1 group compared to the YCW groups 2 and 4 ($p < 0.01$).

3.5 Different YCW fractions differentially modulate innate immune cytokine profiles

As a measure of immune competency, several well-characterised inflammatory (*il17a*, *tnfα*, *ifnγ*) and anti-inflammatory (*il10* and *tgfβ*) cytokines were analysed (Figure 5A). The redundancy analysis (RDA; Figure 5B) summarised the main patterns of variation for each response variable (genes) that can be explained by the matrix of explanatory variables (YCW group). The first two axis RDA1 and RDA2 explained 81.7% of the total canonical eigenvalues. The RDA plot revealed strong positive correlations between the explanatory variables for pro-inflammatory cytokines, *ifnγ* and *tnfα*. In the YCW 1 group, the RDA analysis revealed a positive link with the explanatory variable *il17a*. Indeed, the expression of *il17a* in the YCW 1 group was significantly elevated (+87.0%; $p = 0.002$) compared to the control (Figure 5A). Moreover, the RDA revealed a positive link with both response variables *il17a* and *ifnγ* in the YCW

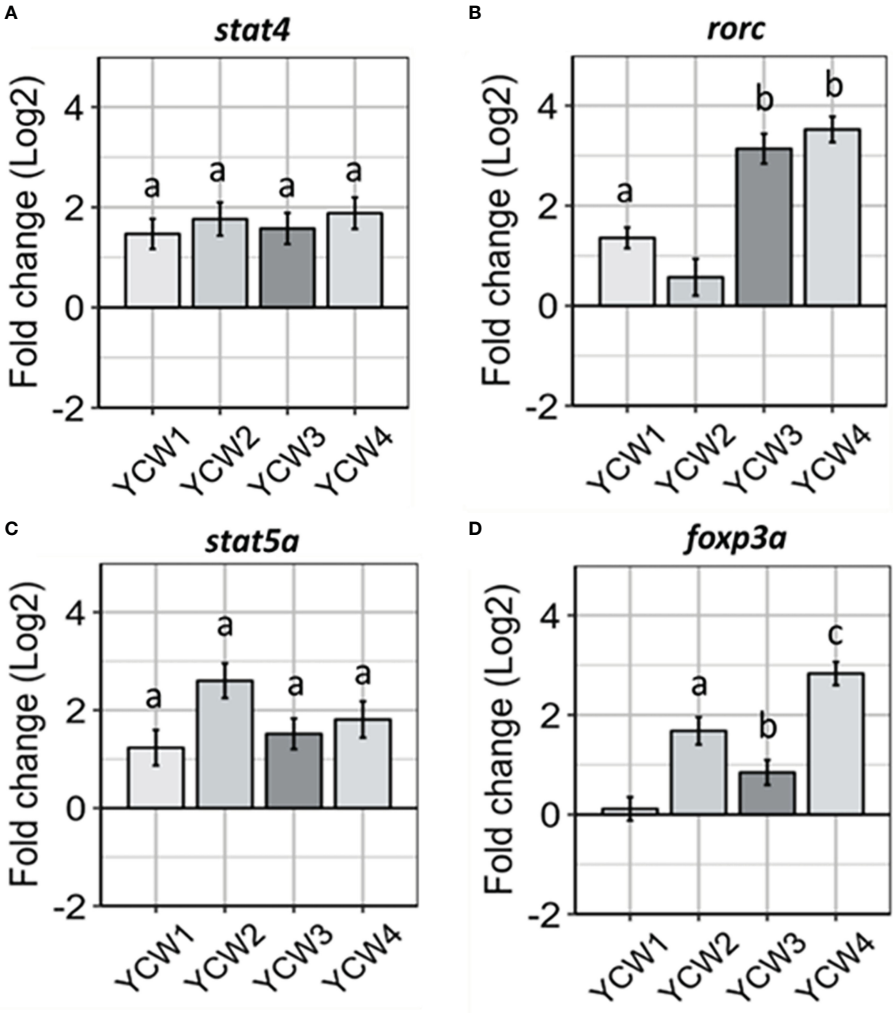

FIGURE 4
Oral administration of different YCW fractions differentially modulate the expression of host transcription factor. Total RNA was isolated from the posterior intestine of zebrafish and gene expression of **(A)** *stat4*, **(B)** *rorc*, **(C)** *stat5a*, **(D)** and *foxp3a*, were evaluated by RT-qPCR. Data expressed as fold-change (Log2) relative to the control and shown as mean ± SEM (n = 6 per group); Presence of a letter highlight significant differences to the control and different letters highlight significant differences between treatments ($p < 0.05$).

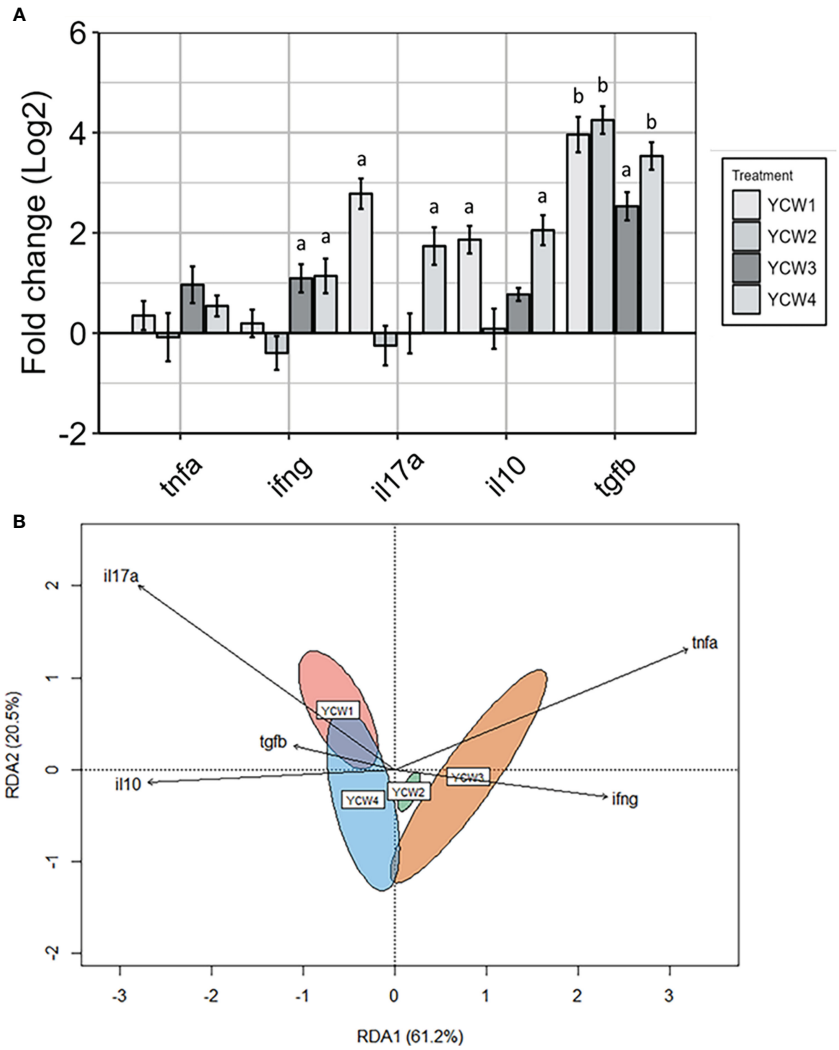

FIGURE 5
Distinct pro- and anti-inflammatory cytokine profiles in response to oral administration of different YCW fractions. **(A)** Gene expression level of *tnfα, ifnγ, il17a, tgfβ,* and *il10* in the posterior intestine of zebrafish evaluated by RT-qPCR. Data expressed as fold-change (Log2) relative to the control and shown as mean ± SEM (n = 6 per group); Presence of a letter highlight significant differences to the control and different letters highlight significant differences between treatments ($p < 0.05$). **(B)** Redundancy analysis was a constrained model indicating a significant difference by permutation in the variation of the explanatory variables (YCW group) for each response variable (genes) ($r2 = 0.81$, $p = 0.001$). Red ellipse = YCW1, green ellipse = YCW2, orange ellipse = YCW3 and blue ellipse = YCW4. Arrows pointing in the same direction indicate positive correlations, and arrows pointing in opposite directions indicate negative correlations. The arrow length corresponds to the variance explained by the explanatory variable. The first two axes explain 81.7% of the total canonical eigenvalues.

4 group. This was supported by significant elevations in the expression of *ifnγ* (+60.3% $p = 0.03$) and *il17a* (+74.8%; $p = 0.002$) in the YCW 4 group compared to the control regime (Figure 5A). Likewise, there was a significant elevation in the expression of *ifnγ* (+57.6%; $p = 0.03$) in the YCW 3 group compared to the control and the RDA revealed a positive link with both response variables *ifnγ*and *tnfα* in the YCW 3 group (Figure 5B).

The RDA revealed strong positive correlations between the response variables for anti-inflammatory cytokines, *tgfβ* and *il10*. Compared to the control group, there was a significant upregulation in *tgfβ* expression in YCW 1 and 4 groups (+94.4%, $p = 0.002$ and +92.1%; $p = 0.002$, respectively) as well as *il10* (+76.1%; $p = 0.006$ and +79.6%; $p = 0.004$; respectively). In contrast, *tgfβ* but not *il10* was significantly upregulated in YCW groups 2 (94.9%; $p = 0.002$) and 3 (86.1%; $p = 0.002$) compared to the control regime.

4 Discussion

Yeast and yeast cell wall fractions derived from *S. cerevisiae* contain functionally conserved molecules acting as microbial associated molecular patterns (MAMPs) that can interact directly or indirectly with pathogens and the host's immune system (37, 38). The current study assessed the structural-functional relationship of four different proprietary yeast cell wall (YCW) fractions from *S. cerevisiae* towards mucosal tissue

response and the intestinal immune response. We documented the discrete biophysical properties of distinct YCWs which were, upon oral administration at the same dosage to zebrafish as a vertebrate model, associated with varying levels of intestinal barrier fortification and distinctive immune molecular signatures from recognition to markers of T-cell differentiation.

Four types of YCW were used with significant differences in mannans and total β-glucan content. Mapping of α-mannans and β-1,3-glucans chains on the cell surface using single-molecule force spectroscopy with functionalised AFM-tips revealed differences in terms of cell coverage and length of these polysaccharides. We highlight two significant YCW characteristics that could be important when considering the potential immune potency of each YCW fraction. Firstly, the proportion of cell coverage and length of α-mannans that decorate the cell surface were different between yeast cell wall fractions. Secondly, the binding of anti-β (1, 3) glucan tip with the cell surface was different between YCW, indicating a difference in the accessibility of β-1,3-glucan at the cell surface which could drive differential recognition by immune host cells and in turn affect their capacity to elicit protection from pathogenic and non-pathogenic agents. The highest cell surface coverage was displayed by YCW4, that was significantly higher compared to all other YCW fractions. Interestingly SEM imaging revealed that YCW4 displayed a rougher outer layer suggesting a more exposed layer and so potential higher exposure of the β-glucan layer. Finally, the mechanical properties of the cell wall β-glucans were studied with a focus on β-1,3-glucans as the AFM-tip only bound to this polysaccharide structure. Elongation forces were described with the WLC model for calculation of the β-1,3 glucan contour lengths, which correspond to the length of the polysaccharides completely unfolded by the AFM tip. The length of β-1,3 glucan structures on the cell surface were different between each YCW fraction; with the highest contour length displayed by YCW2 and being almost double that of YCW1. These apparent structural disparities between each YCW fraction are expected to be due to strain specificities and environmental adaptation to the conditions experienced by the yeast during its propagation. Differences in β-1,3-glucans structure could lead to different response *in vivo* as β-1,3-glucans are classified as biological response modifiers, where the most bioactive β-glucans contain 1,6-linked side chains branching off from the more extended β-1,3-glucan backbone and are referred to as β-1,3/1,6-glucans (39).

To address whether the different structures of the YCW can interact differently with GALT the zebrafish was used as a good comparative model (40) First, the mucosal tissue response was characterised. The intestine represents a major portal of entry for parasites, bacteria and viruses against which goblet cell hyperplasia and subsequent changes in mucin composition constitute an important protective measure against pathogen adherence and translocation across the intestinal epithelial barrier (41, 42). In this study performed under non-challenging conditions, oral supplementation with YCW1, 2 and 3 elevated goblet cell density (GCD) and mucin acidic chemotypes (Figures 2A, B). This agrees with other studies in terrestrial and aquatic farmed species (17, 43–47). Interestingly, acidic mucins have been shown to help scavenge free hydroxyl radicals and increase mucus viscosity (48, 49). Given the apparent elevations in GCD in fish fed the YCW fractions with higher levels of mannoproteins, future studies should focus towards investigating the effects of mannoproteins on GC composition particularly GC chemotypes and mucin composition as this is seldom reported across FFA studies.

Further, the abundance of IEL in the intestinal barrier was measured. In humans, Intestinal IELs are a frontline heterogeneous subpopulation of T-cells and the TCRγδ+ T cells represent about 10% of the lymphocyte population in the small intestine. Their primary function is to maintain intestinal homeostasis and epithelial barrier function by providing immunosurveillance and effector immune functions against pathogen translocation through innate-like mechanisms or as antigen-specific memory T-cells (50). Accordingly in teleost fish, the posterior intestine is an area of high pinocytosis and heightened antigen uptake and surveillance by IELs that are thought to be mainly $CD8\alpha^+$ $TCR\gamma\delta^+$ T cells (51–53). Although in the current study there was no direct staining of the IEL population, staining the intestinal tissue with H&E revealed significantly elevated presence of IELs in the PI of fish fed YCW1, 2 and 3. IELs hyperplasia upon whole-YCW supplementation was previously documented in broilers (54), as well as in the European seabass (55). In contrast to YCW4, YCW1, 2 and 3 had a high mannan content and presented a high level of α-mannans of varying chain-length. The mannan and more specifically α-mannans content and bioactivity would therefore appear important features of YCWs to promote the expansion of intestinal goblet cells (GC), particularly of the acidic chemotype associated with increased mucus viscosity and buffering capacity, and of intestinal IELs in the submucosa. The mechanisms at play in such YCW responses are not fully elucidated. Yeast-derived MOS are ligands to the endocytic mannose-receptor (MR) primarily expressed on macrophages and dendritic cells (56, 57). MR ligation has been associated with in an array of mechanisms including phagocytosis, antigen processing, cell migration as well as intestinal homeostasis and the resolution of inflammatory processes (58). Besides mucin secretion, GCs form goblet cell-associated antigen passages (GAPs) able to deliver luminal antigens to antigen presenting cells in the submucosa for processing and presentation to IELs and other adaptive immune cells (41). In summary, YCW4 that contained exposed β-glucans had little apparent effect on the intestinal barrier responses unlike the mannan-rich YCW1, 2 and 3 fractions which promoted GC and IEL abundance. Such fortification of the intestinal barrier and immune competence can present an effective strategy to reduce host-adhesion and invasion by potential pathogens and suggest enhanced antigen sensing capacity (58). However, the histological appraisal of the intestinal mucosa only provides a limited view on the potentially specific immune functionalities of contrasted YCWs.

To verify the ability of the four different *S. cerevisiae* YCW to elicit a different intestinal immune response, different classes of innate immune PRRs were investigated including scavenger receptors (SRs), microbe sensing toll-like receptors (TLRs) and downstream signal transduction markers. These PRRs have previously been implicated in the detection of microbial associated molecular patterns (MAMPs) from the *S. cerevisiae*

yeast (59–62). The four YCW fractions assessed in this study displayed different affinity to the different PRRs assessed based on their gene expression responses. YCW4 and YCW1 both displayed significant elevations in expression of *tlr2, tlr4* and *marco* despite marked differences in cell wall composition and structure. On the other hand, YCW2 and 3 had similar mannans and β-glucans composition; but elicited distinctive PRR-responses reflecting their contrasted YCW architecture. It therefore appears that the cell wall composition does not adequately predict PRRs recognition such that *S. cerevisae* YCWs containing similar levels of mannans, and glucans could lead to distinctive downstream signalling and immune responses.

Different studies have shown that the structure of β-glucans will influence the recognition and subsequent immunomodulatory effects of this polysaccharide, where large molecular weight and particulate β-glucans are mainly recognised by TLR2 as reviewed by Brown and Gordon (63). Our results appear to be in agreement as YCW2 displayed the largest β-1,3-glucan chain lengths and elicited the highest *tlr2* up-regulation followed by YCW3 and 4. Mannan chain lengths may also have had a significant effect on PRR recognition of YCW fractions 2, 3 and 4. Indeed, Nigou and colleagues (64) reported that fungal extracts presenting longer mannan chain lengths had a significantly higher affinity to TLR2 which modified downstream signalling. Accordingly in this study, the longer mannan chain lengths of YCW2, 3 and 4 compared to YCW1 may also have contributed to the upregulating of *tlr2* expression. Besides TLR2, there are recognised synergistic relationships between dectin-1, a major β-glucan receptor, and TLR4 for recognition of β-glucan and mannan ligands (65–67). Interestingly, YCW fractions 1 and 4 displayed a shorter mannan length (Figure 1D) that could confer better accessibility to the β-1,3-glucan located beneath the outer mannan layer of the yeast cell wall. This may have influenced the potential binding to TLR4 as *tlr4* expression level was significantly elevated in fish fed YCW1 and 4 compared to the control (Figure 3B).

The study also measured the expression levels of the class A scavenger receptor *marco* that is mainly expressed on macrophages and plays a major role in the antibacterial host defences as confirmed in fish (68). Indeed, using MARCO knockout transgenic mice lines, Bowdish and colleagues (69) reported that MARCO was an important receptor required for TLR signalling during *Mycobacterium tuberculosis* infection. Notably, macrophages from MARCO defective mice were unable to secrete pro-inflammatory cytokines in the presence of *M. tuberculosis*. Besides, MARCO is involved in the direct recognition of major constituents of fungal cell walls such as yeasts and eradication of fungal pathogens (70). In this study, the expression level of *marco* was significantly elevated compared to the control in fish exposed to YCW fractions 1, 3 and 4 but not to YCW fraction 2. Interestingly, in relation to YCW structure the contour lengths for β-1,3-glucans were shorter by ~10% in YCW fractions 1, 3 and 4 compared to YCW fraction 2 (Figure 1D). Accordingly, the shorter β-glucan chain lengths may require cooperation of PRRs including MARCO to "tether" the yeast ligands to macrophages and activate either TLR2 or TLR4.

Following recognition of YCWs MAMPs, the subsequent signalling indicated activation of immune receptors as the expression levels for *myd88* were significantly elevated in all fish exposed to the YCW fractions (Figure 3D). In contrast the expression of *traf6*, which mediates IL-1 signalling, was significantly elevated in fish exposed to YCW fractions 3 and 4 only. Notably, TRAF6 has been implicated to play a protective role in epithelial barrier homeostasis and innate protective response in the intestine (71). Likewise, the expression of *tollip 1* gene, an adaptor-protein associating directly with TLR2 and TLR4 and playing an inhibitory role in TLR-mediated cell activation (72), was significantly elevated with YCW fractions 1, 3 and 4 only (Figure 3E). It would therefore appear that although all YCW tested induced the expression of markers for TLR-MyD88 signalling, some but not all fractions show the potential to further regulate TLR-mediated signalling *via* induction of TOLLIP1 gene; again, pointing to an impact of the yeast cell wall architecture on its immunogenicity.

It must be emphasised that yeast β-glucans and mannans are known to interact with an array of PRRs including Dectin 1 and 2, DC-SIGN, TLR2, TLR4, and TLR6 located on various immune cells such as monocytes, macrophages, neutrophils and T-regs (73, 74). Future studies should expand the PRRs target to profile MAMP's-PRR interactions across yeast cell wall fractions of distinctive molecular structure. This was not performed in this study, which focused on gene expression markers for TLR-MyD88 signalling through to transcription factors, cytokine and intestinal tissue responses in order to document the distinctive immune properties of *S. cerevisae* derived yeast cell wall fractions from detection to tissue response.

The main cytokines families in teleosts are the interleukins (ILs), interferons (IFN), tumor necrosis factors (TNF), and transforming growth factors (TGF) produced by several innate as well as adaptive immune cells. Cytokines orchestrate innate immunity and further characterise the adaptive immune response hence have a pivotal role in the clearance of infectious agents (75, 76). From this perspective, the study characterised the gut immune response using molecular markers for characterisation of both effective innate and T-cell mediated immunity.

Herein the results showed that different YCW fractions elicited specific gene expression profiles for transcription factors (TFs) and effector cytokines that reflected mobilisation of effector T-helper cell subsets for Th17, Th1, Tr1 and Th3 (Figure 6). Oral administration of YCW1 and 4 elicited the upregulation of a cluster of genes suggestive of polarisation of naïve T-cells to Th17-like cells including *tlr4*, *myd88*, *stat4*, *rorc* and high expression for *il17a* compared to all other experimental groups. RAR-related orphan receptor gamma (RORγ) is a protein that in humans is encoded by the RORC (RAR-related orphan receptor C) gene and the induction of transcription factor RORγt is a key part of the transcriptional programming required for Th17 cell differentiation. The effector cytokines IL-17 and granulocyte macrophage colony-stimulating factor (GM-CSF) are integral to the recruitment of neutrophils to the site of inflammation, promoting inflammatory processes (77, 78). Th17 cell-mediated immunity (CMI) has been shown to be effective at removing extracellular fungal and bacterial pathogens such as *Klebsiella pneumonia*, *Citrobacter rodentium* and *Candida albicans* (79). Here we show evidence of polarisation of naive T-cells to Th17-like cells in the form of significant upregulation in the

10.3389/fimmu.2023.1158390

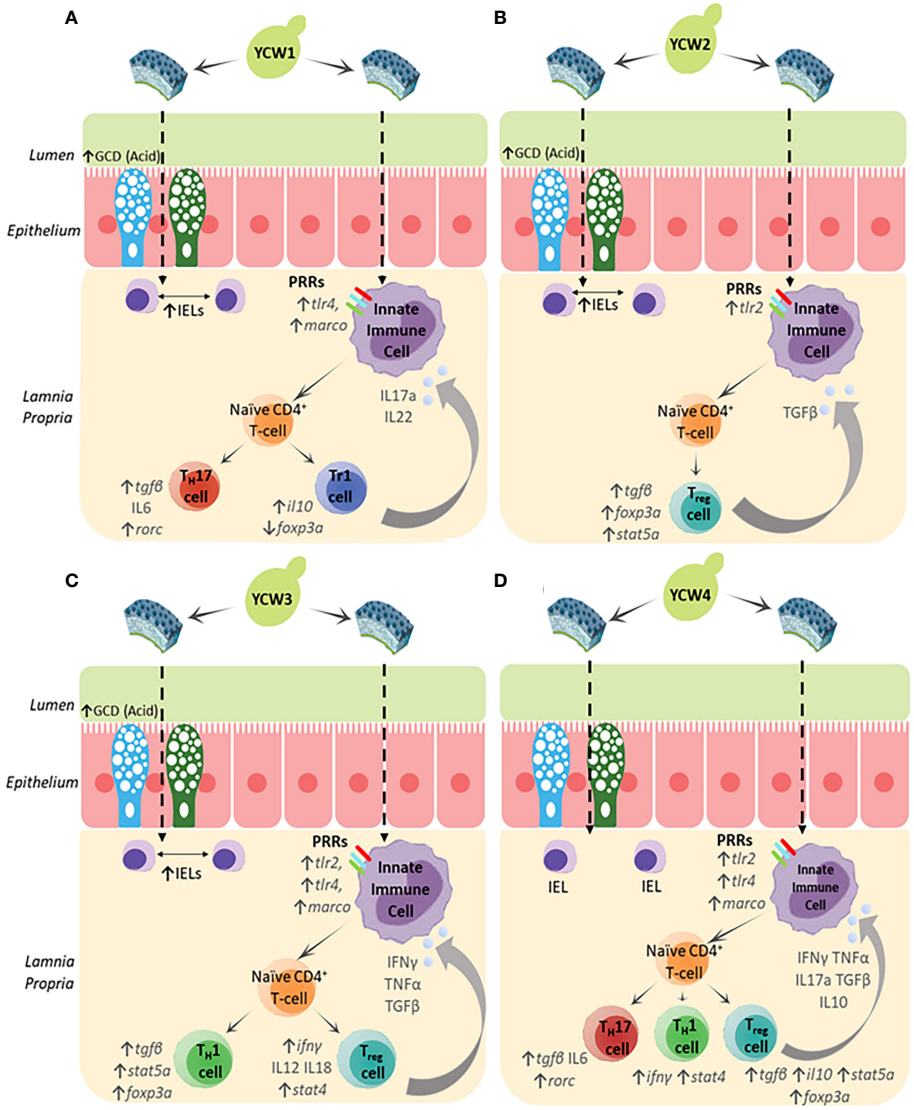

FIGURE 6
The biochemical and molecular characterisation of the YCW fractions **(A)** YCW 1, **(B)** YCW 2, **(C)** YCW 3 and **(D)** YCW 4, demonstrate elevations in specific biomarkers for fortification of intestinal barrier integrity (↑ GCD (Acidomucins), ↑IELs) except for YCW fraction 4. Apparent differences in immune competence markers are proposed to have been driven by differences in cell wall mannan and β-glucan content as well as structure determining potential recognition by different PRRs presented on innate immune cells such as macrophages (Mϕ), dendritic cells and neutrophils present in the underlying intestinal tissue. Downstream signalling suggests potential mobilisation of naïve T-helper cells to effector subsets and drive specific cytokine milieus to control innate immunity.

expression of *rorc* and *il17a* in experimental groups YCW1 and 4 compared to the control group. Interestingly, this is in line with our present findings that YCW1 and 4 elicited elevations in the expression of *tlr4* which has been shown to directly regulate Th17 differentiation (80). However, zebrafish were recently found to present ILC-like cells involved in the mucosal immune response and homeostasis of mammals (81). Like Th17 T-cells, ILC3s require the induction of RORC for activation and produce effector cytokines IL-17a, IL-22 and GM-CSF. Accordingly, PRR recognition of yeast ligands *via* a combination of C-type lectin receptors and, or TLR4 could influence cell signalling and effector cytokines for polarisation of naïve T-cells to Th17-like cells or activation of ILC3s, as shown in studies with human cell lines and mice (82–84). The short mannans chain-length measured in both YCW1 and 4 (Figure 1D) may have constituted an important trait to elicit such downstream immune functions.

The pro-inflammatory Th1 cell-mediated responses are orchestrated through the release of signature Th1 cytokines, IFN-γ and TNF-α (85, 86). Indeed, these cytokines play a pivotal role in the induction of classically activated (M1) macrophages which assist in the clearance of both fungal and intracellular pathogens (87). Furthermore, transcription factors STAT4 and T-bet are important mediators in the differentiation of naïve T-cells to Th1 subsets and STAT4 is preferentially expressed in Th1 cells (88, 89).

Herein compared to the control group, oral administration of YCW fractions 3 and 4 elevated *tlr2* and *myd88* (Figures 3A, D), *stat4* and *rorc* (Figures 6A, B), *tnfα* and *ifnγ* (Figure 5A) expression level together suggesting the potential polarisation of naïve T-cells to Th1-cells *via* TLR2-Myd88 signalling. In contrast, YCW 4 displayed shorter mannan chains but longer β-1,3-glucan chains conceivably allowing for exposure of β-glucans. Indeed, as aforementioned, it was apparent that fish fed the YCW fraction 4 displayed significant elevations in all PRR genes showing the potential for a strong affinity to MARCO-Syk, TLR2 and TLR4-MyD88 signalling. This in part could explain why the expression profile for YCW4 suggest mobilisation of effector cytokines and polarisation of naïve CD4 T-cells to Th1/Th17 subsets.

Regulation of the immune response is important in protecting the host from infection-associated immunopathology, autoimmune diseases and allergy. At the heart of these immunological events are regulatory T-cells (Treg) that are key to orchestrating the immune response and tissue repair. Treg cells are abundant in non-lymphoid tissues particularly at mucosal surfaces where constant exposure to inflammatory triggers must be tightly regulated to ensure homeostasis at a steady state or be ready to engage in potent immune responses when required. In this regard, the study targeted key effector cytokines (IL-10 and TGF-β) and master transcription factors (FoxP3 and stat5a) involved in the polarisation of naïve T-cells to Treg cells (90). Results revealed distinct patterns for stimulation of secondary inducible T regulatory cells (iTregs) analogous to T regulatory type 1 (Tr1) cells and FOXP3$^+$ iTreg cells.

In particular, oral exposure of YCW fraction 1 significantly elevated the gene expression *il10* but not *foxp3a*, (Figures 4A, 6D, respectively) reflecting a mobilisation of FOXP3$^-$ Tr1 cells (91). Tr1 cells are typically located in the intestinal mucosa and their key role is to maintain peripheral tolerance and suppress tissue inflammation to self and non-self antigens. Interestingly, human Tr1 cells have been reported to secrete IL-22 an IL-10 family member cytokines that act on intestinal epithelial cells to promote intestinal cell barrier integrity by driving mucin production and differentiation (92). This agrees with our histological findings of a higher goblet cell density and altered chemotypes in fish fed the YCW1 fraction (Figures 2A, C). In contrast and compared to the control, fish fed YCW2, 3, and 4 fractions significantly elevated the expression levels of *tgfβ*, *stat5a* and *foxp3a* (Figures 5A, 4C, D, respectively). These profiles may reflect a polarisation of naïve T-cells to FOXP3$^+$ iTreg cells that are essential in tolerance to self and non-self antigens (93). To summarise, the markers assessed suggested contrasted effects on iTreg subsets in response to the presentation of different yeast cell wall polysaccharides. Recently, a preparation of mannan/β-1,6-glucan was found to facilitate the induction of Treg from naïve T-cells by a Dectin1-Cox2 signalling pathway (94). This confirms the potential of yeast-fractions as T-reg immunity modifiers as well as the interest of assessing the affinity of YCW fractions to augment specific PRR signalling pathways in future studies.

5 Conclusion

The study documented marked intra-species variability in the molecular properties of *S. cerevisae* yeast cell wall fractions from discrete sources which were associated with contrasted mucosal immune responses upon oral administration in a vertebrate animal model. The study identifies α-mannan content as a potent driver of GCD and IEL hyperplasia, suggestive of fortifying intestinal barrier integrity and immune competence. Further the structural molecular differences of the YCW polysaccharides, in terms of α-mannans and β-1,3-glucans chain-length, are shown to modify the expression pattern of PRR responses.

The resulting downstream transcription factors and cytokine responses suggest the preferential mobilisation of distinct effector T-helper cell subsets for Th17, Th1, Tr1 and Foxp3$^+$-Treg (Figure 6), indicating a particular potential for each YCW fraction against infectious agents and, or non-infectious pathologies (Table 3). Although this study under no challenge condition suggests priming responses on adaptive immune T cells but cannot, yet, be discriminated from the functionality of other cells, such as innate lymphoid cells (ILCs) which are found in the gut mucosa. Further investigation with a time-course of immune response to pathogenic/antigen challenge can be interesting for future investigation of the adaptive immune response. Accordingly, beyond the mannans and β-1,3/β-1,6-glucans content of *S. cerevisae* YCW fraction, the study confirms the importance of considering the molecular structural characteristics of the YCW to apprehend their specific immune properties and ultimately elicit targeted immune-functionalities. This comparative study offers new perspectives in the development of specific *S. cerevisae* fractions towards targeted application in precision animal nutrition.

TABLE 3 Specific functional responses of each YCW fraction related to their effectiveness at clearing different pathogen types and maintenance of immune homeostasis.

Functional responses	YCW1	YCW2	YCW3	YCW4
Pro-inflammatory	Extracellular anti-bacterial/fungal CMI – Th17 response	No response	Intracellular anti-viral/bacterial CMI –Th1 response	Anti-viral/bacterial/fungal CMI – Th1/17 response
Anti-inflammatory	Non-specific tolerance/tissue repair CMI – Tr1 (Foxp3$^-$)	Tissue repair and immune homeostasis CMI – iTreg (Foxp3$^+$)	Tissue repair and immune homeostasis CMI – iTreg (Foxp3$^+$)	Tissue repair and immune homestasis CMI – iTreg (Foxp3$^+$)

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by University of Plymouth animal ethical review board.

Author contributions

MR, DM, EL, MC, and AF contributed to conception and design of the study. MR, MS, and EL organised the data for submission. MR and MS performed the statistical analysis. MR, MS, EL, and EA wrote the first draft of the manuscript. MR, MS, and EL wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

Acknowledgments

The authors would like to thank Ben Enyon, Dr Paul Laurance-Young and Dr Joceline Triner, for their technical support and we thank Lallemand SAS for the provision of yeast products.

Conflict of interest

Authors MS, EA, MC and EL were employed by company Lallemand SAS.

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.

The authors declare that this study received funding from Lallemand SAS. The funder had the following involvement in the study: study design, editing and proofing of manuscript.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1158390/full#supplementary-material

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